Introducing Sites of Specific Recognition into a Modified Chitosan Hydrogel Through Molecular Imprinting – Development of a Specialised Polymer Network as Potential Avenue for Selective Biosensor Detection and Site-Specific Recognition at the Biomaterial Interface

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Abstract

The ability to selectively recognise particular biomolecules of interest is of great import to scientists and clinicians across numerous fields. In the area of biosensors this capability permits sensitive and discriminating detection methods to be created. The specialty divisions of advanced functional materials and miniaturising technologies have greatly expanded on this, but these methods currently remain costly to produce and often require expertise in synthesis, along with the inclusion of intricate transducers and equipment to measure and convert signals. Furthermore, the biomolecular capture locales used in these devices are frequently sensitive to environmental conditions and storage degradation. It is desirable to be able to simply and efficiently create a highly specific substrate that can be used to replace the reliance on expensive and susceptible binding motifs.

The process of molecular imprinting can be used to impart particular recognition sites within a polymeric matrix by the inclusion of a template during polymerisation that mimics the physical and chemical structure of the target molecule. These specific recognition sites can then be used to attain higher levels of preferential adsorption and capture of a target biomolecule. Such specificity can be exploited as the 'sensing' substrate in analytical devices, be used for separation of chemical species, create memory in a bulk material for drug capture and release, and can impart improved functionality on biomaterial surfaces.

We report on an investigation of molecular imprinting using the polysaccharide chitosan, an attractive biomaterial for its strong biocompatibility, stability, and physical versatility. Using chemical modification of a water-soluble chitosan derivative, the polymer was functionalised to be crosslinkable by ultraviolet exposure for free-radical initiation. This provides a new method of using chitosan for molecular imprinting, at physiologic pH that would represent conditions suitable for biomolecule stability. The molecular weight of chitosan was reduced by temporal adjustment of the reaction time for acidic fractionation. Following this, reactive methacrylate moieties were added to side groups along

the chitosan backbone to allow for crosslinking of the polymer chains. The degree of substitution was controlled by the amount of functionalising species added. This ultimately affects the density and mechanical properties of the resultant hydrogel. The interchain network can be adjusted to achieve desired matrix conditions for each application.

Several gelation parameters were investigated to study their effect on imprint recognition. The capture and release of a model template from bulk hydrogels was used to investigate optimisation of the polymerisation system. Incorporation of additional crosslinking agents was not found to offer significant improvement to imprint recognition of the polymer, while molecular weight of the chitosan was observed to affect its recognition; lower molecular weight chains provided stronger interaction and rebinding with the template. This system was further expanded upon with the inclusion of model proteins as larger molecular targets of imprinting, using micropatterning of hydrogel in a thin layer for rapid qualitative observation of binding effect. The results demonstrated a measurable imprinting effect with chitosan gels, evidenced by increased capture of target molecule, which was not significantly improved upon by the addition of methylenebisacrylamide or ethyleneglycol dimethacrylate into the gelation matrix as crosslinking agents. The increased recognition of model compounds seen in this study achieved for larger templates (heparin) and small proteins (albumin), compared with the smaller molecular targets typically employed in MIP investigations, suggests that macromolecules of interest – in a molecular weight range increasingly relevant to scientists – can be pursued successfully as imprinting templates, and that the flexible nature of hydrogels can provide a suitable recognition matrix for an area currently posing a challenge to researchers.

Chemical crosslinking of the modified chitosan was subsequently used to form gel microspheres in an inverse emulsion system. These microspheres were similarly imprinted with model proteins to probe the capability for capture towards larger molecular weight templates. Release of captured target and

competitor molecules was examined through changes in system impedance via dielectric spectroscopy. This provided a sensitive, real-time method for verifying the imprinting effect as a difference between imprinted polymers and control, as well as improved recognition of the model templates over an analogous molecule. Using dielectric spectroscopy provided an enhanced ability to quantify imprinting effect over conventional methods, demonstrating a measurable difference between imprint polymers and non-imprinted controls, at levels not easily detectable by other analytical techniques. The system presents a new prospective avenue for use of dielectric spectroscopy in conjunction with molecular imprinting for rapid, real-time diagnostic devices. The small scale and sensitive capability of the system suggest potential for miniaturisation and incorporation into biosensors.

The conclusions of this study verified a capacity to improve the application of chitosan as a biomaterial through molecular imprinting. We confirmed from the observed results that imprinted chitosan gels possessed levels of both specificity and selectivity to target binding in an aqueous medium. Being largely heretofore an unexplored area of research, the outcomes of this thesis provide valuable insight into furthering this field of investigation, as a important step forward in achieving molecule recognition through imprinting in aqueous media under physiologic conditions, and the specific use of chitosan as a molecular imprinting matrix. In this thesis its potential application as a biomaterial for rapid qualitative and quantitative specific recognition as part of a biosensor is presented, but the scope of its promise extends far beyond this, as adding selective binding capability to chitosan would allow for highly functionalised biomaterial substrates to be created for applications in tissue engineering as well as controlled capture and delayed release of molecules for drug delivery applications.

Résumé

La capacité de reconnaître sélectivement biomolécules d'intérêt particulier est d'une grande importance pour les scientifiques et les cliniciens à travers de nombreux domaines. Dans la domaine des biocapteurs cette capacité permet des méthodes de détection hautement sensibles et sélectives. Les divisions spécialisées de matériaux fonctionnels avancés et les technologies miniaturisation ont considérablement élargi sur ce point, mais ces méthodes restent actuellement coûteux à produire et souvent besoin d'une expertise dans la synthèse, avec l'inclusion de transducteurs et équipements complexes pour mesurer et convertir des signaux. En outre, les lieux de capture biomoléculaires utilisés dans ces dispositifs sont souvent sensibles aux conditions environnementales et de la dégradation de stockage. Il est souhaitable d'être capable de créer de manière simple et efficace un substrat très spécifique qui peut être utilisé pour remplacer le recours à des motifs de liaison coûteux et sensibles.

Procédé selon l'une empreinte moléculaire peut être utilisé pour conférer des sites de reconnaissance spécifiques au sein d'une matrice polymère par l'inclusion d'un modèle au cours de la polymérisation qui imite la structure physique et chimique de la molécule cible. Ces sites de reconnaissance spécifiques peuvent ensuite être utilisées pour atteindre des niveaux plus élevés d'adsorption préférentielle et la capture d'une biomolécule cible. Cette spécificité peut être exploitée comme «détection» substrat dans les appareils d'analyse, être utilisé pour la séparation d'espèces chimiques, créer mémoire dans un matériau en vrac pour la capture de la drogue et la libération, et peut conférer une meilleure fonctionnalité sur les surfaces des biomatériaux.

Nous rapportons une enquête de l'empreinte moléculaire en utilisant le polysaccharide de chitosane, un biomatériau attractif pour sa biocompatibilité forte, la stabilité physique et de polyvalence. Utilisation de la modification chimique d'un dérivé de chitosane soluble dans l'eau, le polymère a été fonctionnalisé pour être réticulable par exposition aux rayons ultraviolets pour

l'initiation de radicaux libres. Ceci permet d'obtenir un nouveau procédé d'utilisation de chitosane pour l'impression moléculaire, au pH physiologique, qui représentent des conditions appropriées pour la stabilité de la biomolécule. Le poids moléculaire du chitosane a été réduite de réglage temporel de la durée de réaction pour le fractionnement acide. Par la suite, des fragments de méthacrylate réactifs ont été ajoutés à l'autre le long du squelette groupes chitosane pour permettre la reticulation des chaînes polymères. Le degré de substitution a été contrôlée par la quantité de fonctionnalisation espèces ajoutées. Cela affecte finalement la densité et les propriétés mécaniques de l'hydrogel résultant. Le réseau de interchaîne peut être ajustée pour obtenir des conditions de matrices souhaitées pour chaque application.

Plusieurs paramètres de gélification ont été étudiés pour étudier leur effet sur la reconnaissance empreinte. La capture et la libération d'un template de modèle hydrogels en vrac a été utilisé pour enquêter sur l'optimisation du système de polymérisation. L'incorporation d'agents de réticulation supplémentaires n'a pas été trouvé d'offrir une amélioration significative pour imprimer reconnaissance du polymère, tandis que le poids moléculaire du chitosan a été observé pour altérer la reconnaissance; chaînes de plus bas poids moléculaire et à condition interaction forte avec reconsolidation du modèle. Ce système a également été étoffée avec l'inclusion de protéines modèles comme cibles moléculaires plus de l'empreinte, en utilisant micromodelage d'hydrogel dans une couche mince pour l'observation qualitative rapide de l'effet contraignant. Les résultats ont démontré un effet mesurable d'impression avec des gels de chitosane, en évidence par une augmentation de la capture d'une molécule cible, qui n'a pas été significativement améliorée par l'addition de méthylènebisacrylamide ou le diméthacrylate d'éthylèneglycol dans la matrice de gélification comme agents de reticulation. La reconnaissance accrue de composés modèles observés dans cette étude réalisée pour les modèles plus grands (héparine) et les petites protéines (albumine), par rapport aux cibles moléculaires plus petites généralement utilisées dans les enquêtes de MIP, suggère que les macromolécules d'intérêt - dans un

intervalle de poids moléculaire de plus en plus pertinent pour - les scientifiques peuvent être poursuivis avec succès en tant que modèles de marquage, et que la nature flexible de hydrogels peuvent fournir une matrice de reconnaissance approprié pour une zone posant actuellement un défi pour les chercheurs.

Réticulation chimique du chitosane modifié a été utilisé par la suite pour former des microsphères de gel dans un système d'émulsion inverse. Ces microsphères ont été imprimées de façon similaire avec des protéines de modèle pour sonder la capacité de capture vers les modèles de plus grand poids moléculaire. Libération de molécules cibles et des concurrents capturés a été examiné par des changements dans l'impédance du système via la spectroscopie diélectrique. Ceci a fourni, un procédé en temps réel sensible pour la vérification de l'effet d'impression en tant que différence entre des polymères et de contrôle imprimés, ainsi que l'amélioration de la reconnaissance des modèles de modèle sur une molécule analogue. En utilisant la spectroscopie diélectrique fourni une meilleure capacité à quantifier l'effet de l'empreinte par rapport aux procédés classiques, ce qui démontre une différence mesurable entre les polymères impression et les contrôles non-imprimé, à des niveaux pas facilement détectables par d'autres techniques analytiques. Le système présente une nouvelle avenue prospective pour l'utilisation de la spectroscopie diélectrique en conjonction avec l'impression moléculaire, pour les appareils de diagnostic en temps réel rapides. La petite taille et la capacité sensible du système donnent à penser que la miniaturisation et l'intégration dans les biocapteurs.

Les conclusions de cette étude vérifiées une capacité à améliorer l'application de chitosane comme biomatériau par empreinte moléculaire. Nous avons confirmé les résultats observés à partir de ce imprimées gels de chitosane possèdent à la fois les niveaux de spécificité et de sélectivité de liaison à une cible dans un milieu aqueux. Étant en grande partie jusqu'ici une région inexplorée de la recherche, les résultats de cette thèse donne un aperçu précieux avancer ce champ d'investigation, comme un important pas en avant dans la réalisation de la

reconnaissance molécule par empreinte dans un milieu aqueux dans des conditions physiologiques, et l'utilisation spécifique du chitosane comme un matrice d'impression moléculaire. Dans cette thèse, son application potentielle comme biomatériau de reconnaissance spécifique qualitative et quantitative rapide dans le cadre d'un biocapteur est présenté, mais la portée de sa promesse va bien au-delà de cela, comme l'ajout de capacité de liaison sélective au chitosane permettrait de substrats de biomatériaux hautement fonctionnalisés à être créé pour des applications dans l'ingénierie tissulaire, ainsi que la capture et la libération contrôlées de molécules pour des applications de distribution de médicaments retardé.

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Glossary

ACVA	4,4-azobis-(4-cyanovaleric acid)
AIBN	azobisisobutyronitrile
AFM	atomic force microscopy
APS	ammonium persulphate
ATRP	atom transfer radical polymerisation
Av	avidin
Av-Fl	avidin-fluorescein
B-B	Box-Behnken
BSA	bovine serum albumin
BSA-Cy5	cyanine 5 labelled bovine serum albumin
C6S	chondroitin-6-sulphate
CAD	computer aided design
CCD	central composite design
ConA	concanavalin *A*
CS	chondroitin sulphate
Cy3	cyanine 3
Cy5	cyanine 5
Cyt C	cytochrome *C*
DA	degree of acetylation
DDAB	dimethyldioctadecylammonium bromide
DEGDMA	diethylene glycol dimethacrylate
DMT-MM	4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride n-hydrate
DNA	deoxyribonucleic acid
dn/dc	differential index of refraction/refractive index increment
DOE	design of experiments
DOS	degree of substitution
Dox	doxorubicin
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DS	dielectric spectroscopy
ECM	extracellular matrix
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EGDA	ethylene glycol diacrylate
EGDGE	ethylene glycol diglycidyl ether
EGDMA	ethylene glycol dimethacrylate
EWC	equilibrium water content
FITC	fluorescein isothiocyanate
GalNAc	*N*-acetylgalactosamine
GC	glycol chitosan
GMA	glycidyl methacrylate
GPC	gel permeation chromatography
HSA	human serum albumin
HSA-Cy3	cyanine 3 labelled human serum albumin
Hep	heparin

HPLC	high performance liquid chromatography
IDE	interdigitated electrode
IgG	immunoglobulin *G*
LUV	large unilamellar vesicle
MAA	methacrylic acid
MBAm	*N,N′*-methylenebisacrylamide
MEMS	microelectromechanical systems
mGC	methacrylated glycol chitosan
MIP	molecularly imprinted polymer
MLV	multilamellar vesicle
M_n	number average molecular weight
MPTS	methacryloxypropyltrimethoxysilane
MW	molecular weight
M_w	weight average molecular weight
MWCO	molecular weight cut-off
NHS	*N*-hydroxysuccinimide
NIP	non-imprinted polymer
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
PDA	photodiode array
PDS	photothermal deflection spectrometry
PDMS	polydimethylsiloxane
PEG	poly(ethylene glycol)
PET	photoinduced electron transfer
PPy	polypyrrole
QCM	quartz crystal microbalance
RAFT	reversible addition-fragmentation chain transfer
RIfS	reflectometric interference spectroscopy
RGD	arginine-glycine-aspartic acid
RT	room temperature
SDS	sodium dodecyl sulphate
SEM	scanning electron microscope
SiN	silicon nitride
SPR	surface plasmon resonance
SPW	surface plasmon wave
TCP	tissue culture plastic
TEM	tunnelling electron microscopy
TEMED	tetramethylethylenediamine
TIR	total internal reflection
TMS	trimethylsilyl chloride
TRIM	trimethylolpropanetrimethacrylate
UV	ultraviolet light

Contribution of authors

This thesis is presented in a traditional monograph style as written by the candidate, under the guidance and collaboration of the supervisor and advisory committee. It represents original scholarship in the investigation of a previously unexplored area of research. The contributions contained herein mark a notable addition to the field and provide insight into potential further exploration. The thesis covers experiments designed and executed by the candidate who was also responsible for data collection and analysis. The design, modification, and fabrication of the molecular imprinting systems were carried out by the candidate, as was the evaluation of imprinting effect under the various systems.

The information contained within this thesis represents several valuable contributions to the fields of biomaterial science and molecular imprinting which have potential for publication in the form of manuscripts which are currently in the process of preparation for submission to peer-reviewed journals. The results reported herein reflect a novel design for molecular imprinting research, as does the approaches and experimental design used to demonstrate the efficacy of the research.

1 Introduction

There is currently a growing interest in the scientific field in research into molecular imprinting – the creation of selective recognition sites in polymeric materials through the use of molecular templates. While the concept and basic chemistry have been well known for some time, more and more groups are beginning to turn to molecularly imprinted polymers for their versatility, ease of use, and customisability. Not only in the areas of separation for specialty chemicals, but in advanced sensors, pharmaceuticals, biochemistry, and medicine – there is a niche to be filled by the scope of imprinted polymers.

Even with numerous ongoing research studies being put forth in recent years, the state of the art of molecular imprinting has still not overcome a vital hurdle: achieving highly selective template recognition of biomacromolecules in an aqueous medium. Naturally, achieving the selective recognition imprinted polymers offer in a water-based environment is a critical aspect for extension of the technology into biomedical applications. Many factors act to hinder this success: a lack of electrostatic and hydrogen bonding available in polar solvents, more complicated chemistries of target molecules involved for interaction, matrices that swell and change conformation with environmental conditions, etc., but these are not necessarily insurmountable. With the right combination of materials and design, it should be possible to develop an aqueous imprinted system for biological use; one with high specificity – representing the so-called gold standard, ‘artificial antibodies.’

We proposed combining the proven biocompatibility of chitosan, the physiologic relevance of hydrogels, and the ‘memory’ of molecular imprinting to create a tissue scaffold material, that could preferentially recruit and bind a particular cell type, to selectively proliferate a desired phenotype within the artificial support. This would be accomplished with modified, water-soluble chitosan, photocrosslinked to form a stable imprint hydrogel. Concurrently, the developed construct should prove a valuable matrix to investigate the binding of smaller molecules, as an avenue for the

development of sensitive biosensors, or in their capture and release for drug delivery applications. In casting the chitosan hydrogel into a molecular imprinting matrix, it was our aim to demonstrate a proof-of-concept for aqueous molecular imprinting of biomolecules and establish a technique for creating the recognition matrix which could be further developed and modified for a wide range of potential applications.

1.1 Rationale and Motivation

The fields of tissue engineering and biosensors are greatly augmented by the use of 'smart' biomaterials. These materials are often finely-tuned to attain a specific interaction with other biomolecules, or to elicit a desired behaviour under certain conditions. They can even be engineered to respond in a desired way to specialised circumstances. Oftentimes, the development of these materials is very complex, and is thus time-consuming, costly, and requires a great deal of expertise. By contrast, the technique of molecular imprinting is simple, straightforward, of low cost, and robust. It offers a high degree of specific recognition towards targeted molecules, yet has been little exploited in the biomaterial field. The resiliency in chemical recognition and capturing of targets applied so often in the separation of stereochemical mixtures lends itself particularly well to the biorecognition which plays a key role in physical processes. In fact, molecularly imprinted polymers (MIPs) are often referred to as 'artificial antibodies' for their selective capability. Our research group saw potential to exploit this same recognition in the form of a polymer targeted specifically at biological applications. The aim of this thesis work was to investigate whether a MIP strategy could achieve the same recognition seen in standalone chemical screening to demonstrate potential preferential binding of a biomacromolecule, with the ultimate aim of forming a major component [possessing specialised recognition] of a more complex biomaterial system.

Owing to a structural and chemical similarity to tissue biomolecules, and with a proven history of strong biocompatible characteristics, the natural polysaccharide chitosan has been studied extensively in recent decades, and reports of its use continue

to grow at a rapid pace. It has been widely incorporated in many forms and polymeric systems, but has not been investigated to any substantial degree as a potential matrix for molecular imprinting. We proposed to use a chitosan-based hydrogel to evaluate whether its compelling biocompatibility could be paired with an ability for molecular recognition. If achieved, this would represent a substantial step forward in the fields of molecular imprinting and chitosan use. Successfully bridging these two areas would result in a material with enhanced versatility for tissue engineering applications as well as incorporation into a biosensor.

Using an hydrogel for the ultimate development of a MIP is not a typical approach for the field. Given that such materials hold two inherent properties which directly contradict the archetypal means by which MIPs attain recognition of target molecules, namely through strong hydrogen bonding and rigid conformation around templates, one does not expect that the results from investigations using a highly flexible and swellable matrix, done in the highly polar solvent of water, will reach easy success. With these factors taken into consideration, any improvement in observed recognition of a target as a result of molecular imprinting under these conditions would be seen as a positive, if unexpected, result. Because of the knowledge gained from such an investigation on molecular imprinting science, its importance on the overall field being as yet unstudied in this fashion, and the potential a molecularly imprinted chitosan hydrogel would hold in further applications, the thesis research was undertaken as a scientific study with full acknowledgement of the inherent difficulties it would involve. Even poor or negative results yielded from this proposed study would be seen as contributing to the overall knowledge of molecular imprinting, and could assist to lay context for improving future studies along similar lines.

Using the framework from an earlier developed strategy,[1] a water-soluble glycolated chitosan was modified to form a photocrosslinkable polymer. The applicability of this material for molecular imprinting was then to be evaluated by crosslinking in the presence of several model templates, covering both smaller and macromolecular size ranges, and evaluating the material's ability to subsequently

selectively re-bind these molecules. To demonstrate the material's versatility, we sought to examine it as a MIP in the form of a bulk hydrogel, thin layer surface, and micrometer-ordered spheres.

A major projected contribution of this research is the successful molecular imprinting in an aqueous environment, together with the examination methods used to verify this achievement. Because the vast majority of technical applications of MIPs exploit template recognition accomplished through hydrogen bonding, the field largely involves selective recognition done in organic solvents. However, attaining this same recognition in water-based media is far more biologically relevant to the goals of both biosensors and biomaterials engineering. A small number of studies have been made with aqueous-soluble polymers, but still the recognition remains inferior compared to that of organic-based MIPs, and applications thereof have been generally limited to chemical separations.

It was envisioned that a MIP created from such a modified hydrogel would present pluripotential function. One of these ultimate ambitions would be to use the chitosan imprint for the purposes of tissue engineering. Through the use of cellular epitopes, the controlled attachment and growth of a specific cell type could be achieved. This is an important factor, since in many biological environments one has to contend with the presence of a multitude of cells wherein only a specifically desired cell should be preferentially 'recruited' out of the mixture. By targeting a substrate to this cell type, one effectively creates a tissue engineering matrix of specifically discriminate growth surface. This can equally apply to the imprinting of particular target molecules into the matrix, which could be selected from such chemicals as growth factors or proteins which mitigate the organisational processes of the cells along desired lines. Were the matrix imprinted with these templates, they could either be easily recaptured to impregnate the growth matrix and delivered to the environment from the hydrogel to support biological development, or could be preferentially 'collected' directly out of the surrounding media from concentrations naturally present, thereby increasing local exposure of the cells to these desired molecules.

1.2 Hypotheses and Objectives

This thesis set out to determine if it was possible to use a strategy of molecular imprinting together with the production of a chitosan hydrogel to improve its function as a biomaterial. The use of chitosan in this fashion remains as yet unexplored in the area of research, and it was conjectured that modifying an already proven biomaterial in this fashion could present a novel step in the development of a 'smart' biomaterial.

A primary goal was to examine whether chitosan could be used as a base polymer for a molecular imprinting approach in an aqueous medium. If demonstrated successfully, this would mark a significant achievement for both molecular imprinting in polar solvents, as well as the specific use of chitosan in imprinting science. It was our presumption that chitosan would form an ideal candidate material for this process, owing to its many functional groups which would allow significant interaction with template molecules (and potential tailoring thereof by targeted chemistries) and its demonstrated versatility in forming stable, crosslinked networks.

Insofar as achieving highly successful molecular imprinting within a water-based system has largely eluded researchers, this study aims to form a cursory report on this ability using model templates. Aqueous MIPs are a foremost topic of the current imprinting research and bridging the gap of selective recognition from organic to polar solvents provides a principal target for many biomedical applications. Part of this objective also entails the use of non-specific biomacromolecules, to validate the goal of molecular imprinting with complex targets, relevant to scientists in a biomedical setting.

It was also foreseen that, as the microenvironment inside the hydrogel near the imprint sites is changed during binding and release events, this effect could be related to a discrete signal change. The intent would be to determine and resolve the degree of capture by the polymer matrix. Using sensitive instrumentation, measurements of the molecular imprint would create a novel biosensor, capable of detecting the presence of target analytes in surrounding media. Thus a secondary objective was to reveal the

capabilities of this developed material as a biosensor. The principle of sensitive, specific recognition and detection is of great import across many fields, and has only been investigated to a small degree using MIPs.

Lastly, a tertiary goal of this project was to establish the versatile nature of the material being used. By demonstrating the imprinting effect in various systems and configurations, it was anticipated that the use of a modified chitosan hydrogel for molecular imprinting could be envisioned as extending to many other applications which could exploit the chemical changes imparted by molecular imprinting presented herein, and ultimately acting as a potential improvement and enhanced property to be added to biomaterials in combination together with other biomedical strategies. Our objective is to show that other biomedically-relevant materials can similarly be considered in molecular imprinting strategies, to open up the field beyond the traditional polymers to which it has historically been limited. Outside of the targets defined herein, the use of a chitosan hydrogel in this fashion validates the ease with which the process can be tailored depending on the needs and aims of a given experiment, for multipurpose study.

2 Literature Review

2.1 Background of Molecular Imprinting

2.1.1 Principle Theory

During the 1960s and 70s, in a period of rapid growth in the combined fields of chemistry and material science, Klaus Mosbach began his pioneering work on secondary metabolism at Lund University. It was there he began investigating enzyme immobilisation and entrapment,[2, 3] wherein he saw the possibility of applying chemical methods to attain this same specific recognition demonstrated in nature. Specifically, Mosbach and colleagues theorised that polymeric materials, by way of their dense network formations, could be used to create substrate-selective receptors.[4] The authors termed this approach of "molecular imprinting"[5] after the ability to take a target molecule, and, through polymerisation, create a site that was complementary to this compound in the surrounding matrix. At nearly the same time, Wulff presented some groundbreaking data on work constructing enzyme models using synthetic polymers at a meeting for academic and industrial polymer scientists in 1972.[6] This had a profound effect in the scientific community, as it proposed something hitherto thought impossible – artificially achieving the selective and specific recognition seen in natural biology – which sparked much debate amongst science researchers, and planted the seed for the beginning of work in the molecular imprinting field.

It has been suggested that Polyakov was the first person to realise the synthetic recognition of polymers, in a 1931 report which showed silica gel could be made having specialised adsorptive properties.[7] This ability provides the keystone of molecular imprinting. The hypothesis behind molecular imprinting theory is that when an ancillary molecule is added into a mixture of monomers prior to polymerisation, natural diffusive forces, Brownian motion, and a self-assembling reaction will cause the normal polymeric constituents to form around the added material, surrounding it, and entrap it inside as the network forms during the polymerisation reaction. If the monomers and/or the guest molecule possess particular functionalities that cause them

to interact with one another, such as by non-covalent forces, then this self-organisation will be of a specific arrangement according to these forces. If the enclosed guest molecule is then subsequently removed from the casting material, an impression will remain in the polymeric matrix originating from the template that was present. This is not unlike common moulding techniques, to the extent one is using the inverse 'impression' created by an 'original' to later re-create that same article, only in this situation it concerns design on an ångström scale, with the moulded cavities formed being the size of the individual source molecules. These cavities, or imprint sites, are then used to match up again with all or part of the molecule that formed then. Like physical moulding, the process of molecular imprinting should yield an exact inverted duplicate, thus only the specific molecule used as a template will be affected as a target. As in the lock-and-key metaphor used to describe antibody binding models, only the particular target will bind precisely to the matching molecular imprint. This process is illustrated schematically in Figure 2-1.

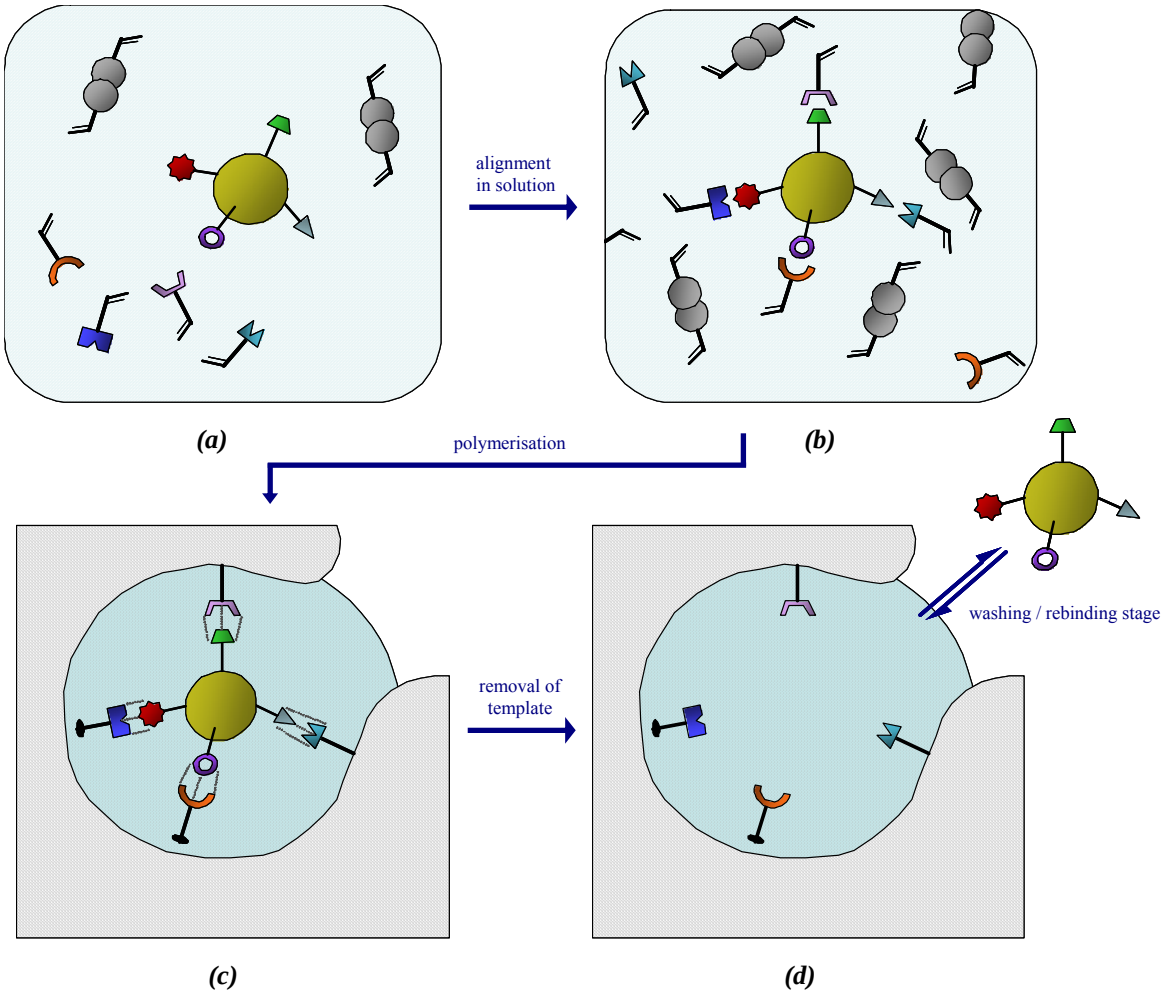


Figure 2-1. Interaction mechanisms and formation of a non-covalent molecularly imprinted polymer. (a) Preparation mix of template with functional monomers and crosslinker in solution, (b) pre-polymerisation organisation (following random Brownian motion) of monomers/crosslinkers as a result of attractive forces, (c) polymerised matrix, encompassing specifically-oriented template according to functional group interactions, and (d) leaching/capturing of target molecule from bulk polymer, maintaining shape of cavity and active regions of interaction complementary to target – the molecular imprint site.

The key to the molecular imprinting approach lies in the fact that, following template removal, the cavity vacated by the imprinted molecule will be a directly matching pattern of this model, and thus theoretically only an exact match for the template will fit precisely into the imprint location.[8] Because of inter- and intra-molecular interactions and the monomer encompassing the template, the vacated site in the polymer would posses a chemically organised surface structure and molecular size and shape fully complementary to the impressed template. In this way, one could theoretically use artificial means to attain the same selective binding as we see with

highly specific biological counterparts; hence the oft-employed term for molecularly imprinted polymers (MIPs) as 'artificial antibodies.'

The advantage of using MIPs is that one can achieve highly specific recognition of target molecules by means which are fully synthetic. Formed from standard polymers and chemical reagents, MIP substrates do not suffer the same sensitivity to environmental factors and conditions as can negatively impact the use of antibodies, making them simple to produce and robust and stable for wide application. Whereas MIPs are synthesised from batch monomers, knowledge of chemistry and polymerisation methods allows them to be specially designed in an as-needed basis to be as specific or as general in targeting as is desired. Polymer chemistries are very familiar to scientists; thus, formation rates of MIPs can be rigidly controlled and interactive imprinted structures designed according to known synthetic strategies. Recent advances in the ease of self-assembled monolayers[9] and the regularity of dendrimer building techniques[10] and supramolecular assembly[11] have been manipulated to assemble molecularly imprinted constructions through advanced design.

2.1.2 Methods for Molecular Imprinting

Molecular imprinting is necessarily dependant upon achieving molecular recognition of the species through the creation of a complementary binding site in a macromolecular structure (e.g. polymer) which is particular to the template molecule. Thus, a natural evolution of the molecular imprinting field is to modify the polymerisation methods in such a way that the polymer network forms a particularly interactive medium about the template. This is often achieved by careful selection of the monomers from ones which bear chemical groups that should show some affinity to functional areas of the template molecule. When employed, the particular interaction of the monomers with the matched functionalities of the template molecule results in a highly specific spatial arrangement, which is then maintained during the crosslinking stage. Thus, with appropriate chemical design, one can obtain a highly ordered structure in the imprinted polymer for a given target molecule. The other primary method of heightening the recognition for a MIP is

to additionally use crosslinkers during polymerisation to construct a dense environmental arrangement. Because molecular imprinting relies heavily on the interaction between the monomers and the target, a tighter polymeric network implies a closer degree of bonding, and potential for a greater number of interaction sites between the two materials. Once the template molecule is successfully separated from the complex, the resultant organisation will possess a complementarity of the target in both spatial hierarchy and chemical group functionality.

In the majority of molecular imprinting cases the interaction between monomers and template involves some form of non-covalent binding between the target molecule and corresponding functional groups in the monomers, e.g. hydrogen bonds, electrostatic attraction of ions, hydrophobic, or van der Waals forces; occasionally metal-ion complexation may also be drawn upon.[12] In these situations, the template molecule – being weakly held in place by the employed interactions – is not fully bound to the surrounding polymer matrix, which makes the removal process easier. The non-covalent approach for MIPs was initially put forth by Andersson *et al*,[13] and since this time most studies of molecular imprinting have been investigated using similar strategies.

Achieving the interactive coordination of a target template with polymerisable monomers is not a trivial task; sometimes weak association in the pre-polymer mix may not be sufficient as a driving force to attain the highly selective sites desired.[14] Further rational design is then needed to prepare the pre-organised structure of the MIP. Under certain circumstances, such as where no interaction of monomers with the template can be achieved due to their set chemical structure or environmental conditions of the reaction, or when the desired polymer does not support these properties, direct covalent binding of the template molecule can be used during formation of the pre-polymerisation macrostructure. This is described as covalent molecular imprinting, and while more chemically complex than non-covalent schemes, is often used for the much improved homogeneity obtained in the resulting MIP. The covalent approach to molecular imprinting was pioneered by Gűnter Wulff,[15, 16] and offers improved binding

over non-covalent methods, at the cost of ease of reversibility.[17] In many typical covalent imprinted polymers, well-established chemistries are used to control the targeted bonds holding the template in place, such as the hydrolysis of ester groups.[18] For the recognition of the target to take place, the reaction should be reversible and stable. An example of one method to exploit this covalent imprint capture is shown in Figure 2-2, wherein a boronic acid group – which bonds strongly to diol moieties – reacts to form the boronate ester in a pH-dependent fashion.[19]

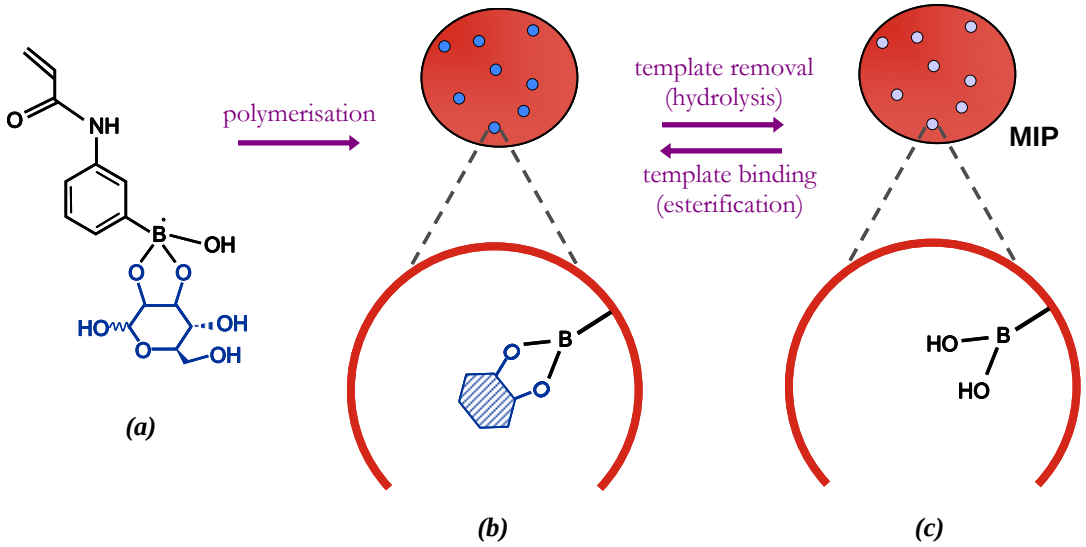


Figure 2-2. Example of covalent approach to molecular imprinting. Following polymerisation of (a) a modified acryl-amidophenylboronic acid monomer (APBA with mannose template attached), (b) a covalent MIP is formed with entrapped template. Through selective adjustment of pH in aqueous media, the boronate ester group is converted to (c) a boronic acid, and can reversibly react with diol moieties in the mannose target to recapture the template at the imprint site. (adapted from Shen *et al.*)[19]

Very few templates are available that confer a fully reversible and efficient covalent binding strategy; however, in cases where the precise control of template positioning and distribution is required, it can be combined with non-covalent re-binding of the target (faster kinetics but lower selectivity). In such a case, the imprinted polymer is formed per typical routine of a covalent MIP, but the template is removed through permanent cleavage of the bond, and is then subsequently recaptured by the MIP via non-covalent interactions. This is often described as the 'semi-covalent' technique.[20] Sellergen and Andersson first applied this merged methodology of molecular imprinting in 1990.[21] Whitcombe *et al.* elucidated the technique further

making use of sacrificial spacers between the template and matrix.[22, 23] Several methodologies for this approach have been subsequently compiled by Alexander *et al*.[24]

Monomer selection is a critical part of MIP design. As the chemistry for achieving selective recognition of the target is reliant on establishing a coordination of the template-polymer complex, monomers must be chosen that will possess some kind of interactive force to make this formation thermodynamically favourable over random alignment in the pre-polymerisation solution. Fortunately, the range of chemical families and functionalities that can be used in this respect is vast, and with the current knowledge researchers can easily turn to established protocols for design and synthesis methods. For specific applications, monomers can be modified as-needed or synthesised from the ground up to suit a desired interaction. Specific chiral monomers can be used to further improve the specialised recognition capability of MIPs.[25]

2.1.3 Template Recognition

The coordination of the polymer network with the template molecule can be modeled as host-guest interaction. This reciprocal pairing is largely based upon the functional groups present in the target having a specific arrangement which can be selectively discerned by the MIP. Typically, the most effectively differentiated molecules are relatively small, e.g. chemical reagents, pharmaceutical analytes, etc., and possess several functional groups. When the imprinted matrix has a strong matching quality to the template, very small differences in molecular arrangements and individual bond orientations can be discriminated.[26] For research into molecular imprinting, there are certain model molecules which can exemplify this difference, and these are often used to test template recognition and selectivity. Examples of these templates are shown in Figure 2-3.

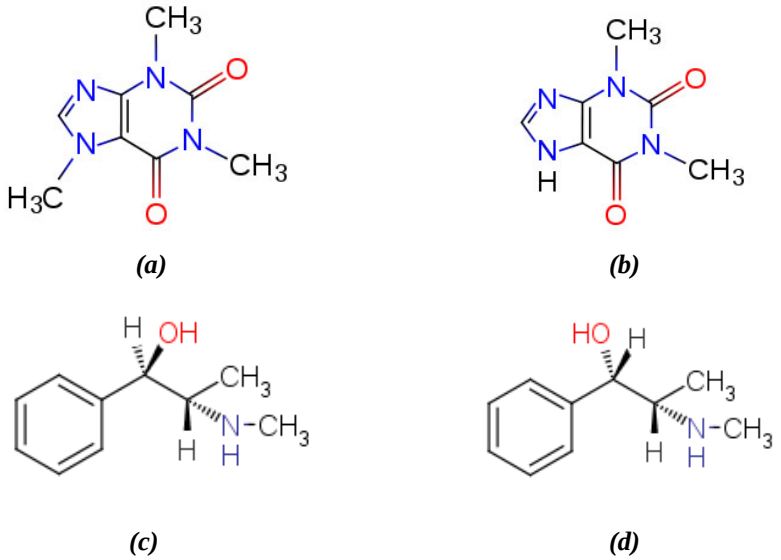


Figure 2-3. Common templates used as binding selectivity models in molecular imprinting studies. Structural analogues (a) caffeine and (b) and theophylline, and diasteriomers (c) ephedrine and (d) pseudoephedrine. A MIP's efficacy is often defined by its ability to differentiate such forms of closely-related compounds.

The ability to selectively recognise particular chemical structures, and even isolate individual stereoisomers from racemic mixtures, is of great interest in the scientific and medical fields. For instance, molecularly imprinted polymers could be used to isolate an active eutomer from the less-effective distomer,[27] which lends itself to particularly useful purposes in drug delivery and pharmaceutical applications.[28, 29] The extracting ability of MIPs offers many purposes in sample preparation for analytics.[30] Feed streams of desired chemicals, like specialised drugs, can be enriched for the specific effective form of interest.[31] It can be used to apply specific binding capability in the development of membranes.[32] A particular recognition for a target biomolecule can be used to enhance the functionality of materials in biomedical solutions.[33] In that MIPs mimic the selectivity of natural antibodies and enzymes, they can be used to design catalysts for transition state stabilisation, a vital part of control mechanics in chemical reactions.[34] There is also the potential to use MIPs as 'molecular screens' to filter out biological fluids and allow identification of minor fraction components.[35]

The most commercial success with MIPs, however, has been restricted to two primary areas. A large proportion of the work with using imprinted polymers, and the

principal marketed application thereof, has been for chromatographic purposes, such as in the separation of racemates[36] and isolation and screening of chemical libraries.[37, 38] Nearly all of these applications are based on solid phase extraction using MIPs.[39] A second customary technique to make use of the imprinted recognition is in chemical detection and sensing.[40, 41] As the demand for rapid biosensors intensifies, MIPs can be used for biomolecules sensitive assays, such as DNA detection.[42] Because of the ease of manufacture and stability involved with MIPs, they can offer considerable advantages over other biological analytical techniques, such as with enzymes or antibodies;[31, 43] still, precise specificity has yet to be realised.[44] While many companies have made use of MIP technology, currently few offer true solutions through imprinted polymer products which reach these recognition goals.[45] They remain, nonetheless, a promising area of research.

A strong advantage of MIPs is their ability to repeatably bind a target molecule of interest. As potentially regeneratable substrates that can be used for multiple applications and remain stable with time, they offer considerable benefit as selective media. The imprint sites created during polymerisation are theoretically permanent, and under the same conditions should allow a continual removal and re-capture of the respective target molecule. The MIP can be regenerated by the physical force of washing out the template from the imprint matrix, or through the addition of a buffer to disrupt the weak non-covalent bonds holding it in place. It is also possible to use 'responsive' polymers or templates that can be released by means of changes in pH,[46] temperature,[47] or light.[48] Intelligent gels have been designed by Miyata *et al*. that demonstrate responsive behaviour to the presence of specific target glycoproteins.[49] Win *et al*. have used a thermoresponsive coating of poly(*N*-isopropylacrylamide) over acrylic MIP beads to act as a 'gate' to control the binding/release of lysosyme.[50] When using such reversible polymer mechanics, it becomes necessary to correctly choose suitable monomers that allow the spatial arrangement to return following a phase transition.[51] This type of on/off systematic binding can be very useful to scientists. It can allow for precisely controlled capture and delivery of a template by simple

alteration of the environment and target release to certain areas of the body/occurrence of biological conditions as needed.

2.1.4 State of the Art and Areas of Improvement

2.1.4.1 General Challenges to Imprinting Efficacy

One of the key weak points with molecular imprinting technology that as yet inhibits it from attaining the same praise and widespread adoption as natural antibodies is that the recognition sites created through imprinting are not uniform throughout the material.[52] The binding sites that result from the polymerisation around the template depend on many factors; and as these elements are not fully uniform in the entirety of the pre-polymer complex and cannot be maintained thusly throughout the crosslinking stage - as it is temporal and spatial process - the recognition will not be the same at each site wherein the template is trapped.

General imprinted polymers will possess a structural heterogeneity, with monomers being aligned 'ideally' in optimum arrangement to the template in some locales, 'partially' aligned in an interacting fashion at other sites, and having little to no complementary shape or functionality in further areas.[53] This results in a heterogeneous distribution of binding sites (and affinity constants) ranging from those of very high affinity to ones of only low recognition.[54, 55] Part of this is as a consequence of the fact that it is common practice to employ a very high concentration of monomer in constructing MIPs, so as to achieve the strong complexation with the template for subsequent binding recognition. As a result, a significant proportion of the excess monomer present will not be involved in forming monomer-template complexes and hence will not produce target binding sites.[56, 57, 58] While monomer aggregation lowers the overall binding capacity of a MIP, under certain circumstances it can be exploited to remove sites of low affinity in non-imprinted polymers (NIPs) as well, which lowers non-specific binding to controls.[59] Moreover, it is also a common procedure to make use of a great excess of template in order to reach a significant degree of imprinting effect.[35] This is also true for applications of capture and release, wherein it is typical to

see a much lower amount of cumulative release compared to the total amount bound initially.[60] However, in addition to eliciting molecular aggregation, this can also lead to an higher distribution of binding sites, and high template concentrations have been seen to lower the selectivity of imprinted polymers.[61] Even without self-aggregation, the presence of the template itself during polymerisation will have some effect,[62] a fact often not accounted for in normal comparisons done against NIP controls. Its effect on the constituents and the polymerisation reaction outside of simple alignment of the matrix cannot be completely negated. Thus, the complex nature of the formulation and pre-polymerisation complex connote that manipulations done to the components will have a profound impact on the recognition – and, hence, effectiveness - of the MIP.

A typical distribution of binding site affinities found in molecularly imprinted polymers is shown in Figure 2-4. This non-uniform dispersal of characteristic binding explains why highly selective recognition is often reported for low concentrations of analyte – at those levels wherein only the high affinity sites are occupied by the template. This can result in a false over-estimate of specific binding in the MIP. However, as analyte concentration increases, both high- and low-affinity sites begin to capture the target. At high levels of analyte, the observed binding properties will be determined largely by the more numerous low-affinity, low-selectivity binding sites. Thus, when using high concentrations of target in solution for analyses, although apparent capacity of the matrix is increased in the MIP, it is at a cost of specificity. Although the technology continues to improve, the distribution of binding sites in most MIPs remains strongly weighted towards those possessing non-specific low-affinity.

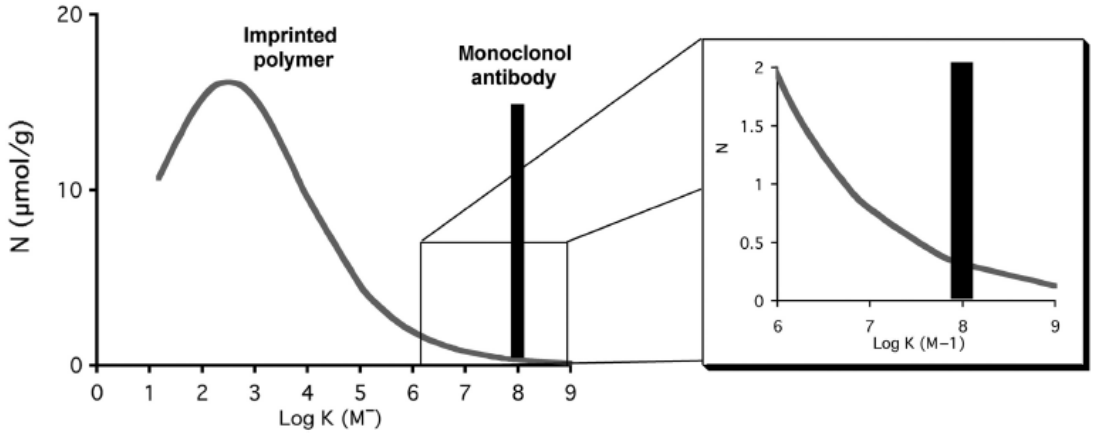


Figure 2-4. Typical heterogeneous distribution of imprint site binding affinities in MIPs, contrasted with comparable binding constants for monoclonal antibodies. Number of binding sites, *N*, having association constant, *K*, as determined using fitting parameters of the Langmuir-Freundlich isotherm equations. (Reprinted from Wu *et al*.[53] as adapted from Umpleby *et al*.[55])

Part of the difficulty in achieving strong, uniform specificity with molecular imprinting arises in that most MIP formulations being reported on in the literature still rely largely on 'blind box' self-assembly of the pre-polymerisation components. Few researchers have explored the interactive system on a molecular level, and as most of the components are added to a solvent for crosslinking in bulk, it remains an unknown variable in formulating MIPs. This is especially important in working with larger biomolecules that posses more molecular intricacies and multiple points of interaction.[63, 64] Progress in computer technology and molecular modeling is one method of predicting the synthesis,[65] bond formation,[66, 67] cavity selectivity,[68] and ligand-binding[69] behaviour via various models,[70] but still remains largely theoretical.[71] The calculated recognition of imprinting effect does not always match the empirical results,[72] but it can nevertheless provide a useful tool for simulation[73] and optimisation[74] of polymer systems. A thorough review of the rational design of molecularly imprinted polymers has been published by Nicholls *et al*.[75]

The complexity of components in a bulk nature for imprint polymerisation creates its own inherent intricacies in MIP valuations. Self-aggregation of the template molecule is also a problem in many systems that is not easily taken into account, and which may be a particular problem in aqueous solvents.[76] Monomer and crosslinker dimerisation is another strong possibility that may occur in bulk systems yet is often

overlooked, and may lead to poor templating efficiency and heterogeneity of recognition sites.[77] The solvent itself, as it constitutes a major portion of the system and interacts with each of the components in turn, as well as providing the means to support the bond formation between network and template molecule, will therefore significantly affect the outcome of the MIP. And yet, many authors place little consideration towards its effects in studies of imprint recognition, outside of merely acting as a porogen for the MIP matrix.[78] In any thoroughly characterised model, the actions of the solvent must be accounted for in determining imprinting effect.[79] Its role is not well understood, particularly when it comes to its influence on the bonding strength for non-covalent MIPs. The solvent's presence thus acts as a point of certain import to regard during the creation and evaluation of MIP techniques.

2.1.4.2 Imprinting in Aqueous Solvents

The great challenge currently occupying much of the contemporary body of molecular imprinting research is realising the specific recognition of MIPs within an aqueous system.[39] As mentioned, the solvent plays a major role in constituting the pre-polymerisation matrix of an imprinted polymer and can have a strong influence on its resulting properties. Polar solvents such as water will inherently disrupt the hydrogen bonds customarily exploited in the creation of most non-covalent MIP constructs – responsible for maintaining the interaction between the template and monomers during polymerisation, as well as the target molecule in subsequent binding studies – and therefore this can severely disrupt the imprint selectivity.[52] Thus, the template recognition must rely on other; often non-covalent, bonds forming in the imprint system, and these are frequently more difficult to achieve and maintain. Nonetheless, a great majority of chemical and biomedical applications involve molecular targets soluble in water, especially for biologically relevant compounds, such as amino acids and therapeutics. Furthermore, a vast number would require functionality of the MIP in an aqueous medium – for target molecule recognition. To begin to approach the replacement of antibodies by their 'plastic' counterparts, we need to overcome the hurdle faced with specific and selective binding in water-based environments. As a

result, investigators must look to the manipulation of other non-covalent bonds, such as electrostatic and π-stacking interactions, to achieve recognition in aqueous media.[80] Some reports of successful chiral selectively and strong target resolution in aqueous solvents have been made, but these are generally limited to the recognition of small molecular templates.[81]

2.1.4.3 Biomedical Application of MIPs

Despite the difficulties in achieving significant recognition with molecularly imprinted polymers, it remains an area of strong scientific interest for its potential to replace the current dependencies on biological systems. Unlike antibodies and enzymes, MIPs offer considerable advantages that make them attractive as a selective medium. Allowing for their polymeric origins, these materials have the potential to be stable in physical and chemical conditions that would normally denature proteins. They are much more resilient against fluctuations of pH, temperature, and pressure, and maintain their structure and composition when placed under physical stresses. These are central properties when considering the requirements of successful biomaterials. It also allows for MIPs to be stored and re-used long term, an important aspect of effectiveness and efficiency for any biomedical device. Some reports suggest selectivity in MIP gels can even be maintained and recovered after freeze-drying.[82]

By way of their formation, MIPs are relatively simple to produce, requiring little user expertise or complex steps of manufacture and purification, a prodigious advantage over their natural counterparts. They can be prepared artlessly in most laboratory situations; the polymers used are generally available and of low cost. When very specialised needs are involved, polymers can be synthesised in a much simpler (and more precise) fashion via known chemistries, and used to fabricate the MIP, in stark contrast to the prominent difficulty of this faced with their biological equivalents. Perhaps most importantly of all, MIPs can be tailored with a great degree of freedom to attain differing degrees of selectively based upon the desired application. With a simple tuning of groups that can interact with the target on the polymer backbone – reactions

well-established in synthetic chemistry – and circumstantial choice of crosslinkers, systems can easily be fine tuned to a specific chemical target, just as common 'recipes' can be used for a family of compounds. This facilitates and improves considerably marketable production, in both versatility and ease of synthesis. It also enables the creation of theoretical MIP 'kits,' wherein scientists could use a starting base polymer, and a selection from a choice of multiple monomers chosen for different functionalities particular to their needs. Some assays have been proposed applying solid phase extraction via MIPs in 96-well plates, which could eventually lead to high-throughput analyses, though currently these remain in development stages.[83]

Interestingly, while most studies of molecular imprinting seek to achieve highly specific recognition of a single target, it is worth remarking that the same principles can be equally exploited to bind a particular series or family of molecules, which may not be necessarily structurally related to the templating compound, but which possess functionality complementary to the imprinted polymer sites. This can be simpler to accomplish, and advantageous when the targets of interest cover a range of molecules that share some common structural element (e.g. a chemical group, amino acid sequence, or oligomer). This multiple targeting of a general group of related chemicals is akin to polyclonal immunoreaction against antigens. Dubey *et al*. demonstrated this using imprinted substrates with functional groups at specific *ortho*-, *meta*- and *para*-orientations, for the selective capture of varying phenolic isomers.[84] While not at the state of achieving the full recognition of antibodies, the parallel development of MIP sorbents is being continually cultivated as an alternative.[85]

2.1.4.4 Assessment and Comparison of Imprint Systems

A primary concern of the field is how to appropriately evaluate any recognition towards the template molecule that is imparted as a result of molecular imprinting. As 'imprinting effect' is a generally undefined property, its measurement in a quantitative sense remains a topic of debate. In typical investigations, molecular recognition is gauged through either the amount of template recaptured by the MIP from a feed source

and its subsequent release from the polymer, or indirectly, by the change in its concentration of the surrounding medium. Unfortunately, neither of these may be an accurate representation of the true affinity of the polymeric matrix towards the target of interest. The changes resultant in any MIP are by nature intrinsic, but cannot be easily determined at an atomic level. This becomes further convoluted by the sensitivity to the technique and conditions at which subsequent evaluations are undertaken. It must also be taken into account that the presence and/or absence of the template itself may have an effect on the polymerisation, beyond creating recognition sites in the matrix. It has been shown that at as much as a macroscopic level, the presence of a template can alter the structural characteristics of imprinted hydrogels.[86] Furthermore, design of an appropriate control substrate is important in any evaluation. As discussed by Baggiani *et al*., using a simple non-interactive base material for a non-imprinted polymer (NIP) comparison (as is the typical approach) may not be considered a strong correlation to reflect the improved affinity of a MIP.[87]

The lack of an agreed upon method that can be used to precisely measure molecular imprinting with a level of repeatability and across an array of different component systems has lead to a degree of stagnation in the development of MIPs, and raises a difficulty in undertaking the comparative assessment of various studies.[88] However, lacking any congruent method of determination, a characteristic assessment can be made evaluating the imprinted polymer versus a control non-imprinted polymer (i.e. made without any template molecule) formed under as close conditions as can be realistically achieved. Negating any contributions from the template during crosslinking, outside of responsive arrangement of the expected pre-polymerisation complex, a comparison of the two polymer forms can give an 'effective' degree of molecular imprinting.

As the molecular imprinting technique has been strongly developed within the field of chromatography, many of the analytical methods used for describing the recognition behaviour are related to the binding capacity of the MIP. Typical methods of examining the binding behaviour include Scatchard plot analysis or binding

isotherms (e.g. Freundlich and Langmuir-Freundlich), both of which are useful for characterising the heterogeneity of binding models having two association constants arising from high- and low-affinity binding sites in MIPs.[89] These affinities are sometimes compared in what is known as an 'imprinting factor,' α, as a relation between binding constants for the MIP and the NIP.

$$\alpha = \frac{k_{MIP}}{k_{NIP}} \qquad \textbf{[2-1]}$$

Whereas MIPs have become ubiquitous for HPLC applications, in terms of the language of typical chromatographic separation, α is the separation factor and k is often defined in terms of retention factor,[90]

$$k = \left(t_R - t_0\right)/t_0 \qquad \textbf{[2-2]}$$

where t_R and t_0 are the retention times of retained and unretained solutes, respectively. The imprint factor (IF) is also describable in terms of overall binding capacities,

$$\alpha = \frac{Q_{\max,MIP}}{Q_{\max,NIP}} \qquad \textbf{[2-3]}$$

with $Q_{\max}$ being the maximum binding value corresponding to the respective polymer formulation, given by

$$Q = \left(C_i - C_f\right) V\ m^{-1} \qquad \textbf{[2-4]}$$

where C_i and C_f are the initial and final concentration of free analyte (in mass per volume units), V is the volume and m the mass of the polymer.[91] As the imprint factor can be considered a ratio of the specific to non-specific binding sites, it is often also referred to as the specificity of a MIP.

The second key quantitative relationship of molecular imprinting is the recognition of the imprint matrix towards its particular template versus other similar

molecular analogue compounds; in other words, a measure of its ability [in binding] to differentiate the target from other analytes. This can be called the selectivity of a MIP matrix, and is expressible as a ratio in terms of the imprinted binding for the target/template molecule to that of a competitive one:

$$\beta = \frac{\alpha_{template}}{\alpha_{analyte}} \qquad \textbf{[2-5]}$$

Advances in technology have allowed some investigation of the complexation events between template and MIP directly, such as molecular tracking through fluorescence,[92, 93] radiolabelling,[94] or ^{1}H NMR titration experiments.[95] Binding assays can be developed to study the adsorption isotherms in MIPs in methods atypical for chromatography. In certain cases, where chemical modification allows, detection of bound target can be linked to the generation of a signal resultant from the MIP-target complex.[40] This can be particularly useful for the development of sensing apparatuses. A common technique to determine selectivity in MIPs is to use dose-response curves, similar to standard immuno assays, to assess the amount of non-labelled ligand required to displace 50% of the template species from the polymers.[52] One must address that certain considerations must be made when using such models towards expressing the affinity in a reliable and applicable sense.[96] These methods notwithstanding, the vast majority of molecular imprinted polymers are defined according the specificity (α) and selectivity (β) obtained from empirically indirect measurements.

Despite the extensive study in molecular imprinting, little is known about the chemical processes leading up to the creation of the target binding cavities, including the interactions of different bond types and the role of the solvent in this arrangement. The chemistry of the pre-polymerisation complex remains largely theoretical, and confined to predictive modeling. It is also rare that comprehensive investigations are done following MIP formation to thoroughly characterise the template sites.[97] For these reasons, it has proven difficult to optimise the reaction conditions during imprinting to achieve the same degree of recognition seen biologically in the immuno response or enzyme catalysis. And although most successful applications with imprinted polymers

have been carried out in organic solvents, achieving an MIP highly functional in aqueous medium remains a foremost goal of research.[98]

2.2 Imprinting of Macromolecules

A major hurdle in ongoing imprinting research is the successful templating of larger biomolecules, such as proteins. Macromolecules are becoming more and more central to the core research of bioscience and medicine, as we become better at elucidating their structure and function, and proficient in the design and synthesis of custom molecules. Because the recognition of such molecules plays such a fundamental role in biology, and given that they are equally important in matters of detection, treatment, and diagnostic applications in medicine, it is unsurprising that they have now become a target of molecular imprinting research. The preparation of synthetic, robust, macromolecular networks with biomimetic molecular imprinting is a key issue of pursuit.[99] The designed complexation and recognition mechanisms towards biological targets is expected to be a new frontier in diagnostics and treatment. Through both organic and inorganic means, researchers continue to work on the adaptation of synthetic imprinting methods to achieve the selective recognition of proteins.[100] A recent comprehensive review of macromolecular imprinting by Kryscio and Peppas provides a detailed background of the current state of protein MIPs.[101]

2.2.1 Protein Imprinting

While rigid molecules and small sequences of amino acids have been imprinted in polymers with some positive results, achieving MIP recognition towards proteins still continues to be an obstacle in the unity of the process.[102] Part of this problem arises from the increased molecular size of proteins. This poses a challenge for obtaining selective recognition, through greater steric hindrance and molecular diffusivity to their larger size and increased steric effects within a polymeric matrix. Both when trying to remove the entrapped template molecules, and during subsequent rebinding events, their considerable molecular dimension creates an impediment to the easy capture and

realignment at the imprint site. This is further complicated by the sensitive and complex nature of proteins. As proteins have such an intricate hierarchal structure, they are easily denatured by even minor conformational changes in their 3D geometry, as a result of slight fluctuation in environmental factors such as pH, temperature, or ionic strength.[212] This transformation makes achieving a successful recognition site difficult, in that, not only are the shape and properties of the site convoluted, it must also be maintained precisely in a single alignment throughout the polymerisation process, and the template protein must be able to hold its 3D organisation throughout the reaction, as well as exactly matching this shape of the imprint cavity during rebinding procedures. When considering the changes that occur in local environments during polymerisation reactions (e.g. presence of free radicals, enthalpy changes, contraction of network chains, exposure to heat and/or light), maintaining protein structure is clearly not an inconsequential matter of minor concern. Add to this that the most common and effective methods of imprinting are done in organic solvents, under conditions which would denature most biological molecules, and a picture of some of the difficulty with protein imprinting begins to emerge.

Tan *et al*. have examined the effect of common imprinting conditions on protein structure and denaturation.[103] The polymerisation methods for MIPs can often involve excesses of heat or UV light to initiate the reaction, and one can perceive how a protein's native structure might not be maintained through the process under these effects. In this case, if just some of the tertiary or quaternary structure is lost, it could then make a positive recapture of the target impossible. Kryscio *et al*. have also used circular dichroism studies to reveal how the commonly employed monomers and crosslinking agents using to form MIPs can impact the conformational stability (disrupting the secondary structure) for macromolecular proteins.[104]

While increased functionality of a template generally suggests more interaction for molecular imprinting, the complexity of proteins can also create a problem in this regard. Ramstrom *et al*. have shown that in larger molecules, certain parts of their functionality may have weaker recognition than others, reducing the stereoselectivity of

the MIP.[26] Furthermore, as complete template removal is more difficult with macromolecules, the resulting binding sites, due to their increased size, are more susceptible to non-selective binding by smaller molecules that possess a part of the larger structure. However, if these concerns with protein MIPs are overcome, the imprinting may provide better recognition among large molecules, as the greater number of sites will reduce the binding of chemical analogues and competitors, and can even separate very specific enantiomers and amino acid sequences from one another.[105]

The measurement of binding with macromolecules also becomes more convoluted than in imprinting systems involving smaller templates. As with antibodies, a binding affinity is often described that reflects a measure of how strongly the template molecule is attracted to the imprint site. This can be modeled as a measure of how well a ligand is bound to a receptor macromolecule. We can define a dissociation constant, K_d, as a quantitative value of this attraction by receptor-ligand equilibrium theory. The formation of the macromolecular–ligand complex can be described by the following equilibrium expression:[99]

$$[M] + [L] \underset{k_r}{\overset{k_f}{\leftrightarrow}} [ML] \qquad \textbf{[2-6]}$$

where $[M]$ represents the concentration of macromolecular binding sites in the MIP network, $[L]$ is the concentration of ligand (template molecule), and $[ML]$ is the macromolecular complex of ligand-polymer. The forward (k_f) and reverse (k_r) rate constants for this equilibrium are used to define the equilibrium binding constant (of the ligand-polymer complex) or association constant, K_a, and the equilibrium dissociation constant, K_d:[99]

$$K_a = K_{complex} = \frac{k_f}{k_r} = \frac{1}{K_d} = \frac{[ML]}{[M][L]} \qquad \textbf{[2-7]}$$

Ligands with high K_a or low K_d will bind very closely to the receptors sites of an MIP, and have high affinities. Weak binding systems are evidenced by very low K_a values or high K_d.

2.2.2 Approaches in Current Research

As the imprinting of large macromolecules still remains a stumbling block with MIP technology, it has been the focus of much additional research in the literature. One proposed method around such difficulty is to use only partial imprinting of the protein, wherein, for example, a peptide sequence or region of the macromolecule is used as the template during polymerisation, and the resultant cavity in the MIP is used as a target for recognition of the whole protein through capture of this smaller sequence or domain in subsequent rebinding experiments.[212] This 'epitope' approach utilises association of a molecular fragment in a polypeptide to bind at a surface domain with the MIP receptor.[210] This is in much the same way as an antibody reacts to a specific site on its corresponding antigen, as opposed to being triggered by the entire molecule. Carter and Rimmer have described how the choice of polymer in this respect is not trivial in supporting protein stability. These authors have stated how particular polymers can be tuned to exhibit a bulk response as a result of physical stimulus, and thereby enhance protein recognition and cell attachment.[106]

Surface fixation is another technique that is attractive when attempting the imprinting of large biomolecules. Templates can be immobilised homogeneously on a surface via chemical bonding or through a sacrificial support.[107] One advantage is that this method permits the use and uniform coverage of insoluble templates. It assures proper 'coating' of the template molecule, and can achieve specific orientation of the target, or templating of a region of interest, but is then limited to two dimensional binding applications.[108] Surface fixation is also beneficial in that it can allow for controlled spacing of template molecules, which minimises aggregation, a concern especially important when working with proteins in hydrophilic media.[109] Liu *et al*. have reported on the oriented fixation of the enzyme methyl parathion hydrolase via genetic modification to add a cysteine peptide linker for increasing MIP homogeneity.[110]

To increase the amount of accessible binding sites for a limited volume of material, surface coatings of MIP can be used, which are, in effect, most effectively

described by two-dimensional (2D) systems on solid supports. Such a plan has been explored by Lu *et al*. on core-shell microbeads of polystyrene, but their results showed high pH-dependency and were not successful for all proteins tested (hemoglobin was recognised but not BSA).[111] Thin monolayers can be applied as imprinted sensor coatings to offer generous accessibility to imprint sites, and operate on partial templating recognition of biomarkers or cells.[9] Dechtrirat *et al*. have combined an immobilisation approach and epitope imprinting to analyses cytochrome *C* imprinting in thin MIP layers.[112]

One of the most novel of reports in recent years has come from Haupt *et al*., who developed a method of using a localised anchor point for ligand attachment, which then can form a seed point to create imprinted microgels. Due to their highly-oriented receptor sites, these MIPs were able to act as enzyme inhibitors towards the template molecule (trypsin), blocking activity following regionalised binding.[113] One of the lone studies involving direct biological assessment was reported by Rosselini *et al*.[114] Using molecular imprinting of a peptide sequence of the glycoprotein laminin, they formed functionalised acrylic particles which were then attached to a basal membrane of polyester. Imprinting of the template sequence led to recognition towards the extracellular matrix (ECM) protein, and the resultant material showed improved selectivity to the peptide and increased cell growth in seven day culture. The ongoing effort to develop artificial antibodies has not come without some success. Some MIPs have been reported to be able to distinguish between source type of a particular target protein, e.g. showing selectivity between bovine and human hemoglobin.[82] While most molecular imprinting research has been restricted to monomers with two vinyl groups, Kempe has shown a strong recognitive capacity for chiral recognition of amino acids using a stationary phase made using tri- and tetra-functional monomers as the crosslinking agent.[115] The ultimate development of fully artificial antibody mimics remains an objective and while some persuasive capacity for recognition has been shown, it remains on a larger scale than immuno reactions of biological counterparts[116] and not as sensitive as antibody-linked assays.[117]

Advances in materials science has also led to protein imprinted polymers being incorporated with many types of synthetic nanomaterials, particularly attractive for receptor/sensing applications.[118, 119] It can also enable formation of highly structured MIPs on a nanometre scale.[120] To accurately measure the binding energy and selectivity recognition of 2- and 3-D imprinted polymers, El Kirat *et al*. used an AFM tip modified with Cyt *C* template to probe the surfaces via force spectroscopy.[121] The synthetic technique of formulation for MIPs lends itself easily to the development of binding arrays for multiple targets; a powerful and attractive potential compared against similar protein adsorbents via other means.[122] Some research groups have worked on the development of MIP coatings for microplates, which would allow incorporation into other standard assay techniques and high-throughput sensing applications.[123, 124] Modern work on system miniaturisation and lab-on-chip designs has led to the exploration of MIPs as potent biosensors. Methods such as potentiometry have the ability to sensitively and rapidly detect selective adsorption of target molecules.[125]

While numerous studies in recent decades have increasingly investigated the use of protein-MIPs, the templates used tend to be limited to a few standard molecules, and careful scrutiny of the studies' results is necessary before drawing conclusive arguments.[126] The methodology of protein imprinting yet remains evocative of strong promise towards the development of specialised biomaterials. As these applications rely on enhancement of functionality they do not have the same need for absolute specificity of chromatographic separations or biosensors.[127]

An innate corollary of MIP technology is the application of this specialised recognition for chemical sensing and detection of analytes of interest. As a natural conclusion arising from their highly specific nature, MIPs can be used to selectively bind the template molecule from a feed source, e.g. a chemical process stream, a biological fluid, or an environmental sample, and then to react in some way that gives signal to indicate its presence. The signal can be visual (e.g. colour change), photometric (e.g. fluorescence, absorbance, reflectivity), electrical, or mass-based. Whatever the means of measurement, with modern advanced equipment very small

changes in the system properties can be detected and converted via transducer to some measure of binding which can be calibrated for a target molecule. There is a strong desire for low-cost, robust, rapid assays for point-of-care diagnostics. While certain strategies currently under research can be applied in this field, few are able to successfully meet all three criteria. Using molecularly imprinted polymers may provide a next stage in development of such tools.

2.3 Monomer and Crosslinker Systems

2.3.1 Functional Monomers

System design and optimisation is a key stage in MIP formation. This includes choice of the family of monomers used or specific functional groups that target chemistry on the template molecules.[128] Many different combinations of functional monomers and crosslinkers have been selected for application towards a variety of templates for biomolecular imprinting.[99] Interactive chemistries must be chosen particular to the desired bond type, strength, and target chemistries of the template. Under aqueous systems, where strong polar forces inhibit the *H*-bonding typical for traditional MIPs, the reliance of proper modeling of the motifs is even more critical. The use of this in molecular imprinting with traditional monomers such as methacrylic acid and vinylpyridines has been presented elsewhere.[129] Byrne *et al*. have extended this discussion to include specific recognition of amino acids in proteins.[216] Beyond this, however, lies the opportunity to add to the polymer network specific molecular 'tags' to interact with chemical groups on certain proteins. Work with these 'artificial' protein-recognising groups has been described by Schrader *et al*.[130, 131] They have developed structural elements against arginine and lysine,[132] as well as recognition of the Arg-Gly-Asp (*RGD*) peptide.[133] Through this unique method of manipulating sequential monomers with protein-binding sites,[134] the authors developed a procedure for a molecular switch towards a full protein.[135] The absence of crosslinkers in these systems has led to a series of protein-recognition polymers that act as 'induced fit' receptors towards their targets.[134, 136]

2.3.2 Crosslinkers

When considering the molecular mechanics of forming the pre-polymerisation complex, it becomes evident that the way in which the polymer forms around the template molecule is of major import in achieving a viable MIP. As previously discussed, crosslinkers are often employed in conjunction with the monomers to form the imprinted matrix. Often, as exemplified by the more traditional MIPs, the crosslinkers form a critical element of the MIP (in fact, constituting a larger dominant portion by mass of the total polymer in some cases). In these situations, the monomer's primary role is as the interacting segment, responsible for coordination with the template molecule. The crosslinker forms the bulk of the polymeric matrix, binding all of the monomer, and itself, together.

In other situations, the crosslinker may be employed to improve the matrix characteristics, although the monomer itself is capable of forming its own network alone. This can be done to achieve properties that are desired for the polymer (e.g. mechanical strength, porosity, molecular weight between crosslinks), increase the reaction rate, or improve on imprint recognition in the MIP. In some cases, the MIP constituent polymers can be synthesised directly in the presence of the template, before forming the MIP.[137] This can allow a greater degree of interaction with functional groups. At other times, such as when applying the covalent approach to imprinting, they are bound directly to the monomers.[138] Gore *et al*. have shown that in some cases, incorporation of a template analogue into the crosslinking structure can increase the binding capacity of an imprinted matrix to its target, in this case cholesterol.[139] This investigation was also noteworthy in that it presented a study of increasing hydrophilicity of the network via this crosslinking, for studies done in an aqueous media, while simultaneously suppressing non-specific interactions.

Numerous types of crosslinkers can be used in the composition phase of molecular imprinting. Several classifications of them can be made, based on structure (e.g. hetero- or homobifunctional), polymerisation type (e.g. free radical, chain growth, ionic, ring opening, etc.), solubility (aqueous/organic), and initiation method (e.g.

temperature, chemical, or ultraviolet light). Crosslinking is generally done to form covalent bonds in the polymer, though conceptually physical crosslinking is also an alternative that could be employed. While not as stable mechanically and thermally, physical crosslinks offer a greater degree of freedom in material properties, and can be reversible.

A great majority of the crosslinkers used in molecular imprinting studies rely on the presence of double bonds in the chemistry, which are typically broken to allow the electron sharing covalent bond with the target monomer. As such, many also can indirectly react with themselves, and so the reactions are not nearly as specific or as controlled as in living-radical polymerisations.[140, 141, 142, 143] Most common among crosslinkers chosen for MIPs include those with acrylate or acrylamide derived functionalities. These are especially common for imprinting studies targeting protein recognition.[144]

2.3.2.1 *N,N'*-methylenebisacrylamide

In that the vast majority of reported imprinting systems involve acrylamide chemistry, *N,N'*-methylenebisacrylamide (MBAm) comprises a significant percentage of the overall amount of crosslinkers used for MIPs. This symmetric dimer of the monomeric acrylamide molecule is widely available, to the point of ubiquity among laboratories. It is chiefly employed as a constituent of polyacrylamide gels used for electrophoretic separation of proteins and nucleic acids in molecular biology. However, due to the ease of crosslinking, and controllable properties of polyacrylamide using MBAm, it has become a crosslinker of choice for many molecular imprinting applications.

Yu and Mosbach have suggested that amide functional groups have the potential to provide strong hydrogen bonding ability, even in polar solvents like water.[145] While not the first to exploit amides in molecular imprinting, they propose that differences in dielectric constants and dipole moments, and atomic charges of the oxygen and hydrogen, together with empirical evidence of improved enantiomeric resolution, might

be cause for switching the commonly used methacrylic acid to and amide functional monomer.

2.3.2.2 Ethylene glycol diacrylate

The second most common and equally well-studied family of crosslinkers in molecular imprinting concern those from the family of acrylates/methacrylates. Because of the extensive knowledgebase surrounding it, these are usually based on ethylene glycol esters. The library of available compounds is extensive, including di- and tri-esters, or even longer molecules, depending on the crosslinking needs. The simplest form can be considered acrylic acid, which can combine with itself or other monomers in any number of variations of homo- or co-polymers. Ethylene glycol diacrylate (EGDA) is one of the most commonly used in MIPs. Poly(ethylene glycol) chemistries offer considerable ability to modify the crosslinkers as necessary. Most commercially available forms are homobifunctional, and have a two or three glycol unit spacer. While acrylates prove to be effective crosslinkers and have rapid kinetics, the solubility of many of these molecules in water is lowered because of the vinyl group, and is also heavily affected by chain length.

2.3.3 Initiation of Polymerisation

The initiation of crosslinking in imprinted polymer systems can generally be divided into two categories: those using chemical initiation and those in which exposure to UV creates the free radicals necessary to start the crosslinking reaction. The former case is very common in acrylamide systems especially, and very frequently employs the use of ammonium persulphate as a strong oxidising agent to create the radical species, often in conjunction with *N,N,N′,N′*-tetramethylethane-1,2-diamine (TEMED) as a catalyst. Azobisisobutyronitrile (AIBN) is sometimes employed as a radical initiator, however, is insoluble in water, and thus requires the additional use of organic solvents or alcohols.

When it is available, the use of ultraviolet light to start the crosslinking reaction can be very convenient. It avoids the need for adding an extra reagent to the pre-

polymerisation solution, which could otherwise further complicate the interactions. Radical initiators are generally highly reactive also, so avoiding their use simplifies the procedure and improves process safety. Using photocrosslinking also importantly allows for control over the polymerisation area. This offers two primary advantages. Critically, it allows *in situ* polymerisation, which means it can be used to create an MIP directly inside any desired shaped mould, or even to allow direct polymerisation in a biological setting, such as in an exposed area during surgery. Secondly, this method allows the user to precisely control the exposed area, which is decidedly beneficial for patterning surfaces through techniques like photomasking. By using an opaque filter during UV bombardment, for example, which has been patterned (e.g. die-cut) in a desired fashion, the polymerisation is limited to areas whereupon light was exposed. The remaining [unexposed] areas can conceivably be washed away as uncrosslinked solution, and what results is a polymer in the inverse/negative configuration to the mask design. This is incredibly useful for making MIPs conformed to specialised applications, such as sensor arrays or microfluidic channels for the sorting of proteins or cells.

2.4 Hydrogels

Hydrogels as a particular class of materials are of great interest to biological scientists and engineers because of their chemical and physical similarity to physiologic tissues. These hydrophilic polymer networks have been applied in many biomaterial applications, as their swelling and degradation can be tailored to coincide with a desired natural response in the body. Using hydrogels for molecular imprinting is not without its difficulty, however. In addition to the hindrances of achieving the selective recognition for MIPs in polar solvents as was previously discussed, gels are generally relatively weak and loose networks, whose physical nature does not easily lend itself to the rigid alignment and strict conformations required for template-polymer association. These same polymer branches may also expand and contract in response to environmental changes and solvent concentrations, which makes preserving the imprint site problematical. Moreover, they are generally uniform networks, and as such may not

thermodynamically favour formation of a specific arrangement around the target molecule to support interaction. However, with proper monomer selection, stoichiometric control, and crosslinking, it is possible to establish gels which can form irreversible arrangements around a template, and which will show an ability to re-complex with the specific target through the imparted 'memory' of molecular imprinting.[146] One of the advantages to exploring hydrogels is that this same property of swelling/collapsing which can create difficulty in maintaining imprint sites can be exploited as means of on/off controlled binding for state-dependent memory.[147]

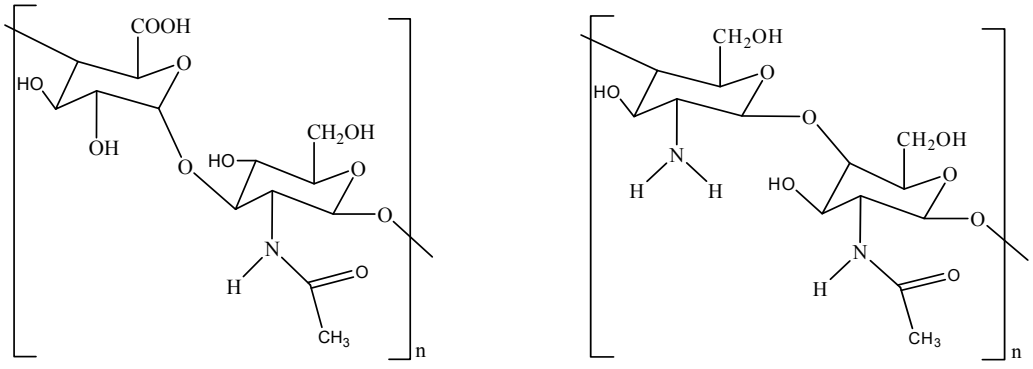
The most commonly used gels in molecular imprinting are of the acrylamide family.[51, 148, 149] Despite their aqueous chemistry, remarkable selectivity has been seen using polyacrylamide columns for the chromatographic separation of proteins.[150] Another primary application of imprinted hydrogels is as vessels for controlled drug release.[151, 152] This includes, for example, application as soft contact lenses, where achieving slow release of therapeutic compounds can overcome the difficulties of ocular lacrimation and low patient compliance seen in other delivery methods.[153] Of particular note are the studies on timolol imprinting in crosslinked hydroxyethyl methacrylate lenses by Hiratani and Alvarez-Lorenzo.[154, 155] This group has reported achieving sustained drug release with imprinted lenses in *in vivo* studies.[156] Later, using norfloxacin as the template, they optimised rational design of the delivery system with titration calorimetry, and achieved high loading and reproducibility independent of lens thickness.[157] The delivery of insulin at specific pH conditions has also been investigated with imprinted gels.[158] It has been suggested that molecular imprinting in hydrogels can be combined with stimuli-responsive physical behaviours, thus leading to a polymer network which would act as a 'feedback-controlled' delivery system.[159] The ultimate goal would be to achieve concentration independent release long-term, and evidence suggests that this might be achievable with MIPs.[153] It remains to be seen whether the improved recognition and diffusion hindrance imparted by a MIP would offer considerable advantages over standard drug delivery hydrogels.

2.5 Chitosan in Molecular Imprinting

Recently, natural polymers have been investigated as host matrices for molecular imprinting, including amylase,[160] cellulose acetate,[61] and chitosan.[161, 162, 163] Disaccharide films can act as imprint matrices for proteins while also limiting their susceptibility to denaturation.[164] Of these, chitosan in particular is of great interest as a biomaterial.

2.5.1 Chitosan

Chitosan is a cationic linear polysaccharide derived from the *n*-deacetylation of chitin, the second most abundant biopolymer in nature, after cellulose. Chitosan is the general term given to the family of copolymers of *n*-acetyl-*D*-glucosamine and *D*-glucosamine, coupled through $\beta(1\rightarrow4)$ glycosidic linkages. Most commercially available chitosans have a degree of deacetylation (glucosamine/*n*-acetyl-*D*-glucosamine ratio) ranging from 50 to 90%.[165] Chitosan is an abundant polysaccharide, with a structural similarity to hyaluronic acid (see Figure 2-5), and is derived primarily from the shells of crustaceans, though it can also be sourced from the exoskeletons of other arthropods as well as the cell walls of fungi.



(a) ***(b)***

Figure 2-5. Chemical structures of (a) hyaluronic acid, and (b) chitosan.

Several studies have examined chitosan's biocompatible, bioactive, haemostatic, and antimicrobial properties.[166, 167, 168, 169] This makes it a valuable material in soft-

tissue engineering and wound dressings.[170] Further to its positive biointeraction, it is an acutely good biomaterial, as a result of its chemical properties and physical versatility; it can be formed into sponges, gels, fibres, porous scaffolds, sheets, membranes, and beads,[165, 171, 172, 173] via such methods as precipitation, freeze-drying, crosslinking, and electrospinning. Manipulation of chitosan to form biomaterials is often done through the modification of physical properties such as solubility, crystallinity, functionality, and charge. As with many natural polymers, chitosan is biodegradable, breaking down into glucosamine units through enzymatic cleavage, though degradation takes place at a fairly slow rate. This can be a hindrance in some applications, but also makes it a useful molecule for long-term biomaterial uses such as tissue replacement or extended drug delivery. Chitosan is gradually degraded by lysosyme in the body, and to some extend the rate of degradation can be controlled by the degree of acetylation of the molecule.[174, 175]

Controlled degradation rates are an essential parameter in many polymer engineering designs. To some extent, both the aqueous solubility and degradation rates of chitosan can be altered by chemical modifications of the structure. Many research groups have investigated the grafting of hydrophilic groups, such as carboxymethyl, acyl/alkyl, and succinyl substituents, to the amine or hydroxyl groups in the chitosan backbone.[176, 177, 178, 179] Other groups have investigated the use of larger molecules, such as poly(ethylene glycol), sialic dendrimers, and poly(vinyl alcohol).[180, 181, 182] While these alterations can impart improved properties of chitosan, it is also desirable to leave some portion of the free amino groups unreacted, as they impart many of the advantageous biological properties of chitosan, and also provide sites for further chemical grafting, such as for drug delivery of peptides.[183]

Because of its relative abundance and biological properties, chitosan has realised extensive use in industry.[184] Aside from a variety of food and drug and agricultural uses, chitosan has been approved by Health Canada for application in bandages and by the FDA in an arterial closure device.[185, 186] Currently, 260 patents have been issued by the U.S. Patent and Trademark Office that specifically relate to chitosan, of which 252

fall in the period since 1976.[187] Its highly biocompatible nature in conjunction with its physical properties, specifically its ionic nature, makes chitosan the basis for many approaches in tissue engineering.[188]

A primary difficulty with chitosan is that it is poorly soluble at physiologic pH. This severely limits some applications, especially as a biomaterial, and makes processing and manufacture of it necessitate the use of organic acids. Naturally, the aqueous solubility is affected in large part by the molecular weight of the chitosan, smaller molecules being more soluble.[189] The polymer can be fractionated into smaller molecular weight residues in order to improve this quality.[190] The water solubility of chitosan is also affected by its degree of crystallinity; highly acetylated/deacetylated forms which have a much organised crystal structure will have much lower solubility in water than a blend of both forms.[189]

Although not widely studied, one of the derivatives of chitosan possessing good water solubility is the glycolated form. The addition of glycol groups to its functionality increases the hydrophilic nature of the large polymer, and imparts an improved interaction with the polar molecules of water that helps to overcome its organic nature and increased chain length. Unlike its parent molecule, glycol chitosan has not been reported as in-depth in the literature. It has currently not been well characterised or classified, but its proposed structure will presented in a more detailed fashion in subsequent sections. Glycol chitosan has been fashioned into various materials, including cross-linked hydrogels, physical networks, membranes, and microspheres.[191, 192, 193, 194] It has also been used for sustained model drug delivery.[195] Its aqueous solubility, apparent biocompatibility, hydrogel-forming ability, and the presence of chemical sites on its backbone to allow for further functionalisation make glycol chitosan a worthy choice for use as a material engineering matrix. For these reasons it was selected as a model polymer to use for the MIP scaffold in this design.

2.5.2 Use of Chitosan in MIPs

To date, chitosan polymers have been studied to a very limited degree in molecular imprinting applications. Generally, chitosan has been incorporated in MIPs only as a structural support for the recognition matrix.[196, 197, 198, 199] In some cases chitosan has been used for imprinting in combination with other materials, such as acrylamide,[197, 198] in sol-gel hybrids,[200, 201] or resins.[202] Xia *et al*. demonstrated improved recognition in using the combination of chitosan and polyacrylamide in an interpenetrating network (IPN) over either material alone,[203] while a similar study evaluated the difference between copolymerising the two materials versus the IPN.[204] In the case of MIP recognition from chitosan alone, reports in the literature are limited to the field of chemical adsorption and treatment.[162, 163, 205, 206] To the authors' knowledge, outside of this aspect of the field, this report represents one of the few studies done on molecular imprinting using chitosan alone[207] and the sole such for large biomolecular template recognition.

Many factors warrant careful examination with respect to imprinting in a chitosan matrix, primary of which is achieving the memory effect in a polar solvent like water. Generally, the more polar a solvent is, the worse the recognition obtained in the MIP.[208] Because non-covalent imprinting relies heavily on hydrogen bonding for alignment during polymerisation and subsequent template pairing, realising the same recognition in aqueous media is a major hurdle to this work. Oral and Peppas showed this difficulty in trying to achieve imprinting of a hydrophilic molecule (glucose) in an hydrogel.[209] The resulting levels of non-specific interactions were very high, and in order to reach a significant level of imprinted recognition, the template levels drawn on during polymerisation had to be excessive. Selectivity in aqueous phases depends on ionic strength, pH, and water content of the media.[210] This notwithstanding, the addition of electrostatic interactions has not been shown to increase the adsorption characteristics within MIPs in some cases,[211] and thus it would suggest that the dominant form of receptor-template interaction remains a combination of hydrophobic and hydrogen bond formation.[212] Piletska *et al*. suggest that the inconsistent recognition seen in polar solvents results not necessarily from ion pairing between the

template and functional monomers, but the ionisation properties of the functional groups at and near the binding cavity.[213] Furthermore, while relatively higher success has been seen with molecular imprinting using organic solvents, and the vast majority of research is focused in this area, the role of the solvent remains poorly understood in the confines of the chemistry during imprinting.

While the nature of the solvent is key to the imprinting process, a large number of peripheral factors also affect the creation of an imprint site during polymerisation. The physical conditions during imprinting have been shown to affect the template recognition, in that the best results are seen at or near the temperature[205] and pH[214] of the polymerisation. This fact can be manipulated to achieve tailored release of the template molecule at desired pH ranges[160] or control release profiles.[27] The concentration of crosslinker present during the polymerisation step has been thought to be a critical aspect in MIP recognition, and intuitively one would expect this to be the case, as a tighter, rigid network would seem to result in more specific binding sites. However, Rampey *et al*. suggest that it is not as crucial a factor as was once believed,[215] and Hiratani *et al*. have shown that, above a minimal level, release of the imprinted molecule was sustained independent of crosslinker amount used.[149] This result has subsequently been supported by the experiments of other authors.[147, 213,] So, while tighter networks seem to lead to improved imprinting effect, it may be as a result of crosslink density (related to the crosslinker length) and not as much to the concentration.[216] Hjerten *et al*. have reported on the effect of monomer concentrations, as well as monomer to template ratios.[150] When dealing with imprinting polymers, such as chitosan, the amount of polymer and its molecular weight affect the resulting MIP structure, and therefore affinity for the template.[217]

The physical properties of the proposed chitosan network will also cause difficulty in achieving MIP recognition. While very beneficial for mimicking the chemical and physical properties of tissues, hydrogels are very loose networks of polymer that often possess very high equilibrium water contents. The same degree of inter-chain porosity and flexibility that allows for hydrogels to be such proficient

approximations for cell and tissue growth and drug delivery also means they will lack the tight-knit interactive forces relied upon for target recognition in a MIP. Likewise, hydrogels are known to change conformation and size as they swell and contract in response to environmental changes. Therefore, one would not expect an hydrogel to be a medium ideally suited to molecular imprinting – far from it. For a material made out of chitosan in this fashion will require overcoming some palpable challenges, which may manifest themselves in little-to-no measurable change following molecular imprinting. Testing the matrix empirically will require close examination and a degree of scrutiny in analysis.

Recently, Rechichi *et al*. have proposed the use of imprinted polymers to enhance cellular adhesion for tissue engineering.[218] The use of a material particular to a desired cell type and application is a key factor in the biomedical field, and an imprinted substrate presents an ideal model for controlled bio-stimulation. To be an effective tissue engineering matrix, the imprinted polymer should recognise and support the growth of the desired cell type. Some groups have evaluated the imprinting of entire cells - mostly bacteria and viruses - with some success in sensing strategies.[219] In particular, this has been the focus of much work by Hayden and Dickert, who, in 2001, demonstrated surface imprint patterning and selective yeast strain detection on polyurethanes.[220, 221] One of the first reports involving imprinting of bacterial strains was by Aherne *et al*.[222] This method of bacterial "surface lithography" for MIP beads was slightly improved upon by Harvey *et al*. a decade later.[223] Backsay *et al*. have used acrylamide to develop bacterial-specific "antibodies" that could be electrophoretically separated according to cell-binding ability.[224] Wandelt *et al*. demonstrated how a polymerisation system used for conventional MIP recognition could also be adapted to the surface imprinting of yeast cells.[225] Ren and Zare have suggested from their studies with bacteria 'stamped' into curing polydimethylsiloxane that the key interaction providing selective binding in the cell-imprinted polymers is related to chemical recognition.[226] This implies the action of specific binding forces, rather than mere simple macro-scale physical changes in the polymer. The imprinting of viruses has been investigated by Bolisay *et al*., who showed binding selectivity in a tobacco mosaic virus

MIP.[227] In their continued series on artificial gel antibodies, Takatsy *et al*. have also used imprinted acrylamide beads for electrophoretic identification of viruses in solution.[228] Interestingly, the authors suggest that the recognition is based on a "full-body" imprint of the virus, thus offering an improved selectivity over the smaller antigen site recognition in conventional protein antibodies.

A final area of great import in evaluating the imprinting within the imprinted matrices is measuring the degree of recognition (and specificity) for the template molecule. It is imperative that the methods used be capable of accurately detecting the difference between the template and other molecules. This is not a trivial concern, as the measurable binding within the polymer can be masked by many non-specific interactions. This is especially true of large molecules with many potential sites of interaction, such as proteins. Most often, separation methods such as HPLC are used, wherein the increased interaction of the template allows for temporal separation and detection of the analyte. This remains a cumbersome, costly, and time-consuming process. A simpler method is to detect the presence of the template *in situ* optically, such as by UV absorbance or fluorescence tagging. A problem that arises with these methods is that one must sacrifice considerable analytical capability (high limits-of-detection), and if the difference between MIP and NIP recognition is small (such as with low concentrations of analyte) then any signal change may be lost within the inherent noise. To improve upon this, more precise detection methods are needed, which still allow for real-time detection of binding.

2.6 Detection Methodologies in Molecular Imprinting

Proper quantification of molecular imprinting remains a challenge in the field.[126] Because the amount of high affinity sites in the bulk of a MIP is expected to be low, accurate detection of a template molecule within the network is a great challenge towards verifying the efficacy of the imprint. Especially with such materials as proteins, which are expected to rebind specifically in small quantities (e.g. on the nanomole scale), the detection becomes quite difficult. An ideal situation would allow

investigators to not only measure the amount of binding with a template, but also to localise a target within the MIP.

One of the preeminent of methods currently available to scientists would be using radio-labelled compounds,[229] however, these molecules can be quite costly, in addition to the demands of working with radioactive materials. Alternatively, it is possible to exploit the specific interaction of the target molecule with the surrounding matrix to create an environmental change that is detectable, such as with photoinduced electron transfer (PET) sensors.[230] This is limited to very specific chemistries, and thus the materials that can be chosen for the MIP are narrowed to a degree. The next most logical solution would be using a fluorophore as a detection method to specifically localise bound targets. This, too, requires additional modification of the template, and may not be sufficiently sensitive to be quantitative. An indicator-displacement strategy can be used to discriminate the target binding.[231] One solution put forward by Sunayama *et al*. is to instead incorporate the fluorescent moiety into the polymer, and use quenching caused by the binding of the target to imprint sites as a reporter for successful capture.[232] This was also exploited by Cywinski for a nucleotide sensor.[233] Observing these guest-host interactions showed promising results, but still relies on optical (fluorescence) spectral change and additional modification of the monomers.

In many cases, MIPs may comprise or be incorporated as part of a chemo/biosensor, a rapidly growing field of biotechnology. In these cases the binding or capture of a template can be used to affect some change on a transducer, which is then processed as a signal. As sensitive detection methods, these synthetic recognition systems are a very attractive means to replace their biological counterparts. They offer lower costs, higher physical/chemical stability, easier preparation and better engineering possibility than biological receptors.[234] It is possible to work backwards and use some of the characteristic methods used for real-time analyses and therapeutic monitoring to gauge the amount of recognition of imprinted matrices.[235]

For a simple first-stage analysis of molecular imprinting, the conventional approach is to use bulk imprinting of a more common molecule that allows for easy detection, such as through UV absorbance;[126] the rationale being to use something in present in larger quantities, thus rendering the overall signal and changes therein more easily detectable. Assays with bulk imprinted hydrogels have achieved some incredible sensitivity, capable of detecting template levels in the femtomolar range.[236] Imprinting in bulk polymerisation customarily necessitates grinding of the polymer and leaching of the template, and with the incorporation of large quantities of print molecule this should allow simple detection and confirm whether imprinting was successful in the chitosan. However, in most imprinted systems, the differences between MIP and NIP are often quite small, and while it may be a robust, simple method, UV absorbance does not provide great sensitivity for detection. It nevertheless remains an uncomplicated tool in the analytical kit, often on-hand in most laboratories, and so provides a useful starting point from which to launch discovering groundwork in conducting a detecting assay.

In the course of this study, several techniques were investigated to examine the applicability of a chitosan MIP for use in a biodetection system. Two such techniques, namely quartz crystal microbalance (QCM) and surface Plasmon resonance (SPR), represent some of the most common and relied upon technologies for accurate and sensitive measurements of biomolecule binding activity. A third method, reflectometric interference spectroscopy (RIfS), was also examined, whose use is not as conventional in analytical detection strategies nor in the field of molecular imprinting. As the results from these experiments were not fruitful in elucidating the recognition behaviour of a chitosan MIP, the reader is directed to the Appendix for further detail on these particular examinations. The focus herein will remain on reports pertaining to the application of dielectric spectroscopy to the MIP examination, supported by simplistic trends generated by observations done through UV absorption.

2.6.1 Dielectric Impedance Spectroscopy

As technology grows, newer methods are constantly being developed and applied to aid in the observation and characterisation of complex systems. Dielectric spectroscopy (DS) is one such innovation which has recently shown much growth, particularly in the fields related to biomedical sciences. A major part of this evolvement can be linked to the principles of DS which allow it to be non-invasive to the measured samples and also permit real-time analysis of interactions. While simultaneously being incredibly precise and capable of monitoring changes in heterogeneous samples at both a microscopic and molecular level, DS is a versatile technique offering great promise for future sensing systems.

DS relies on measuring the permittivity and conductivity of an interfacial space, as a result of polarisation within the media from the produced alternating current (AC) field. By using frequency scans of the electrical field, the displacement of current provides spectra of the dielectric change, from which distinguishing dispersions and relaxations can be read. These fluctuations are then used to characterise the medium. The displacement of the electrical field in the material can be defined by the equation:

$$\vec{D} = \varepsilon \vec{E} \qquad \textbf{[2-8]}$$

where $\vec{D}$ is the electrical displacement field
ε is the absolute permittivity
$\vec{E}$ is the electric field

From this expression, the permittivity of the field can be derived from $\vec{D}$. It can alternately be found by the following relation:

$$\vec{D} = \varepsilon_o \vec{E} + \vec{P} \qquad \textbf{[2-9]}$$

where ε_o is the permittivity of vacuum
$\vec{P}$ is the polarisation of the medium

The polarisation is related to the electric field by:

$$\vec{P} = (\varepsilon_r - 1)\varepsilon_o \vec{E} \qquad \textbf{[2-10]}$$

where ε_r is the relative permittivity, a unitless measure of the polarisability of the material (and hereafter referred to as ε).

The dielectric field which forms as a result of the applied current provides the impetus through which the spectra are obtained. As a result of the reaction to the ambient field, the response of the system is measured and broken down into frequency ranges that describe it. Generally, the polarisation effect can be separately ascribed to four mechanisms:

- electronic polarisation inside neutral atoms as the electron cloud is displaced relative to the nucleus; this displacement reaches an equilibrium between the restorative forces of the atom and the electric forces of the induced field
- orientational polarisation resulting from the spatial rearrangement/alignment of a molecule according to the electronegativity of the constituent atoms
- atomic polarisation that arises when charged atoms and ions are displaced; and,
- interfacial polarisation occurring as charge mobility leads to a redistribution of electrons in the material and accruing in an accumulation of charge towards discrete zones.

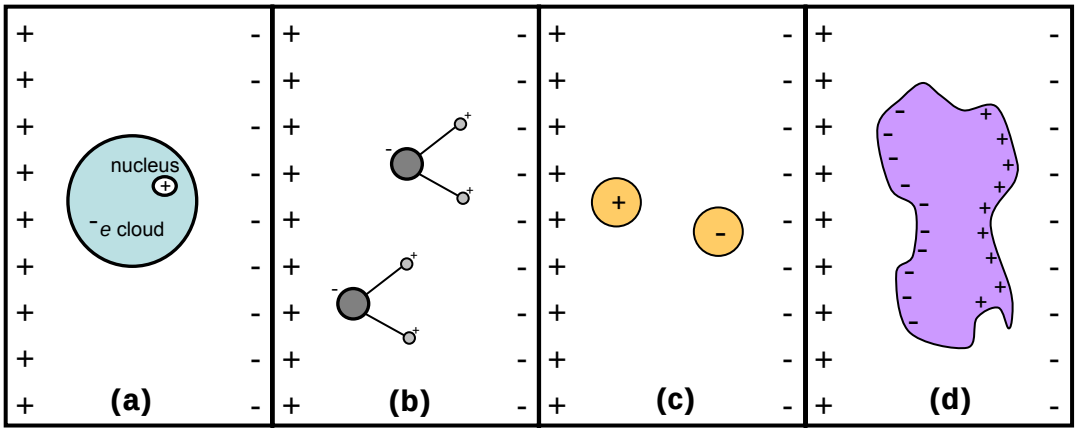
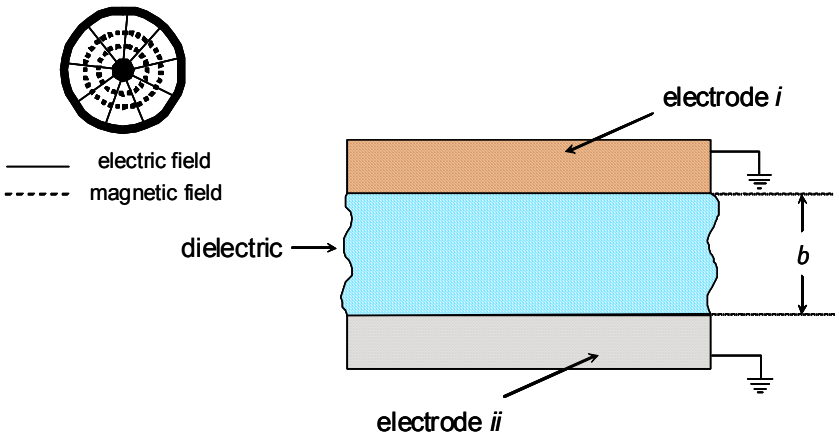


Figure 2-6. Schematic representation of polarisation mechanisms experienced in a dielectric system: a) electronic polarisation, b) orientation polarisation, c) ionic polarisation, d) interfacial polarisation.

Whereas the effects of electronic and ionic polarisation are only significant at high frequency and infrared field frequencies (> 10^{11} Hz), respectively, the large focus of dielectric spectroscopy involves mechanisms due to interfacial and orientational polarisations. Because the masses involved in these cases are much larger than their counterparts, the field induction effect is considerably slower. These resultant transitions within the electric field are observable at the lower frequencies, i.e. prevalent at < 10^{11} Hz.

The dielectric spectroscopy of a sample can be measured by modern instrumentation. For example, a direct current (DC) impedance analyser, such as that shown in Figure 2-7 *(a)*, can be used for precision measurements of impedance by applying a frequency range (e.g. 40 Hz to 110 MHz) to measure permittivity and conductivity changes. While this is a powerful tool in the analysis of circuits and electrical components, with the use of in-fixture devices it is equally capable of performing efficient and accurate measurements on buffered aqueous systems. Functional electrodes can be built and attached to the instrument for a variety of designs and operating conditions.

(a) *(b)*



(c)

Figure 2-7. Depiction of DC impedance analyser. (a) HP/Agilent 4294A Precision Impedance Analyzer[237], and (b) spring clip electrode fixture for same.[238] (c) Schematic representation of electric field in liquid measurement cell.

2.6.1.1 Complex Permittivity

The dielectrical properties used for measurement in this case are related to the complex permittivity of the system. The complex permittivity is described by following relation:

$$\varepsilon^*(w) = \varepsilon'(w) - j\varepsilon''(w) \qquad \textbf{[2-11]}$$

where $j = \sqrt{-1}$, ε' is the real permittivity, ε'' is the imaginary permittivity (also referred to as the loss factor), and ω is the angular frequency of the applied field.

Under the application of an electrical field, the permittivity measurements arise from polarisation within the material. If the field is static, the polarisation occurs in alignment with the field. At lower frequencies of an AC field, the polarisation vector remains in the same direction as the electrical field vector, and is referred to as the low-frequency limit, or static permittivity. When the field frequency is increased, the polarisation vector does not respond identically to the AC field vector, and thus there is a detectable lag where the two are out of phase with one another. This behaviour is demonstrated conceptually in Figure 2-8.

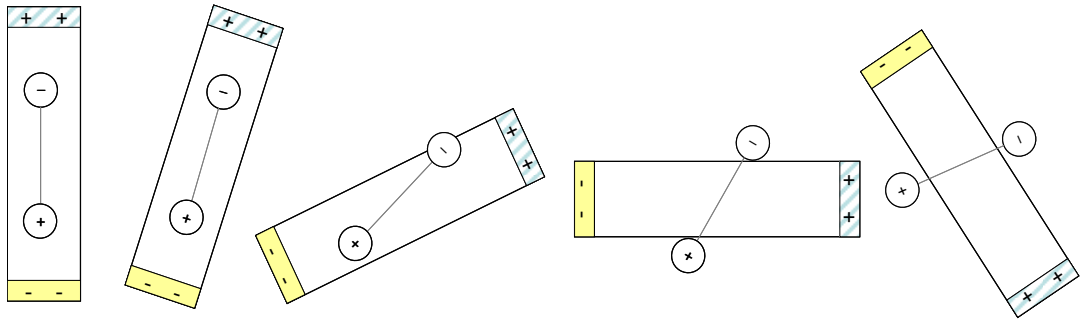


Figure 2-8. Phase lag behaviour experienced between a dipole and the shifting frequency of an applied electrical field.

As a result of this induced polarisation, there are two types of energy loss within the system:

1. energy losses from frictional damping mechanisms during the retardation/relaxation processes
2. ohmic losses that arise if the material being analysed contains numerous free charge carriers, such as ion in an electrolyte solution

Together, these two forms of loss can be accounted for in the loss factor:

$$\varepsilon'' = \frac{\kappa - \kappa_1}{\varepsilon_o \omega} \qquad \textbf{[2-12]}$$

where κ_1 is the low frequency limit of conductivity, or DC conductivity, and κ is the total conductivity.

The real permittivity component, also referred to as the dielectric constant, is defined as the relation between the amount of energy stored per unit volume in the medium and the applied electric field surrounding it. For the majority of solids and liquids the value is greater than unity (e.g. $\varepsilon'(\omega) > 1$).

Finally, the phase of the complex permittivity is often referred to as the loss angle, φ. The tangent of this value represents the amount of power loss in the medium, or loss tangent.

$$\tan\varphi = \frac{\varepsilon''}{\varepsilon'} \cong \frac{\kappa}{\omega\varepsilon} \qquad \textbf{[2-13]}$$

It is important to note that at very high electrical field frequencies (approaching microwave wavelengths) the effects of the interfacial and orientational polarisations decrease. This causes an increase in the loss tangent, and a corresponding decrease in the real permittivity.

2.6.1.2 Dipole Relaxation

Following the application of an electrical field, the dipoles excited by this force will begin to exhibit relaxation according to an exponential trend. To model this pattern, the first-order Debye equation may be used:

$$\varepsilon^*(\omega) = \varepsilon_h + \frac{\varepsilon_l - \varepsilon_h}{1 + j\omega\tau} \qquad \textbf{[2-14]}$$

where ε_l and ε_h are the static and infinite frequency permittivities, respectively, and τ is the characteristic time constant. This relation assumes no interaction between dipoles.[239] Values for these constants have been compiled from empirical data, and are tabulated for a number of common mediums.[240, 241, 242]

When dealing with heterogeneous mixtures, such as complex colloids or biological tissues, certain other models, as that developed by Asami, can be used to describe the system.[243] These approximations account for deviations from the Debye conditions as a result of the existence of a distribution for relaxation times, or the occurrence of several Debye relaxations in the system. The most commonly applied variant model is the Cole-Cole equation:

$$\varepsilon^* = \varepsilon_h + \sum_n \frac{\Delta\varepsilon_n}{\left(1 + j\omega\tau_n\right)^{\beta_c}} + \frac{\kappa_l}{j\omega\varepsilon_o} \qquad \textbf{[2-15]}$$

where ε_h is the high-frequency limit of relative permittivity, $\Delta\varepsilon$ the relaxation intensity, τ the relaxation time $(1/(2\pi f_c))$, f_c is the characteristic frequency, κ_l is the low-frequency limit of conductivity, β_c is the Cole-Cole parameter ($0 < \beta_c \leq 1$), and n represents the number of relaxation terms. The Cole-Cole parameter is an experimentally determined factor, where the Cole-Cole approximation is reduced to a simple Debye model when $\beta_c = 1$.

2.6.1.3 Electrode Polarisation Effects

One of the hindrances to conducting measurements of permittivity in media is polarisation that occurs near the electrode.[244] Electrode polarisation phenomena arise as a result of charge build-up at the interface between electrode and media, resulting in the formation of an electrical double-layer.[245] The existence of this double layer interferes with normal measurement of permittivity, and this error becomes more significant and impedes the measurement spectrum at frequencies below 100 MHz.[245, 246] The electric double-layer is affected by certain conditions, such as surface topography, chemistry and area of the electrode, as well as the chemical composition of the sample being analysed. Because of these dependencies, there is no singular technique to correct for the double layer interference across varying experiments; there are, however, certain generally accepted methods available to model the electrode polarisation double-layer.[245, 247, 248]

2.6.1.4 Temperature Effects

When conducting measurements of complex permittivity, the temperature of the medium plays a fundamental role in the system response, and as such must be accounted for in any empirical study. It is important to note these direct correlations as temperature increases:

- the strength and extent of molecular bonds decrease
- the static and optical permittivities decrease
- the dipole is more free to oscillate at higher frequencies
- drag against the rotating molecules is reduced

For small-scale systems, temperature control can be maintained by conducting the measurements inside an incubator. In larger devices it may be necessary to incorporate a thermocouple and temperature controller within the unit to reduce the fluctuations as much as possible. With setups involving high volume transfer of fluid, it is also possible to integrate the heating and cooling directly to the feed and allow this to regulate the measurement chamber. When the detection cell is of fairly low volume (e.g. less than a few millilitres), temperature effects are mitigated by the system reaching faster equilibrium with the ambient environment, so small fluctuations of the system present less of a concern.

2.6.1.5 DS in Polymeric Systems

2.6.1.5.1 Films

Some polymers, including chitosan, in solution behave very much like a polyelectrolyte; in the particular case of chitosan, this is owing to the presence of amino groups along the main chain. This has been reflected by studies of the dissolved polymer with dielectric spectroscopy by Bordi and others, in which the influence of pH and counterions on the dielectric relaxation have been reported.[249, 250] As a bulk material, however, the number of charged groups present may be reduced, owing to the modification of side groups during polymerisation, and the transport phenomena and

physical properties will be necessarily different. It has also been proposed that, because chitosan films alone act as only weak conductors of electricity (on account of strongly bonded protons), in order to increase this mobility, the addition of dispersed ions in the solvent phase can be used (e.g. H^+, CH_3COO^-).[251, 252] The conductivity of chitosan films shows a temperature dependence according to an Arrhenius relationship, for a range up to 50°C and possibly beyond.[251, 252] This can be observed by variation of relaxation time as:[253]

$$\tau = \tau_o e^{(E_D/kT)} \qquad \textbf{[2-16]}$$

where τ is the relaxation time, τ_o is the pre-exponential factor, E_D is the activation energy for the relaxation process, and k is the Boltzmann constant. In the case of deviation from the Debye relation, a distribution of relaxation behaviour in the conductivity may be modeled by the Kohlrausch-Williams-Watts (KWW) law:[253]

$$\Phi(t) = \exp\left[-\left(t/\tau_\sigma\right)^\beta\right] \qquad \textbf{[2-17]}$$

where τ_σ and β are the relaxation time and Kohlrausch exponent, respectively.

Osman *et al*. have described the important role of a plasticiser, as may be commonly added to polymer systems, has on this effect via transitions of the glass transition temperature, T_g. Obtaining the bulk resistance, R_b, from a Cole-Cole plot, the room temperature electrical conductivity is given by

$$\sigma = \frac{t}{R_b A} \qquad \textbf{[2-18]}$$

where σ is the conductivity, t is the film thickness, and A is the cross-sectional area. The addition of plasticiser, in this case ethylene carbonate, caused an increase in the dielectric constant of the film, while also aiding in its ability to dissolve a salt ($LiCF_3SO_3$). It may also increase ionic motility, by decreasing cation-anion coordination of the salt which reduces barrier potential to the ionic motion.[251]

As many other factors can influence the sensitive measurements in DS, it is important to understand each of the processes taking place during sample analysis. Nogales *at al*. demonstrated the importance of sample conditions and history, as the presence of water adsorbed in equilibrium with cast chitosan films can produce two distinct dielectric processes.[254]

2.6.1.5.2 Spheres

Spherical particles dispersed in a medium are an ideal target for DS analysis, owing to its sensitive ability to measure heterogeneic systems. In such a system, the dielectric behaviour depends on the dielectric constants for the surrounding media, ε_a^*, the dielectric value of the spheres, ε_i , and the void fraction, ϕ. This is shown schematically in Figure 2-9.

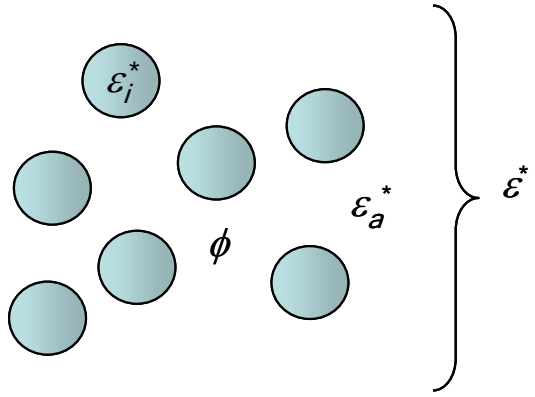


Figure 2-9. Diagram showing dielectric model of spherical particles dispersed in a fluidic medium.

The complex permittivity of the whole suspension is defined as:

$$\varepsilon^* = \varepsilon - \frac{j\kappa}{\omega\varepsilon_o} \qquad \textbf{[2-19]}$$

where ε and κ are the permittivity and conductivity, respectively, ε_o is the vacuum dielectric constant, and $j = (-1)^{1/2}$. In densely packed systems, the total complex permittivity is modeled using the Hanai equation:[255, 256]

$$\left(\frac{\varepsilon^* - \varepsilon_i^*}{\varepsilon_a^* - \varepsilon_i^*}\right)\left(\frac{\varepsilon_a^*}{e^*}\right)^{1/3} = 1 - \phi \qquad \textbf{[2-20]}$$

Because ε_a is generally known, the virtue of Hanai's derivation is that the formula allows one to determine the phase parameters (ε_i, κ_i, κ_a, ϕ) from the measured dielectric parameters (ε_l, ε_h, κ_l, κ_h).

The distance between the particles is important, as the electrochemical interaction between them will depend on whether their separation is on the order of the Debye length, λ_D, or less. The interfacial interaction between particles is also affected by the surfaces, as smooth or rough particles will exhibit different responses.[257] As seen in Figure 2-10, the characteristic interaction region of two particles with radii a is a sphere with radius $(a\lambda_D)^{1/2}$. The electrical double layer is defined as the overlaying region of interaction with dimension $2\lambda_D$. Ionic transfer and accumulation in this small area determines the overall; interfacial relaxation behavior of the system.

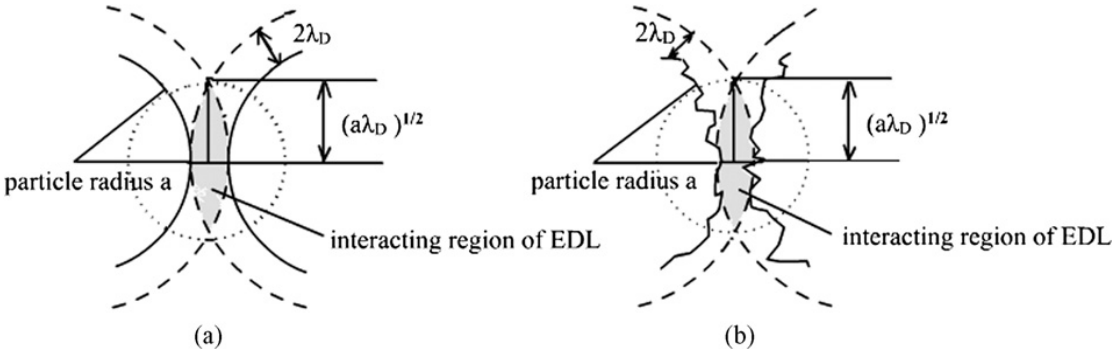


Figure 2-10. Interfacial electrokinetic models for (a) smooth particles and (b) rough particles. EDL: electric double layer.[257]

Herein the relaxation time is designated as the time that ions spend on transferring a distance of $2\lambda_D$, under the application of an electric field. It is relatable to the relaxation frequency by Equation 2-21.[257]

$$\tau = \left(2\Pi f_o\right)^{-1} \qquad \textbf{[2-21]}$$

where τ is the relaxation time and f_o is the relaxation frequency. Ni and Zhao have used these relations to investigate glutaraldehyde-crosslinked chitosan beads suspended in water.[257] Empirically-measured data is fitted to the Cole-Cole plots to determine dielectric parameters, which are then substituted into the Hanai formula for the phase parameters. The authors used DS to show the parameter dependency of crosslink density with dielectric parameters and phase parameters. This allowed them to make conclusions with respect to the effect of glutaraldehyde crosslinking on morphology, surface roughness, and inner structure of chitosan beads.

Generally, one expects to see two relaxations characterised by two relaxation frequencies for particle dispersions; however, it is often the case that only one is observed in practice. It has been established that the low frequency dispersion of colloidal particles can be attributed to electrolytic conductivity.[258, 259] The authors suggest that the relaxation observed at low frequencies is due to surface conductance and is given by:

$$f_s = \frac{ukT}{\pi R^2 e} \qquad \textbf{[2-22]}$$

where R, u, k, T, and e, are the radii of particles, ionic mobility, the Boltzmann constant, absolute temperature, and the electric charge of counter ions, respectively. Calculating the theoretical value of f_s for the experimental conditions can allow one to identify whether a measured dispersion is from surface charge conductivity or interfacial polarisation.[257]

The dielectric behaviour of chitosan particles has been further investigated by Zhao *et al*.[260] wherein the authors were able to characterise the heterogeneous mixture of chitosan microspheres in electrolytic suspension. It was demonstrated that the dielectric relaxation curves obtained by fitting the Cole-Cole equation were broader than normal Debye behaviour, and the conductivity was best modeled according to the Hanai relation[261]:

$$\kappa_h = \left(\varepsilon_l - \varepsilon_h\right) 2\pi f_o \varepsilon_v + \kappa_l \qquad \textbf{[2-23]}$$

where κ and ε are the permittivity and conductivity, respectively, corresponding to values denoted by the high- (h) and low- (l) frequency limits. A strong pH dependence of the relaxation was observed, with peak relaxation intensities occurring in the pH range of 2-6. As densely packed microspheres, the behavior of the system was found to very closely mimic that of ion-exchange resin beds. By assuming the microspheres to be a mixture of water and polymer, the individual permittivity of a microsphere can be determined from:[260]

$$\varepsilon_i = f_w \eta_w + \left(1 - f_w\right)\varepsilon_p \qquad \textbf{[2-24]}$$

where ε_i is the individual permittivity, f_w is the volume fraction of water in the microsphere, and ε_w and ε_p are the permittivities of water and the polymer, respectively.

In order to examine the interfacial polarisation of chitosan microspheres, Zhao *et al*. added gradually increasing amounts of KCl into the surrounding solution. The internal conductivity, κ_i, began higher than the external conductivity, κ_a, and as more salt was added, the ratio of the two decreased exponentially up to a limiting value. At low concentrations, the distribution of ions is focused near the fixed charge of the polymer, but as more counterions become available, the distribution between the internal water phase and external balances out.[260] Interestingly, because the limiting value of κ_i/κ_a was seen to be less than one, it suggests that the internal conductivity of the chitosan microspheres is determined not from moveable ions alone, but other possible conduction mechanisms, such as proton transport.[249]

2.6.1.5.3 Porous Scaffolds

Few studies have thus far examined the dielectric properties of chitosan in more complex 3D organisations. Because most studies have been focused on elucidating the principles and mechanics of action, little work has been done on designs more practical towards biomedical applications. Bagnaninchi *et al*. have used DS to observe the porosity and cell growth in gel scaffolds of chitosan.[262] While preliminary, this study demonstrates the potential for on-line monitoring of cells inside growth matrices via DS. In a later study, the authors subsequently applied complex permittivity measurements to monitor cell growth on chitosan scaffolds as an *in vitro* pre-tissue model.[263] Monitoring the depolarisation factor via the Hanai function allowed real-time observation of cell attachment and morphological changes. Similarly, by measuring total cell volume fraction, overall growth can be estimated in a sample non-destructive fashion. Proliferation and differentiation was then assessed under different media conditions, for direct-response observations. A key finding of this study was that, because of the sensitivity of DS, the inclusion of other material inside the measurement cell is detectable, thus proving the estimation of the additional formation of extracellular matrix, a key component for tissue engineering applications.

2.6.1.5.4 Dielectric Spectroscopy of Molecularly Imprinted Polymers

While conductivity changes have been used to observe template binding,[264] there have been few studies of using electrical impedance to measure the effectiveness of MIPs.[265] One of the first such reports came in 2002, in which Panasyuk-Delaney *et al*. proposed an imprinted chemosensor towards creatinine.[266] Generally these studies applying DS to MIPs have investigated non-biomolecules and/or have used electropolymerised polymers.[267, 268] Furthermore, most reports concerning MIP sensors are also limited to investigations of AC impedance spectroscopy with electrochemical cells.[269, 270] Kan *et al*. evaluated the imprinting of hemoglobin in polypyrrole MIPs, however used a very harsh protein removal stage and subsequently found a high cross-reactivity with lysosyme.[271] Some flow-through sensors based on MIP methodology have been used, which could be incorporated for bio-detection as part of an in-line monitoring device.[272]

Chitosan has been investigated in a very limited fashion in this regard, only being incorporated with graphene in an eletrodeposited film for the detection of dopamine, in which case the chitosan served only as a structural element to support the π-π interaction of the template with graphene.[273] Peeters *et al*. recently looked at the determination of histamine in bowel fluids, but this involved ground MIP/NIP incorporated into a conducting polymer on electrodes.[274] In another noteworthy study, Viswanathan *et al*. created a polyphenol MIP on a surface of gold nanofibers for the detection of epithelial ovarian cancer antigen-125.[275] The sensor exhibited accurate sensitivity and good reproducibility to doped human serum samples, however the use of a solid coating of non-conductive polymer for the NIP makes imprinting effectiveness difficult to gauge. Using a similar technique, Cai *et al*. showed that for ferritin-imprinted carbon nanotube arrays, a thin layer coating of polyphenol allowed near single template molecule thickness, and the number of individual binding sites could estimated. These particular arrays provided great potential towards clinical diagnostic tools, as the MIPs demonstrated sensor selectivity between species of proteins as well as viral strain types.[276]

Whereas using molecular imprinting with filtration membranes can allow for controlled permeation of solutes, Kochkodan *et al*. exploited this principle for the selective capture of small molecule analytes, which the authors could then incorporate into a portable sensor device based on dielectric permeability.[277] While their device was limited in that the capturing and drying stages were independent of the measurement, the MIP coating and optimisation thereof provides useful information for the screening of target molecules via molecular imprinting. One of the few studies to use both traditional imprinting polymers and DC impedance metering (via balancing bridge technique) was described by Morelli *et al*.[278] By monitoring the impedance phase and gain with sub-micron MIP particles (towards phenylalanine amino acid) entrapped in a poly(*l*-lactic acid) matrix, they showed a strong recognition factor towards the template as detected in real time in PBS buffer. Still, the potential towards using the sensitivity of electrical impedance for monitoring of binding events in MIPs, particular in hydrogels, remains promising. This is exemplified by the submission of a patent from Bayer and Peppas describing the particular recognition and capture of a triggering molecule.[279]

3 Imprinting Challenges and Design Motivations

The initial inspiration for this project was to use a water-soluble chitosan as the primary monomer/polymer to form an aqueous-based 'smart' biomaterial, and investigate the ability of molecular imprinting in this novel medium. From this starting point, as a result of the versatility of the hydrogel, several different strategies could be adopted as to how this material could best be employed for imprinting. Possibly the simplest method would be to use straight crosslinking of a modified chitosan, with the template entrapped within the bulk matrix. This follows the most straightforward of MIP synthesis protocols, in which the template is mixed together with one or more functional monomers, a crosslinker, a polymerisation initiator, and the solvent.[280] Following radical polymerisation, there remains a bulk polymer with the template localised at the areas of specific interaction. Depending on the degree of crosslinking, molecular weight, and physical form of the resultant polymer, this methodology may be a disadvantage to achieving good recognition, because of greater hindrance to the diffusion of large molecules within the 3D matrix.

An alternate strategy is to maximise the overall surface area of the MIP, thereby reducing the dependence on diffusion into/out of the gel, and allowing for the imprinting of large templates, e.g. biomacromolecules. Rather than making use of a bulky hydrogel for the MIP, the polymerisation could be manipulated to create a thin layer of imprinted material supported by a solid substrate. As has already been presented, this is a common technique for many applications of MIPs, and substantially mitigates the amount of raw materials required while also increasing access to imprint sites, particularly for large biomolecules. As a thin coating, an imprinted polymer could be used to coat a biosensor, create bioassays, or functionalise the surface of a biomaterial. Fukuzawa and Ishihara have described an artificial membrane with imprinting modification (cell adhesion through fibronectin templating) to target cell capture.[281]

While the thin layer of MIP may be a relevant model for coatings or biosensor applications, to further bridge the gap with biomaterials, an implantable or injectable system may offer additional advantages; for this one might consider smaller gel spheres that could easily be loaded or injected. Because the chitosan hydrogel and gelation system offer physical versatility and the polymerisation of it can effected by several different approaches, the currently proposed system affords flexibility in regards to the application method. It may also be prudent to consider the use of chitosan in conjunction with another polymer, either in the form of an interpenetrating network of both, or use co-crosslinking of the two. This would allow selection of secondary matrix polymers with properties that would augment those of chitosan alone.

Another principle objective of the research was to demonstrate successful improvement in the template recognition from molecular imprinting in aqueous media. Achieving a level of high specificity and selectivity in polar solvents remains a vital goal for scientist working with molecular imprinting. While some modestly successful reports have been made, these are still generally confined to small target molecules and charged species.[282] We aspired to design and construct a method capable of realising MIP recognition of larger, biologically relevant molecules. Following this line of investigation, a polymer choice for the research should provide the ability to explore the aspect of macromolecular imprinting.

3.1 Bulk Polymerisation

An initial stage of investigation was necessary to confirm that a chitosan polymer can form an imprintable network when crosslinked. A preliminary study using a photocrosslinkable chitosan and model chemicals was proposed to meet this aim. A secondary objective was to examine the effects of polymer and reaction conditions on this imprint recognition. Sequentially, these studies could provide insight into the potentiality of peptide imprinting using similar methods.

The vast majority of molecular imprinting studies in the literature invoke bulk formation of the polymers. This is especially true of those preparations targeted as molecular sorbents.[283] As conventional means for chromatographic screening and detection, such MIPs can be used for sensitive and fairly rapid assays.[43] Bulk polymerisation is oftentimes followed by grinding and sieving of the polymer monolith, for applications wherein the MIP is packed into a column for chromatographic separation.[284] This is a rather crude production method, and results in inhomogeneous polymer particles of varying size.[115] This type of inelegant packing also becomes very difficult in applications where thin capillary columns are used for chromatography.[285] Furthermore, while access to imprint sites may be mitigated by the crushing of the polymer, there is also the potential when undertaking this procedure to disrupt binding sites in looser polymer networks. Thusly, for the purpose of an hydrogel as used in this study, which is relatively loose network, and for which only a cursory study is desired, a simple bulk monolith should provide sufficient available binding locations and suitable transport properties to template molecule such that an estimate of imprinting effect is discernible.

3.2 Thin Gel Surfaces

Some of the drawbacks of bulk polymerisation can be negated by using other methods to form the polymer, such as turning to MIP membranes[286, 287] or surface coatings,[288, 289] which offer sizable binding area to volume ratios. These techniques can increase the surface area accessible for imprinting and binding, and limit the use of excessive template, which is often expensive, and cannot be fully recovered. As one of the key hindrances to imprinting larger biomolecules remains diffusion into and out of the polymer network to reach the imprint sites, it is conceivable that certain systems will whave a size-limited function to template recognition. One method to resolve this is to focus on imprint creation localised at the surfaces of polymers. Outside of overcoming the diffusion difficulties, there are a few immediately evident advantages to this procedure. The maximisation of the surface-to-volume ratio considerably reduces the amount of material (both polymer and template) required, thus lowering the cost of the

overall MIP manufacture, and cutting the amount of raw template needed, which can be hard to come by and expensive. Thin layers of polymer can be used for coatings of other biomaterials already being applied for supportive or other structures, thus adding a molecularly imprinted functionality to change the interfacial biological response. For larger molecules which would remain in the proximal boundary regions of the surface, this effectively increases the site-to-MIP ratio, which means increased imprinting per volume. This would also result in a greater unit change in binding and release events, yielding a higher signal when the MIP is employed as a sensing device.

Thin MIP coatings have created some complexity for researchers. Numerous methods, such as 'sandwiching' the pre-polymerisation mixture between two substrates,[290] have often been used, but these have difficulty in creating thin, homogeneous layers of polymer. Other techniques such as electropolymerisation[291] or surface initiation[292, 293] can be explored, but these require specialised conductive substrates and complex chemistries, respectively, which severely limit their mass application. Whereas MIPs are typically highly crosslinked networks, solutions of them are often too viscous to efficiently spread to a uniform layer by techniques such as spin-coating. One possible method is to use imprinted microparticles dissolved in a coating material which can then be cast into thin layers.[294] Another alternative is to add the MIP components solubilised to a surface and in use *in situ* polymerisation. This can also pose a challenge with traditional imprinting mixtures, as the crosslinking can often demand long reaction times and elevated temperatures, both of which, combined together with the high surface area of exposure, can lead to very rapid evaporation of the volatile solvents commonly used. Phase separation in this case would lead to incomplete polymerisation and inhomogeneity of the film. Achieving a fine balance of monomer solubility and concentration with reaction rate and solvent evaporation is necessary to enable uniform coatings. These physical constraints must be coupled with the already significant formulation issues for creating MIPs, to reach a suitable level of thickness, density, and porosity, to allow binding recognition. Schmidt and Haupt have reported on some methods of controlling phase separation kinetics to achieve MIP films of good thickness control and porosity with photopolymerisation.[295]

The use of thin-layer MIPs has been a rapidly growing niche of the field, particularly in the subject of biosensors. Small amounts of polymer can be used in conjunction with a solid substrate to support a large imprinted region. Kan *et al*., for example, achieved highly sensitive and specific dopamine recognition by using a MIP coating on carbon nanotubes.[296] A novel approach to creating nanowires from the pores in alumina membranes has proved to lead to highly selective MIPs,[297] though the design system does not fully allow for imprinting of larger biomolecules or cells.[298] Some authors have explored using simple non-covalent interaction to adsorb protein templates on solid surfaces which can then be impressed via 'micro-contact printing' into the polymer solution.[299] This produces a very thin sensor layer on a substrate with relative ease and utilising only minimal amounts of material.

3.3 Microparticles

One approach which shows great promise from both analytical (detection) and drug delivery standpoints is the use of imprinted micro- and nanospheres. With very high surface areas and surface-to-volume ratios, such MIPs can maximise the potential binding, while also allowing flexibility with being injectable, packable, shapeable, etc. Depending on the desired size of the particles, different polymerisation methods can be chosen (see Figure 3-1).

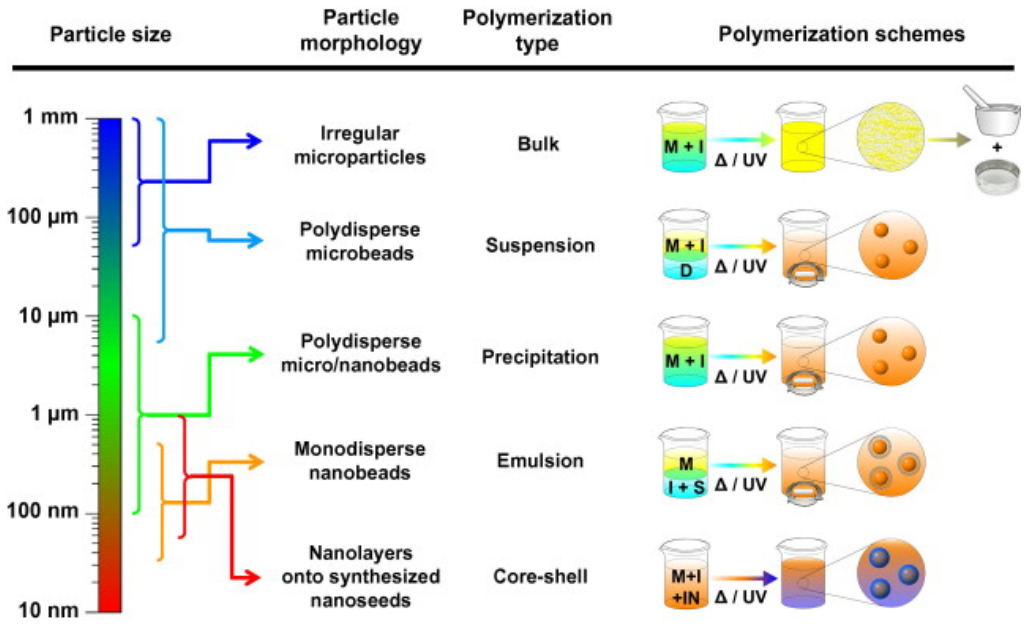


Figure 3-1. Fabrication methods used for small-scale MIP particle formation (reprinted from Díaz-Díaz *et al.*).[118]

Microspheres and nanospheres are usually produced by emulsion polymerisation, which incorporates an added advantage that might be of benefit to this particular research project. Because imprinting has been shown to be more effective in organic solvents, and as many drugs and other molecules of interest in molecular imprinting are hydrophobic, it can allow equally good imprinting at the interface with hydrophilic solutions, which can later be used under aqueous conditions for rebinding of targets, such as in the body. An innovative methodology was put forth by Tovar *et al.*, which makes use of a combined template-surfactant molecule, allowing improved tuning of the interface and eliminating the need for an added emulsifier.[300]

3.4 Additional Considerations

3.4.1 Crosslinking Methodology

A primary objective of this research was to demonstrate the ability for molecular imprint recognition in a water-based system that would be useful for biologically relevant applications. Chitosan was chosen as a strong candidate polymer for this goal. For optimum stability of the template and broad use in biological settings, we wished to

maintain an environment as close to natural/physiologic conditions as possible, which included a pH near neutrality. In order to obtain successful polymerisation of the polymer in aqueous media at pH 7, glycol chitosan, a water soluble derivative of the polysaccharide, was selected for use as a starting material. Unlike the unmodified form of the molecule, glycol chitosan does not require a low pH solution to aid in solubilisation of the polymer. Based on previous research, this polysaccharide could be further modified to form a photocrosslinkable material, according to the procedure of Amsden *et al*.[301] UV-initiated crosslinking of the polymer was selected as it has shown to be more effective in terms of resultant MIP selectivity compared with thermally-cured polymers.[302] In addition, photocrosslinking was chosen for its versatility, allowing direct *in situ* polymerisation, relative ease, and rapid polymerisation times. The molecule glycidyl methacrylate was used as modifying agent to act on the glycol chitosan side groups for subsequent crosslinking in forming the hydrogel network. The methacrylate epoxy ring undergoes ring-opening, and can reduce non-specific hydrophobic interactions.[39] It has also been used as a co-monomer in past MIP syntheses.[303]

3.4.2 Removal of Template

It was also necessary, in the course of this research, to closely observe the washing stage used for template removal from the MIP. Most studies of molecular imprinting utilise an acid wash to free any template from within the polymeric matrix. A weak acid such as acetic acid is usually sufficient to dissociate the bonding between polymer and template, though some studies have used more harsh methods. Often the acid wash is also coupled with the use of a surfactant, such as sodium dodecyl sulphate, to aid in disrupting the interactive bonds holding the template in place and help in solubilising it for removal from the MIP. However, such acid/surfactant treatments, while effective in removing most of the template molecules, may also cause undesired changes in the polymeric matrix, and could result in alteration of the imprinted binding sites, leading to a reduction in binding capacity, or a loss of specificity as a result of "false-imprinting."[217] In point of fact, any condition which causes changes to the imprinted

polymer network, e.g. swelling or contraction, can lead to a loss of true imprint site recognition.[8, 15] This may account for the commonly reported occurrence of seeing more template uptake post-crosslinking than was initially imprinted inside the polymer.[304] It is nonetheless important to use a treatment that can eliminate the trapped template, as sometimes even robust methods require repeated wash cycles to fully remove any leachable template.[227] This is especially true of large molecules like amino acids and proteins, whose native size hinders simple removal from the polymer.[212] Thus, it is a concern to develop a washing step that is efficient to remove the majority of trapped template molecules, but will not induce any chemical changes at the host sites or to the polymer backbone. Because the interactions between template and the chitosan matrix are fairly weak, and the matrix for this hydrogel in its swollen state is quite open (i.e. high porosity and interconnectivity), we judged a simple extended wash with aqueous buffer and repeated changing of the media (to maintain an infinite sink condition) to be sufficient to remove any template from the gel.

4 Materials and Methods

4.1 General Reagents

Except where otherwise indicated, all reagents used were purchased from Sigma-Aldrich or Fisher Chemicals. Water used for dissolution, dialysis, and forming buffers/reactant solutions was of Milli*Q* Type I purity, obtained from a Barnstead NANOPure Diamond filtration unit (Barnstead/Thermolyne, Thermo Fisher Scientific, Waltham, MA). Human serum albumin was purchased from Sigma-Aldrich (Oakville, ON). FITC-conjugated *IgG* protein was purchased from Abcam (Toronto, ON). Fluorescently labelled proteins human serum albumin-Cy3 and avidin-fluorescein were obtained from Protein Mods (Madison, WI). pH adjustments of solutions were accomplished via addition of either 1 M HCl or NaOH solutions, and verified on an Accumet AB15 pH meter, accurate to 0.01 units. Machining of acrylic parts and assemblies was done in the Departmental workshop (for construction of the impedance flow cell), or contracted out from the machine shop in the Department of Mechanical Engineering (Teflon gelation mould).

4.2 Analytical Equipment

Light-contrast and visual confirmation of microsphere size and surface patterning was done using a Nikon TE2000-U Eclipse microscope (Nikon Instruments, Melville, NY) equipped with Retiga 2000R QImaging CCD digital camera (QImaging, Surry, BC). Fluorescence images were taken on a Zeiss LSM 510 confocal scanning microscope (Carl Zeiss, Jena, Germany). Aqueous solutions were concentrated at low vacuum via rotary evaporation on Büchi R-200 rotavapor with B-490 heating bath set to 38^{o}C (Büchi Corporation, New Castle, DE). Samples for nuclear magnetic resonance (NMR) and gel permeation chromatography (GPC) characterisation were prepared by freezing solutions at -20^{o}C overnight, followed by lyophilisation on a Thermo Savant ModulyoD freeze dryer (Thermo Scientific, Waltham, MA) attached to VLP2000 vacuum pump and VLPOF110 recirculating filter. Dry chitosan was stored in a humidity-free environment until used. UV absorbance measurements were conducted with a μQuant

MQX200 plate reader (BioTek, Winooski, VT) in standard 96-well tissue culture plastic microplates. Particle size analyses were conducted on a Brookhaven Instruments Corporation ZetaPALS combined zeta potential/phase analysis light scattering particle analyser (Brookhaven Instruments Corporation, Holtsville, NY). Glass surfaces for binding and gelation were first treated with 100% ethanol followed by DI water and dried under N_2 stream. The surfaces were then further cleaned by plasma treatment inside a PlasmaEtch PE-50 benchtop plasma cleaner (Plasma Etch Inc., Carson City, NV), under low atmosphere (100 mTorr) and oxygen feed for 2 min. Scanning electron microscope (SEM) images were obtained on a Hitachi S-4700 field emission gun scanning electron microscope (Hitachi High Technologies America, Inc., Shaumburg, IL). Samples were dried and sputter-coated with gold-palladium under an argon atmosphere prior to SEM analysis.

4.3 Experimental Polymer Design

4.3.1 Purification

Flaked glycol chitosan (GC) as received was first dissolved in water (75 ml) and purified through filtration with coarse (P8, 20-25 μm pore size) cellulose filter paper (Fisher Scientific, St. Laurent, QC) to remove insoluble impurities. The filtrate was then dialysed against water (1 L) for eight hours in molecular weight cutoff (MWCO) 50 kDa dialysis tubing (Spectrum Labs, Rancho Dominguez, CA), changing the membranes and surrounding media at four hours. This further removed smaller dissolved molecules and impurities from the chitosan solution, and narrowed the molecular weight distribution of the GC. Following purification, the GC was characterised in terms of its molecular weight and functionality by running GPC and NMR analyses, respectively (per procedures given below).

4.3.2 Initial Molecular Characterisation

An important factor when working with multifunctional polysaccharides is accurate determination of the structure and functionality of the molecule, as the type and number

of groups present on the individual sugar units can have a profound impact on the ultimate properties of the material. Chitosan is a cationic chain of *n*-acetyl *D*-glucosamine and *D*-glucosamine residues, coupled through a β (1-4) glycosidic bond, and as such it is desirable to determine to what degree each of the corresponding moieties is present in the polymer chain (typically referred to as the degree of acetylation, DA). Glycol chitosan, chosen for its enhanced water solubility, has also been substituted to some degree by the incorporation of hydrophilic glycol groups. Thus, it was sought to discern the accessibility of free amine groups and elucidate the structure of an average glycol chitosan molecule constituting the polymer chain.

Purified, lyophilised glycol chitosan was characterised by ^{1}H NMR. Analysis was conducted at the Québec/Eastern Canada High Field NMR Facility, using a Varian INOVA 800 MHz spectrometer equipped with an elevated temperature HCN triple resonance probe. Samples were dissolved in D_2O (Cambridge Isotope Labs, Andover, MA) at a concentration of 15 mg/ml and pre-heated for six hours prior to analyses to improve solubilisation of the polymer and attain a clear spectrum. Spectral data was collected at 75^{o}C using Varian software, with trimethylsilane as an internal reference for chemical shifts. For observation of the effects of shifting the ^{1}H NMR spectrum, e.g. to isolate individual peaks of interest suffering from overlap, pH adjustments of dissolved samples were made by adding small amounts of either 1 M HCl or 1 M NaOH solutions to deuterated NMR samples.

4.3.3 Chitosan Modification

4.3.3.1 Molecular Weight

A critical factor towards impacting the ultimate gel obtained following crosslinking is the molecular weight (MW) of the chitosan before polymerisation. The MW will affect the physical and chemical properties of any resulting material, and can play an important function in fine-tuning the gel parameters to suit desired target applications. The chain MW will also play a key role in efficient template recognition during molecular imprinting. Glycol chitosan, as received from Sigma Aldrich, possessed an

average molecular weight of approximately 80 kDa. As it was theorised that a lower MW polymer will lead to a lower molecular weight between crosslinks and higher crosslink density, and thus, create smaller imprint spaces to yield ancillary recognition around the template molecule, the MW of the chitosan was adjusted as a factor in evaluating imprinting effect. Reduction of polymer MW was achieved through acidic de-polymerisation with $K_2(SO_4)_2$ at elevated temperature (70°C).[305] The process for fractionation of glycol chitosan was adapted from procedures proposed by Allan and Peyron and Hsu *et al*.[306, 307] The reaction schematic is shown in Figure 4-1.

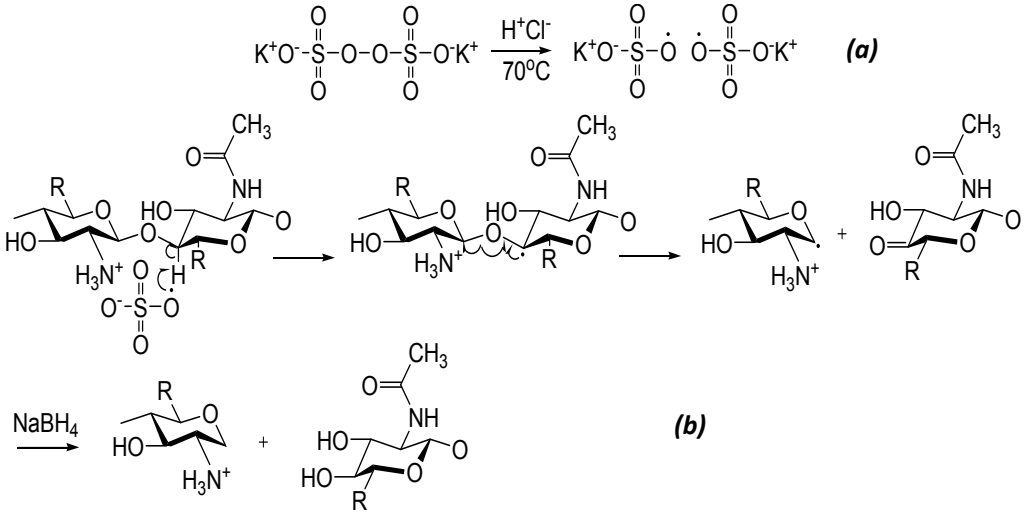


Figure 4-1. Proposed reaction mechanism for the acid-initiated fractionation of glycol chitosan by potassium persulphate.[308] (a) Acidic initiation at elevated temperature of potassium persulphate into reactive radical species. (b) Radical attack of chitosan chain groups by reactive sulphate groups, cleaving polymer molecule between residues.

At certain time points during fractionation of the polymer, the reactions were ceased by cooling to room temperature and reduced for 30 min with $NaBH_4$. Finally, they were neutralised and remaining free radicals quenched by bubbling under a low flow rate of air for 24 h. Collected samples were concentrated to a small working volume (~ 30 ml) on a rotovap. Reaction by-products and residuals in the reduced MW chitosan were removed by dialysis against 1 L water using MWCO 1 kDa regenerated cellulose tubing. Dialysis was run for a four hour period to purify fractionated chitosan polymer, with a change of filtration membranes and media after two hours. By this procedure, the molecular weight of glycol chitosan was adjusted to an approximate

range of 12-30 kDa. Molecular weight of fractionated polymer was assessed through gel permeation chromatography (GPC) analysis obtained using on a Polymer Laboratories GPC-50 Plus, equipped with UV and dynamic angle light scattering (DALS) at 0 and 45^{o} detectors. The columns used were PL aquagel-OH. Samples were eluted in 0.2 M AcONa/0.3 M AcOH buffer at 30^{o}C and 1 ml/min. Lyophilised chitosan was dissolved in fresh mobile phase at a concentration of ~1.25 mg/ml. Refractive index increment (dn/dc) values were calculated empirically by the software for each sample using the known concentration dissolved.

4.3.3.2 Chemical Group Substitution

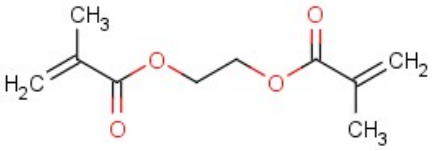
To facilitate gelation into a hydrogel, GC was modified with photoreactive methacrylate groups via addition of glycidyl methacrylate (GMA) to aqueous GC solution at elevated pH.[301] Briefly, dissolved GC was raised to pH 9.00 through the addition of NaOH, to which varying amounts of GMA were then added (typically ranging from 0.2-0.3 % v/v). The functionalisation reaction was allowed to proceed under stirring at room temperature (RT) for 48 h. Following completion the solution was neutralised to pH 7 and products were dialysed against water (1 L) for 8 h, exchanging the surrounding sink media and dialysis membranes at the halfway point. The degree of crosslinking of the hydrogel depends heavily on the amount of functional groups added to the chitosan backbone. Successful addition of functionality and degree of substitution with methacrylate moieties was monitored by ^{1}H NMR spectroscopy. Spectra were obtained in D_2O at high temperature (80^{o}C) with an 800 MHz Varian spectrometer.

4.4 Crosslinking Optimisation

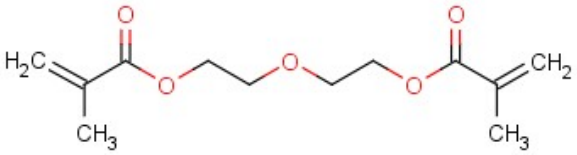
In examining the efficiency of imprint recognition, it is inherent to consider the physical nature of the polymer matrix. This is going to be dependent on the nature of the crosslinks formed; the type, length, and amount of crosslinking agent used will affect the properties of the network created. The conditions of gelation can also significantly impact the characteristics of the polymer mass resulting from crosslinking.

As the process is a chemical reaction, environmental factors that may affect the reaction kinetics, such as temperature or concentration, will change the determining rate and alignment of the polymer crosslinks formed.

Two commonly used crosslinking agents were investigated as additional chemicals to help bridge the chitosan gel network. Both are also initiated via free radical reaction, and target the double bond functionalities added to the chitosan chain groups via GMA. Ethylene glycol dimethacrylate (EGDMA) and diethylene glycol dimethacrylate (DEGDMA) are very common agents used in polymer crosslinking. They are also used extensively in molecular imprinting formulations. As can be seen in Figure 4-2, their structures are very similar, with the latter having one more ethylene glycol group, making the molecule slightly longer and causing a reverse orientation of one of the methacrylate end groups.



(a)



(b)

Figure 4-2. Chemical structure of two crosslinking agents, (a) EGDMA and (b) DEGDMA.

While both of the crosslinkers succeeded in achieving rapid crosslinking of the polymer, their limited water solubility (particularly for DEGDMA) proved problematic. The crosslinkers tended to remain in a poorly miscible organic phase, and post-crosslinking could be seen to have largely self-reacted in ‘droplets’ of PEG polymer suspended separate from the larger bulk of the chitosan hydrogel.

The time allowed for the crosslinking reaction was a critical factor impacting the stability of the hydrogels formed. Whereas the functionalisation and photocrosslinking systems used enable relatively rapid gelation of a bulk solution, taking place in just a matter of minutes, this is not necessarily the ideal method for this particular application (i.e. molecular imprinting). Rather than quickly forming a rigid polymer, for optimum imprinting it is desirable to achieve a polymer network that forms uniformly at a consistent, controllable rate. This should create a better organisational structure around the template (i.e. pre-polymerisation complex) and result in a more permanently stable physical gel. It is also necessary to allow sufficient time in solution for interaction of the monomer/polymer reagents and template to form the pre-polymerisation associated complex. As the creation of the MIP is not crucially a time-sensitive process, sacrificing very rapid formation in favour of an improved gel structure is not of great concern, and is balanced by the augmented benefit of stronger gel imprinting achieved.

The rate of photopolymerisation of the chitosan solution is moderately controllable by external factors such as the degree of ultraviolet exposure and temperature. The amount/intensity of light bombarded on the initiator and the length of exposure time will affect the number and rate of free radical production. The solution temperature affects the evaporation of solvent from the system as it polymerises, and alters the diffusion rates inside the media. Maintaining the other conditions of the solution constant allows for a practical adjustment of the crosslinking time. Furthermore, in the photocuring box being used, both of these states can be varied singularly by controlling the distance of the sample away from the light source.

While a few different light sources were investigated for the photocrosslinking of the chitosan hydrogel, the Dymax curing chamber was found to provide the most effective method. Although this particular unit does not provide for the ability to adjust the intensity used (having exposure control only through an on/off shutter) it was possible to vary the distance from the light source through the use of internal shelving and supporting structures placed underneath the sample to be cured. A problem

encountered was that the high intensity bulb used in the Dymax curer creates elevated temperatures, and for many trials in close approximation to the source, this caused a portion of the solvent to evaporate rapidly from the polymer solution, before thorough crosslinking could occur throughout the chitosan. What resulted were thin, weak films of polymer, that would often break down during re-swelling due to insufficient amount of crosslinks in the matrix. However, there was some issue in previous experiments where other light sources did not provide enough of an intensity in the chitosan solution to generate the free radicals necessary to initiate polymerisation. This was a contributing factor in the choice of the Dymax system, as its higher luminosity provided enough exposure even at the bottom of the curing chamber. An optimum distance of approximately 20 cm from the shutter was found to be adequate to balance the solution exposure to UV light with sufficient distance to limit the heat at the polymer surface, and was used for subsequent gelation experiments.

5 Results for Chitosan Polymer

5.1 Characterisation and Conversion Results

5.1.1 Chemical Structure

Nuclear magnetic resonance (NMR) was used to determine the functionality for each batch of the glycol chitosan used in experiments. In order to obtain the best resolution of the peaks of interest, for the purpose of identifying functional groups and integrating for quantification, initial studies were investigated using pH-adjusted deuterated solvent. This was done to examine the shifts in the NMR spectrum of certain proton peaks with protonation/deprotonation, to observe whether an optimum condition existed for running the spectroscopy wherein the particular peaks of interest for analysis would be most deconvoluted from neighbouring peaks, allowing the clearest identification of distinct functional groups and obtain accurate integration values thereof. While concentrations and pre-treatment conditions were held constant, small adjustments to the dissolved sample pHs were made through the addition of HCl or NaOH. An overlay of the effect this addition had on the spectra of an example purified glycol chitosan sample (identical batch, prepared in similar fashion at different pH levels) is shown in Figure 5-1. From this, it was evident that running the ^{1}H NMR of dissolved samples with no further adjustment in pH provided the optimum spread of peaks for discriminating the individual groups and observing the chitosan molecular structure.

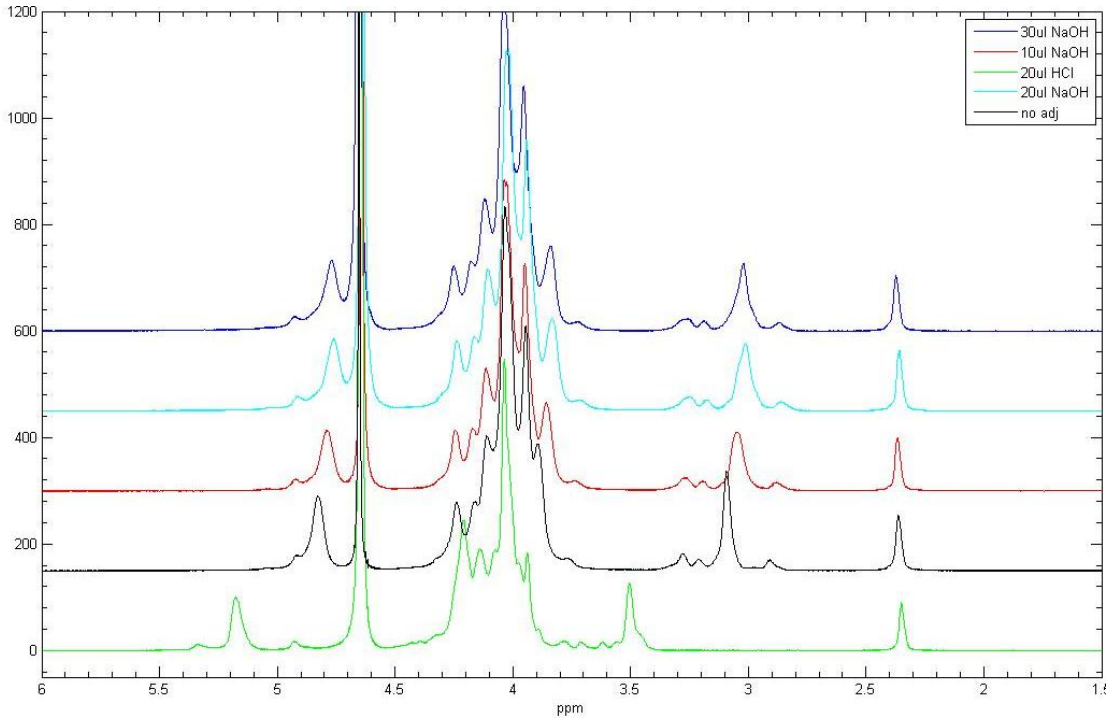


Figure 5-1. Upfield and downfield shifts in proton peaks of ^{1}H NMR spectra with small adjustments in pH for a purified glycol chitosan sample (run in D_2O at 80°C). Deuterated polymer solutions for NMR were pH-adjusted by adding incremental amounts of 1 M solutions of acid or base, as shown.

Based on the NMR results of purified samples of glycol chitosan used in this work (n = 12) and those of previously published studies by the author, including ^{13}C and coupled magnetic resonance spectroscopy, the proposed chemical structure of glycol chitosan is shown in Figure 5-2.[305] This suggested molecular structure is slightly different than the commonly accepted formula for glycol chitosan in that, as depicted, in addition to the acetylation of the amine groups along the GC backbone, there is the possibility of single and biglycolation at these sites.

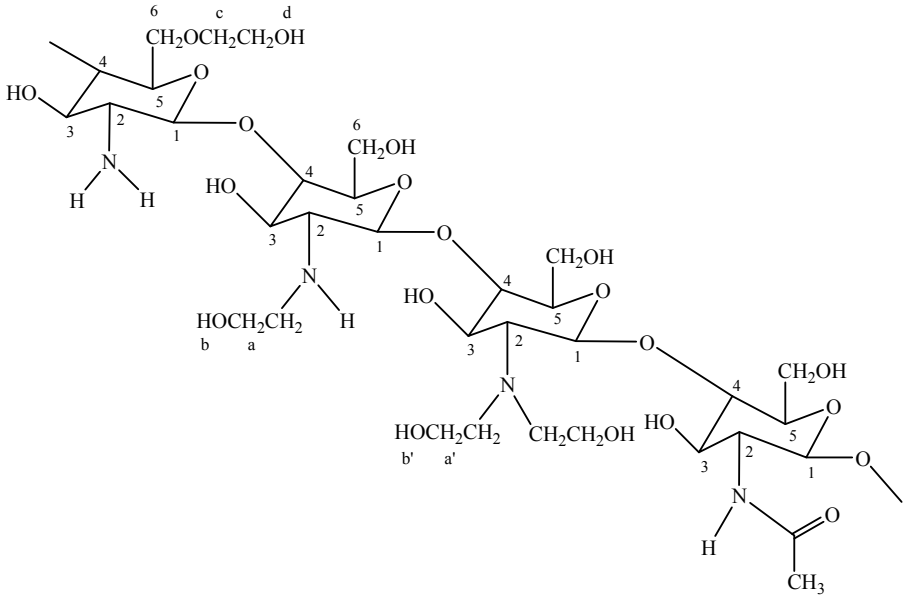


Figure 5-2 Chemical structure of glycol chitosan depicting side group substitutions, as proposed from summary of NMR studies.[308]

Each sample of GC was collected and lyophilised for NMR characterisation, and spectra were confirmed to conform to an identical structure. The ^{1}H NMR of backbone protons for glycol chitosan following the purification procedure is shown in a representative sample spectrum in Figure 5-3. The corresponding protons of the glycolated groups for the same sample are noted separately in Figure 5-4 for clarity, and to illustrate the change for nitrogen-adjacent protons specific to the glycolated case.

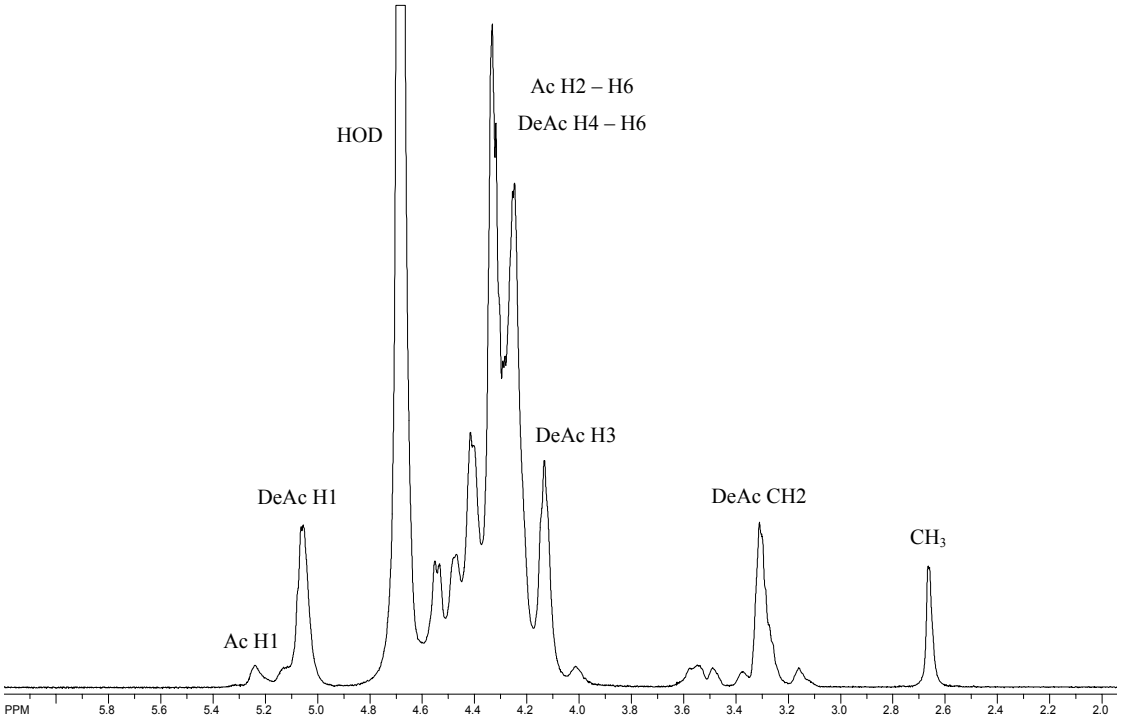


Figure 5-3. [1]H NMR spectrum of a purified glycol chitosan sample with specific peak assignments for saccharide unit backbone protons common to chitosan polymers. (Ac = acetylated residues, DeAc = deactelyated residues)

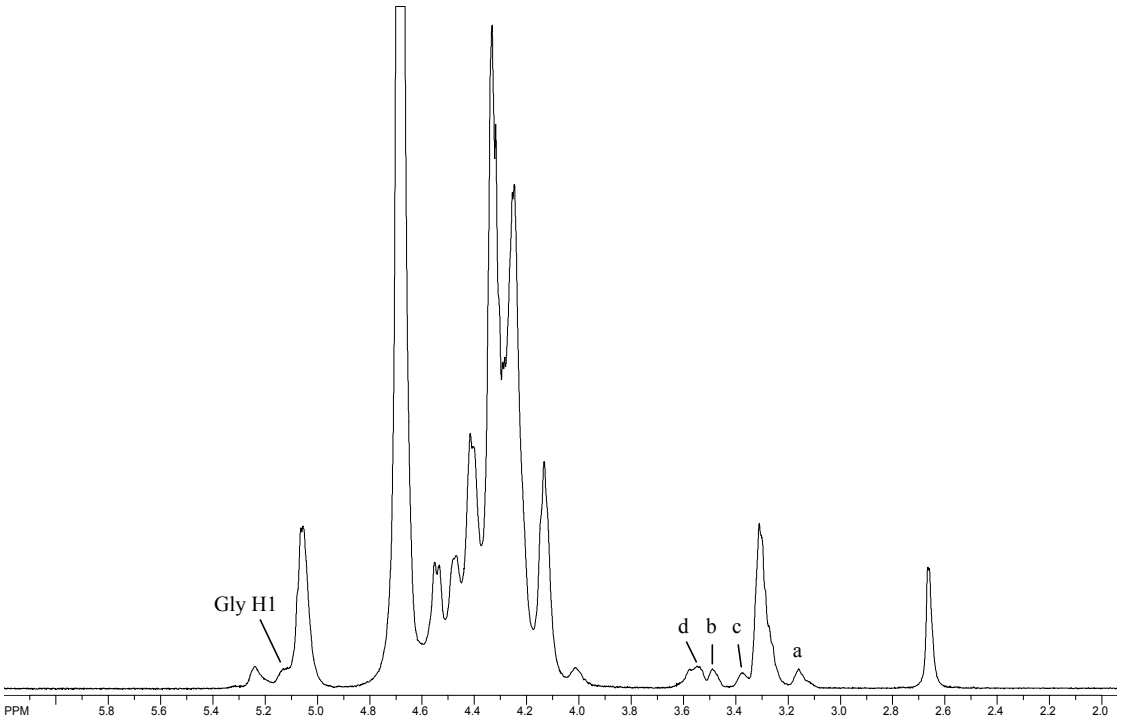


Figure 5-4. [1]H NMR spectrum of a purified glycol chitosan sample with peak assignments for glycolated H1 protons and protons adjacent to nitrogen (a-d). (Gly = glycolated residues)

Table 5-1. Peak assignments of functional groups derived from ^{1}H NMR spectra and corresponding integrations for glycol chitosan from the above shown sample.

Chemical Shift (ppm)	Proton Assignment	Approx. Integration
2.67	CH_3	1.00
3.16	Gly a	0.24
3.30	DeAc H2	2.23
3.38	Gly c	0.24
3.49	Gly b	0.25
3.54	Gly d	0.49
4.02-4.60	Ac H2 to H6 DeAc H3 to H6	28.17
4.70	HOD	63.07
5.05	DeAc H1	2.36
5.13	Gly H1	~ 0.2 (heavy overlap)
5.22	Ac H1	0.31

Using the integrations seen in Table 5-1, it is possible to estimate the degree of acetylation for the glycol chitosan polymers. The protons arising between 5.05 and 5.22 ppm must be due to H1 proton for all of the respective cases, therefore a sum of all integrations will give an approximation of the number of residues per glycol chitosan chain. Accordingly, a ratio of the methyl protons of the acetyl group to this value should represent the degree of acetylation (DA), as illustrated by Equation 5.1.

$$Degree\ of\ acetylation = \frac{I_{CH_3} \big/ 3}{I_{H1_{TOTAL}}} x\,100 \qquad \textbf{[5-1]}$$

Based on this calculation, the sample spectrum shown above (Figure 5-3) gives an approximate degree of acetylation of 11%. This value was representative of most samples, as all glycol chitosan trials evaluated typically fell within a range of 9-13% DA. The residues constituting the remainder of the percentage of total polymer chain would be contributed by cases of primary, secondary, and tertiary amination [refer to Figure 5-2].

5.1.2 Polymer Chain Length

5.1.2.1 Initial Purified Samples

The molecular weights of the glycol chitosan following purification ranged from 70 kDa to 84 kDa (M_w), with an average value of 78 kDa ($^+$/- 4 kDa, n = 12). dn/dc values used for MW analysis for raw, purified and fractionated samples, calculated based on dissolved concentrations, averaged to 0.164 (n = 23). This value also reflected that estimated from the software, suggested based on the input amounts and chromatogram results, and is in close agreement with the commonly accepted dn/dc value of chitosan as reported by Rinaudo.[309] Table 5-2 gives exact values of the results from gel permeation chromatography for a few sample batches of purified GC. Some deviation between chitosan samples made under the same conditions was observed, but was not an unexpected result. When working with chemical reactions, particularly those involving polymers, even the most controlled reaction conditions and purification steps can lead to a distribution in resultant MW of the products. Even without any instrumental or reading error, it can very difficult to achieve precise control over reactants. This can be further confounded by distributions seen in even slight concentration differences or column integrity when running GPC.

Average polydispersity index (PI) values obtained through GPC analysis were near 1.51 with a standard deviation of 0.22 (n = 23). Most of the large variation in both weight average and number average molecular weights was due to sample disparity between batches of GC as received from the manufacturer. Glycol chitosan was obtained from both Sigma-Aldrich and Wako Chemicals. While the structure of the GC was independent of supplier and did not change from batch to batch, as confirmed by ^{1}H NMR, there were consistently two distinct batches of molecule sizes; one being near 75 kDa (M_w) and the other centered around 125 kDa (M_w). Because there were no detectable chemical or other differences between each lot, other than molecular weight, they were treated in the same fashion. Each individual glycol chitosan sample was, however, characterised by both NMR and GPC before and after each modification, and any changes in molecular weight accounted for. Whereas it was aimed to have a

polymer of lower molecular weight for the molecular imprinting studies, it was decided to focus the analyses and formation of the MIP with the lower starting MW batch of glycol chitosan.

Table 5-2. Example results from GPC analysis illustrating MW range and reproducibility of chitosan test samples following purification procedure. Each run shown represents an average of three injections onto the column.

Sample ID	Run #	M_n [Da]	M_w [Da]
1	*i*	59000	74000
	ii	49000	77000
	iii	54000	77000
2	*i*	55000	80000
3	*i*	51000	81000
	ii	49000	74000
4	*i*	52000	78000
5	*i*	59000	84000
6	*i*	51000	79000
7	*i*	52000	83000
8	*i*	46000	70000
9	*i*	51000	75000

5.1.2.2 Depolymerisation for MW Reduction

As a method to narrow down a nominal modification procedure of the chitosan, such that an effective molecular base for an imprinting polymer could be achieved and be fine tuned on a per need basis, a pattern of molecular weight reduction of the polymer through reactive chemical fractionation was developed. A better understanding of the relationship of degree of reduction in the initial chain length with depolymerisation treatment would allow for the eventuality of honing the procedure to attain a specific desired MW, as might be well-suited for targeted molecular imprinting applications. As, in the presence of acidic free-radical attack, the chitosan chain is susceptible to cleavage along its glycosidic bonds, the reaction will be ongoing provided the reactants remain available, until the solution is neutralised and/or quenched. By stopping the reaction at different time periods, we cease the further breaking of polymer chain groups, and can obtain a segregated distribution of polymer molecular weights. Figure 5-5 depicts the pattern of change in chitosan MW with varying depolymerisation

reaction periods. As can be seen from these results, little further change was observed to occur in the MW of the polymer with longer reduction periods beyond two hours. It appears that this was self-terminating reaction through either consumption of one or more of the reactants or limitations of polymer chain length. These results are in agreement with those of a previously published assay on the glycol chitosan depolymerisation.[305]

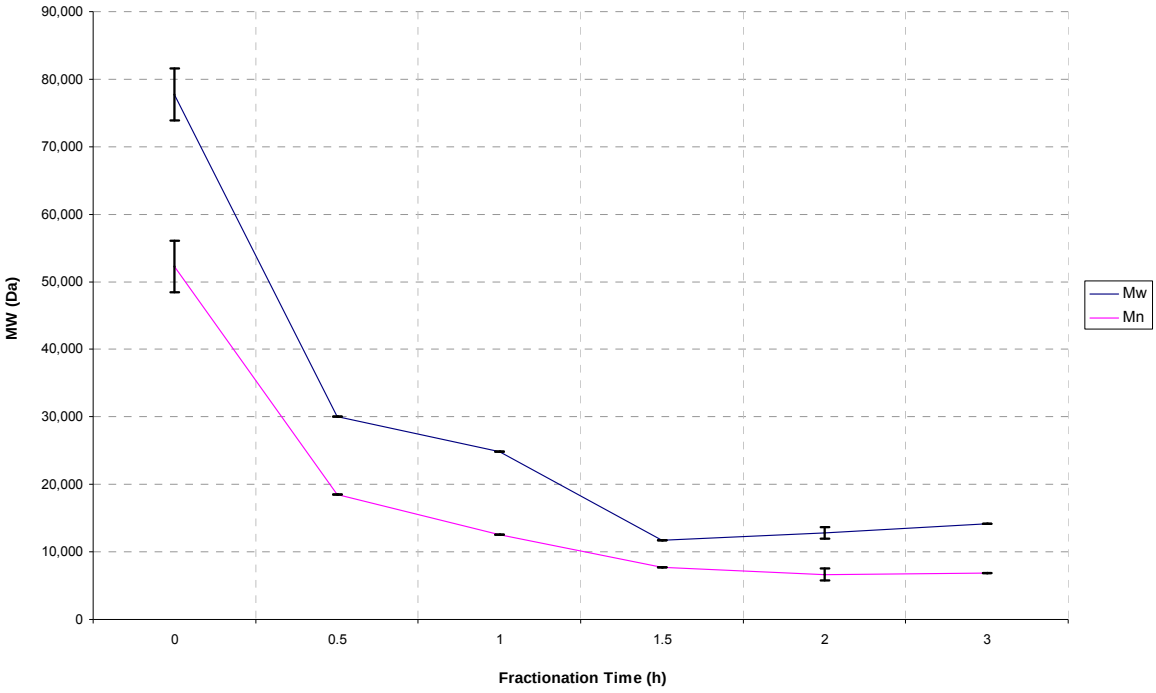


Figure 5-5. Effect of depolymerisation time on chitosan molecular weight. M_w = weight average molecular weight; M_n = number average molecular weight, as determined from GPC analysis.

5.1.3 Functionalisation

^{1}H NMR spectra were used to confirm the successful addition of free radical-reactive moieties to the polymer backbone, i.e. GMA incorporation onto GC functional groups. Studying of the chemical structure and the integration of each group also allowed for quantitative determination of the number of methacrylate bonds added to the glycol chitosan. An example spectrum of a typical methacrylated glycol chitosan (mGC) sample can be seen in Figure 5-6. Similar spectra were obtained for other methacrylated samples (n = 8), from which the degree of functionalisation could be determined. The

peaks at 6.21 and 5.88 ppm represent the two alternate positions of the terminal methacrylate protons (a1), while the peak at 2.48 arises from the methyl protons of the methacrylate group (a2), respectively, of the methacrylated GC residues following reaction. Peaks attributed to residual unreacted GMA show up at 6.73, 6.32, and 2.54 ppm, not seen in this spectrum, indicating that the purification process was successful, and that the peaks observed arise from protons of methacrylate groups attached to the glycol chitosan backbone. The degree of substitution of the GC molecule is found by averaging the integrations of protons of the methacrylate group and dividing this by the total contribution of the H1 protons, i.e. for the acetyl, glycol, and aminated cases. These are identified by the peaks at 5.25, 5.21, and 5.09 ppm, respectively.

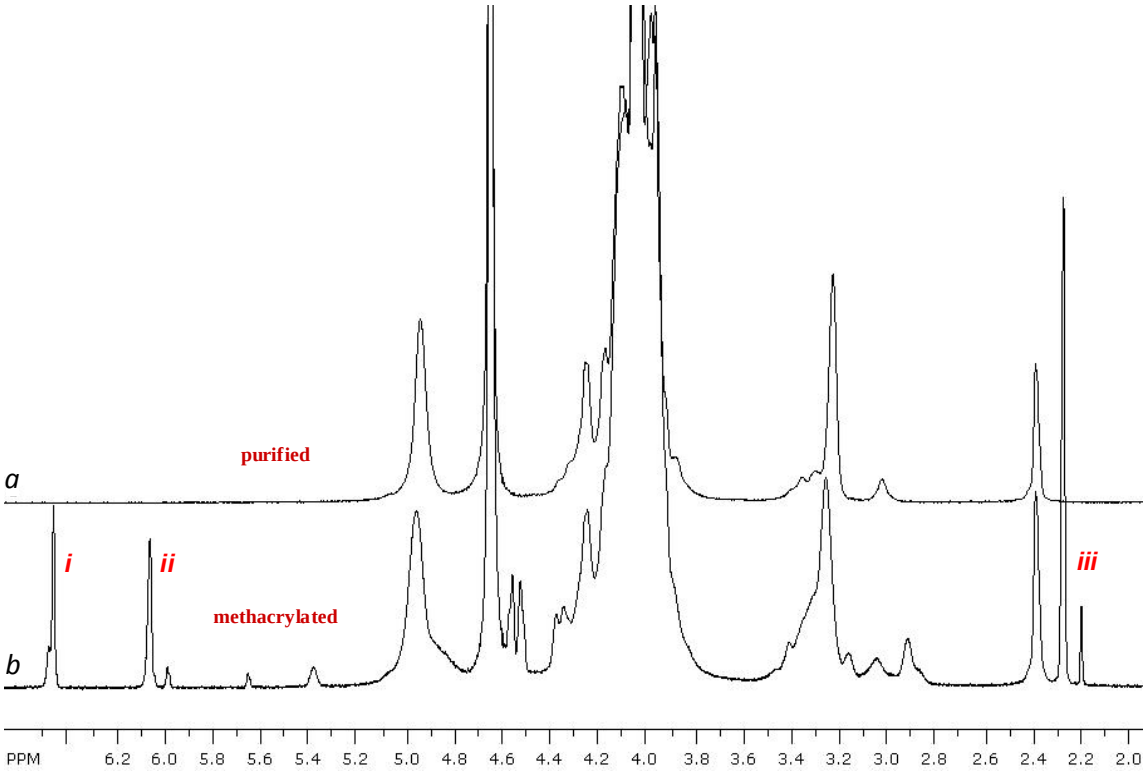


Figure 5-6. ^{1}H NMR demonstrating functionalisation of (a) purified GC polymer, top spectrum, and (b) following addition reaction with glycidyl methacrylate, lower spectrum. Peaks at *i*, *ii*, and *iii* represent the terminal protons of attached GMA moiety on the chitosan molecule.

Based on the above spectra, the formulated structure for modified glycol chitosan following the addition of the methacrylate moieties is shown in Figure 5-7.

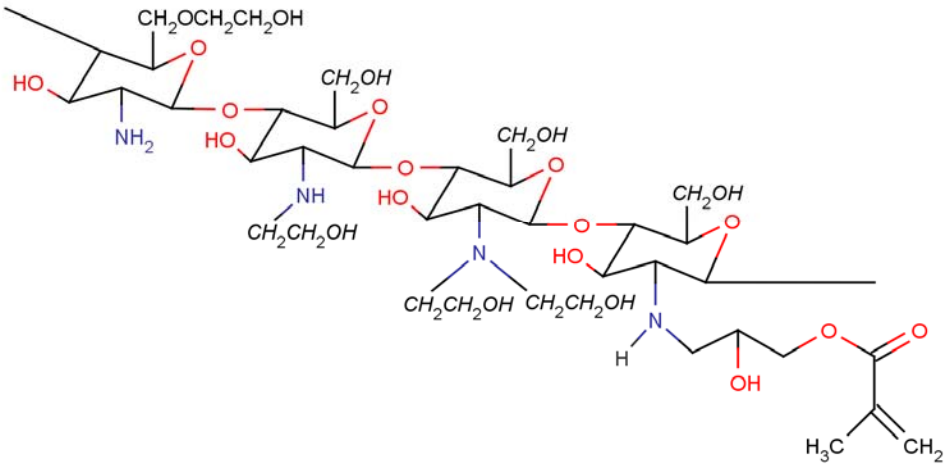


Figure 5-7. Chemical structure of glycol chitosan following GMA addition, as determined from a combination of single nuclei and coupled NMR studies.

While a higher degree of substitution (DOS) of the chitosan side groups would allow for improved crosslinking of the chitosan (i.e. more reactive sites present to form interchain bonds), over-substitution of the chitosan backbone with GMA leads to a loss in water-solubility, as amine groups are consumed and a more hydrophobic organic group is added, thus reducing the hydrophilicity of the polymer molecule. The optimal DOS for GC was found to be approximately 15%. This sustained a strong availability of double bonds in the modified chitosan for crosslinking the polymer into a stable gelled network, but preserved a sufficient degree of water solubility for the material.

6 Bulk Imprinting Study

A preliminary study was undertaken to evaluate the imprinting efficacy of the modified chitosan hydrogel in a bulk form. As a first investigative step, this simple method was inspired by a drive of three chief objectives: i) to evaluate the bulk imprinting model as an examination design for further MIP studies, ii) to determine the molecular recognitive ability of a chitosan hydrogel imparted through molecular imprinting, and iii) to identify some of the key factors that affect the imprint recognition, and to use these parameters for future optimisation of the polymer system. To meet these three desired objectives, an experimental protocol was developed to research each of these aspects in an overlapping fashion. A simple bulk imprinting study was conceived using a model template entrapped inside the chitosan matrix, whose release and recapture by the bulk polymer would be used to judge selective recognition towards the template imparted by the imprinting procedure. A few select factors were chosen for examination that were hypothesised to potentially affect this recognition, such as polymer molecular weight, template concentration, type of crosslinker used, and crosslinker concentration.

6.1 Experimental Groundwork

As initial model template for this study, the anthracycline doxorubicin (Dox) was chosen. This molecule was selected for the high number of electronegative functional groups it possesses that should theoretically give it several points at which it would allow for interaction with positive charges of the polymer. In addition, its red colour allows for easy identification in solution/gel, which provided a simple visual method to gauge residual presence of entrapped template in the gel. The chemical structure of doxorubicin is shown in Figure 6-1.

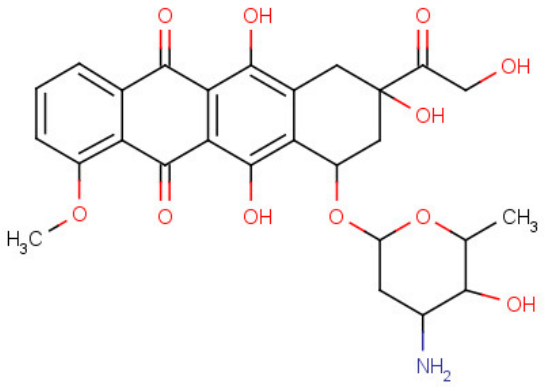


Figure 6-1. Molecular structure of doxorubicin, used as a model template molecule for bulk imprinting in chitosan.

The initial study undertaken involved bulk gelation of modified chitosan in the presence of the template molecule and with use of a crosslinker, ethylene glycol diglycidyl ether (EGDGE). Solutions of functionalised chitosan in water were concentrated to ~0.05 g/ml. Polymers were aliquoted into light-protected vials, along with template molecule (doxorubicin HCl), crosslinker (ethylene glycol diglycidyl ether), and initiator (Irgacure 2959), and thoroughly mixed. A 22 mm glass coverslip was placed in the bottom of a 24-well TCP plate, and activated polymers solutions (350 ml) were added to the wells. The solutions were gelled overnight under low-intensity UV light in a laminar flow hood. After gelation, MIP gels were soaked in water (2 ml each) for 12 hours, changing out solutions in 2-hour intervals to remove template and sol matter. They were then immersed in solutions of template at a concentration of 0.01 mg/ml. Release experiments were conducted in water for a 15 h period. All washings were done under gentle rocking to allow unbound material to fully dissolve and leach out of gel matrices. The degree of imprint capture was assessed via rebinding of template molecule (Dox), through indirect changes in UV absorbance of the analyte media. Each sample (i.e. differing pre-polymer formulation) consisted of an *n* of 3 (individual aliquots), and the average absorbance measuremements from multiple wells across samples was used. Absorbance units were converted to estimates of concentration of doxorubicin in solution using a standard calibration curve. The gelation conditions used were varied with samples, in order to evaluate the effect of each on post-polymerisation imprint recognition. These are summarised in Table 6-1.

Non-imprinted controls (NIP) had identical gel make-up to corresponding MIPs, but with the replacement of template solution by equivalent volumes of Milli-Q water.

Table 6-1. Example experimental design setup for examining parameters used for gel solutions in bulk MIP trials. These initial samples set out to observe effects of varying MW, template amount, and crosslinker volume; components predicted to affect the properties of MIP formation and hence target recognition.

Gel ID	Chitosan Sol'n.	GC Vol [μL]	Temp. Amt. [μg]	Xlink Vol. [μL]
MIP 1	high MW	350	100	0
MIP 2	high MW	350	500	0
MIP 3	high MW	350	800	0
MIP 4	high MW	350	1250	0
MIP 5	high MW	350	500	70
MIP 6	high MW	350	500	100
MIP 7	high MW	350	500	160
MIP 8	high MW	350	500	200
MIP 9	low MW	350	100	0
MIP 10	low MW	350	500	0
MIP 11	low MW	350	800	0
MIP 12	low MW	350	1250	0
MIP 13	low MW	350	500	70
MIP 14	low MW	350	500	100
MIP 15	low MW	350	500	160
MIP 16	low MW	350	500	200

The results of this study seemed to indicate some imprint recognition, as the bulk chitosan MIPs showed an ability to capture and release the model template. There also appeared to be a stronger binding of target for the lower MW polymer starting samples. Unfortunately, the gelation from this first cursory set of trials was inconsistent, and there were not enough replicates conducted to be able to reach any definitive conclusions from it. As such, it was only used as a guideline for constructing the model parameters of investigation in subsequent investigation designs. Though crude, this analysis provided an important basis to use in appropriate experiment selection in the proceeding stages.

6.2 Statistical Design of Experiments

As the preliminary bulk imprinting study showed promise towards examination of the formulation conditions on MIP properties, but only limited conclusions could be drawn from the results, a second bulk experiment was set up to attain more detailed information about the imprinting ability, and the specific affect of polymerisation parameters on ultimate template recapture. Given the high number of variables involved in forming the pre-polymerisation mixture and crosslinking, in order to obtain the most information from a minimum number of runs, statistical design of experiments (DOE) was selected as the manner of study for setting up the bulk polymerisation for the chitosan MIPs. These types of experimental designs involve the use of highly structured experimental runs and coordinated testing procedures in order to use mathematical models to estimate (and observe) the system effects. For a more detailed overview of statistical design and the experimental setup, the reader is directed towards the explanation provided in Appendix B.

This was an important step in developing the protocols for testing of the chitosan MIP, as it allowed for simultaneous examination of the effects and interactions of varying parameters during polymerisation on the recognition ability of the MIP produced. Whereas scrutiny of all of the individual effects involved in forming the MIP alone would require rigourous testing, extensive control of conditions, and numerous costly experiments, the same information can be obtained more efficiently in a fewer number of runs through DOE. It also permits the eventuality of optimisation of reaction conditions/formulations from the experimental model derived from such experimental design.

A selected number of statistical experimental designs have been used for the study of MIPs, such as full two-level factorial,[310] Doehlert,[311] and Plackett–Burman.[312] Because MIP synthesis is based on a complex arrangement of specific molecular components, it naturally lends itself to many combinatorial screening and chemical library synthesis methods,[313] particularly involving intricate modeling.[314, 315] However, as the process of chemical assembly during imprint formation is still very convoluted,

in many situations a simple empirical study can be of equivalent benefit to observe imprint effects. A comprehensive study by Rossi and Haupt demonstrates how simply choosing two factors to examine (e.g. the proportion of crosslinkers ethyleneglycol dimethacrylate and trimethylolpropane trimethacrylate used in polymerisation) can provide a significant degree of information in few runs for the composition and synthesis of MIPs.[316] Advances in manufacturing are moving many synthetic processes to rapid and automated systems; the creation of MIP formulations can take advantage of these methods.[317] An interesting approach proposed by Navarro-Villoslada and Takeuchi combines the process of chemical library screening with rapid throughput synthesis to prepare and evaluate through multivariate analysis a two-level fractional factorial design for MIP binding.[318] Dirion *et al*. applied a similar high-throughput screening method including a library of 80 polymeric components.[319]

Unsurprisingly, as with other polymerisation systems, the type and amount of crosslinker will impact the properties of the resultant material. This includes the ability for template recognition, as emphasised by Hillberg *et al*.[320] Hence, this aspect forms a very logical starting point for investigation of parameter effects. Two additional crosslinkers were included for evaluation as part of this research study. The first, diethylene glycol dimethacrylate, acts as a homobifunctional crosslinker which will react in the presence of free radicals induced by UV exposure. In contrast, the second crosslinker chosen was ethylene glycol diglycidyl ether, which crosslinks both hydroxyl and amine groups that would be present on the chitosan polymer. It is also well understood that the amount of template used in the pre-polymerisation mixture will lead to a particular degree of binding sites following MIP formation.[321] The template concentration was therefore chosen as the additional prime factor to evaluate in this study.

A modified central composite (Box-Behnken) design was set up for experimentation. Four variables were investigated, namely chitosan MW, template concentration, diethylene glycol dimethacrylate (DEGDMA) concentration (UV crosslinker), and ethylene glycol diglycidyl ether (EGDGE) concentration (chemical

crosslinker). Each variable was examined at three discrete levels estimated to encompass the relevant experimental range, with the addition of three centre points runs. Variables were coded and experiments randomised to eliminate as much external error as possible. The experimental design is summarised in Table 6-2.

Table 6-2. Experimental values of pre-polymerisation mixture conditions tested at three levels for various chitosan MIP gel formulations in the B-B design.

Experimental Condition	Level 1	Level 2	Level 3
polymer molecular weight [M_n]	low (16000)	med (33000)	high (78000)
template concentration [mg]	12.5	25	62.5
DEGDMA concentration [µl]	0	60	105
EGDGE concentration [µl]	0	6	15

6.2.1 Bulk Gelation of Chitosan MIPs

Template (Dox) was added to concentrated glycol chitosan solutions (~0.05 mg/ml) together with photoinitiator and crosslinker(s). The additional crosslinkers used were added to the solution in ratios expected to reflect their final composition in the gel, as well as crosslinking nature (free radical vs. chemical). The amounts of template added to the MIPs were chosen to be representative of relevant concentrations for template as part of a bulk gel, covering a range of imprinting efficacy. For non-imprint gels, the addition of Dox solution was replaced by equivalent volumes of Milli-Q water. After thorough mixing, initiated polymer solutions were gelled in Teflon moulds (see Figure 6-2) by 5 min exposure to high-intensity UV light in the Dymax 5000 UV photocuring chamber. Following polymerisation, bulk chitosan gels were soaked in water (10 ml each) for 60 hours, changing out solutions in 12-hour intervals to fully remove template and sol matter. Lack of residual template release from MIPs was confirmed by UV absorbance (480 nm) of external solution on a microplate reader (μQuant). Crosslinked polymers (MIPs and NIP controls) were then immersed in solutions of Dox template at a concentration of 0.08 mg/ml. All soakings were done under gentle agitation on a single axis rocking table to allow unbound material to dissolve out of gel matrices.

Based upon the relatively weak detection results seen in the initial bulk imprinting study, it was decided to extend the observational period for the expanded investigation for a longer timeframe, to see if further template release could be elucidated. After either a 70 h or a 15 day period, template release from the bulk gels was analysed by absorbance of aliquots (n = 8) of soaking solutions. Statistical significance of the results was evaluated using a Student *t*-test, for two samples assuming unequal variance.

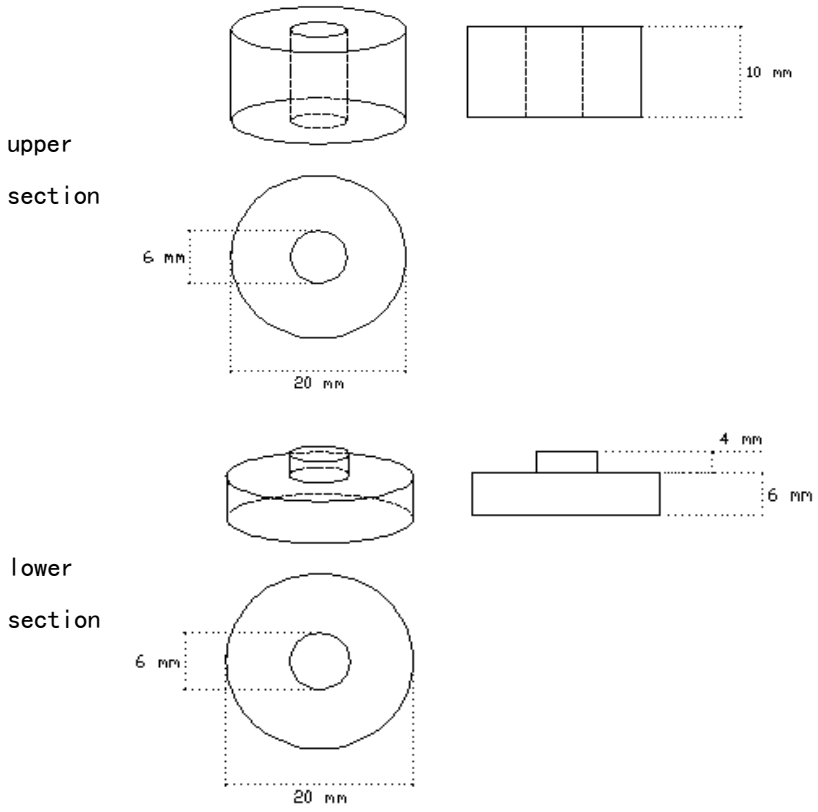


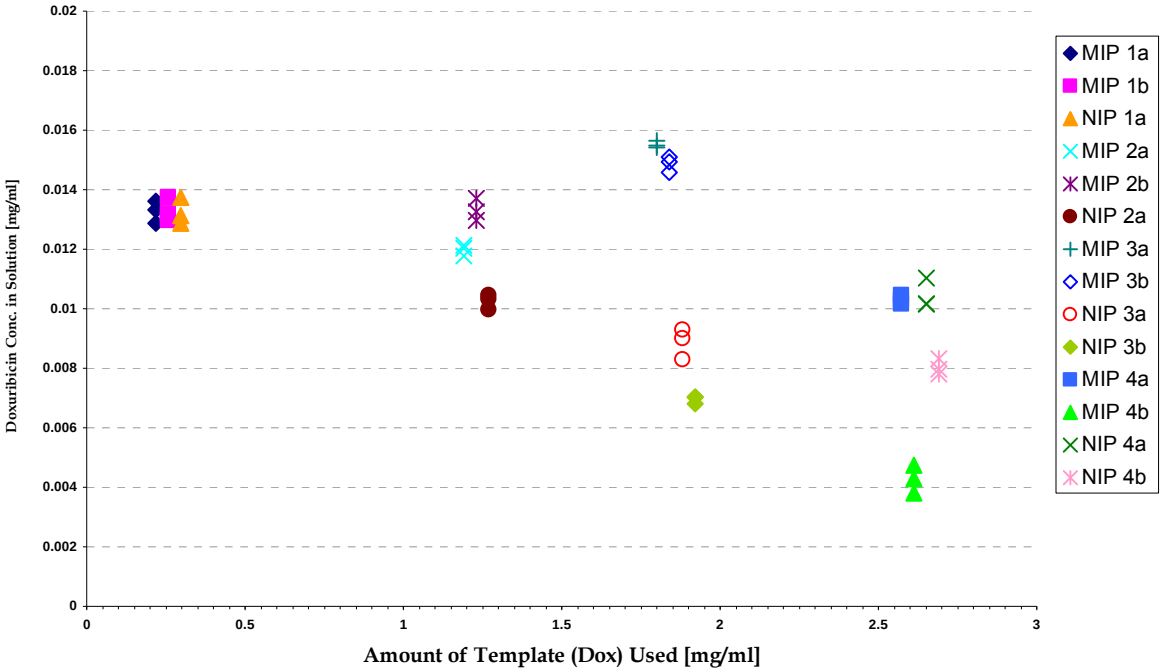
Figure 6-2. Schematic of two-part Teflon mould constructed for the gelation of bulk chitosan cylinders.

6.3 Results for Imprinted Polymer Hydrogels

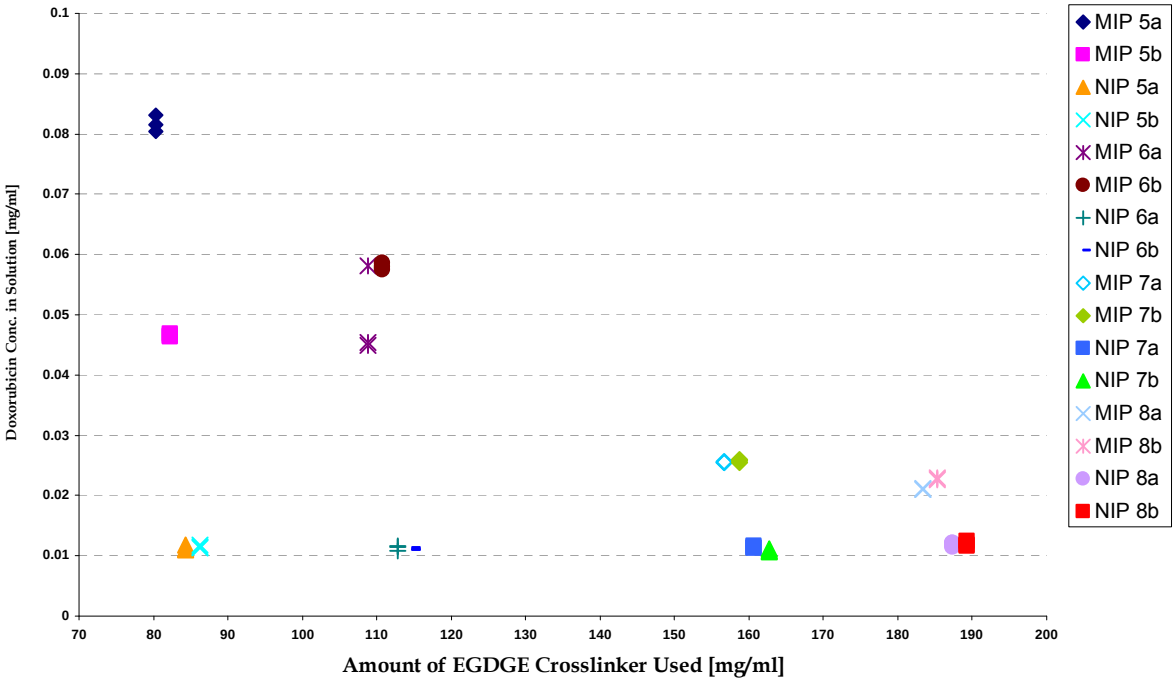
6.3.1 Initial Bulk Imprinting Study

A summary of the imprinting results in bulk chitosan gels, as estimated by the change in absorbances of soaking solution following template (Dox) release after 15 hours, are shown below, in Figure 6-3 and Figure 6-4, for the higher (unfractionated) MW

functionalised GC and for the reduced MW GC, respectively. In each Figure, *(a)* represents results for examining the doxorubicin release from the gels when the amount of template used in the pre-polymerisation formula was altered, under normal crosslinking, while *(b)* demonstrates the release results from those gels which had the template held constant, but which investigated the effects of change in concentration of an incorporated additional co-crosslinker to develop strengthened crosslinking in the network and binding of target molecule.

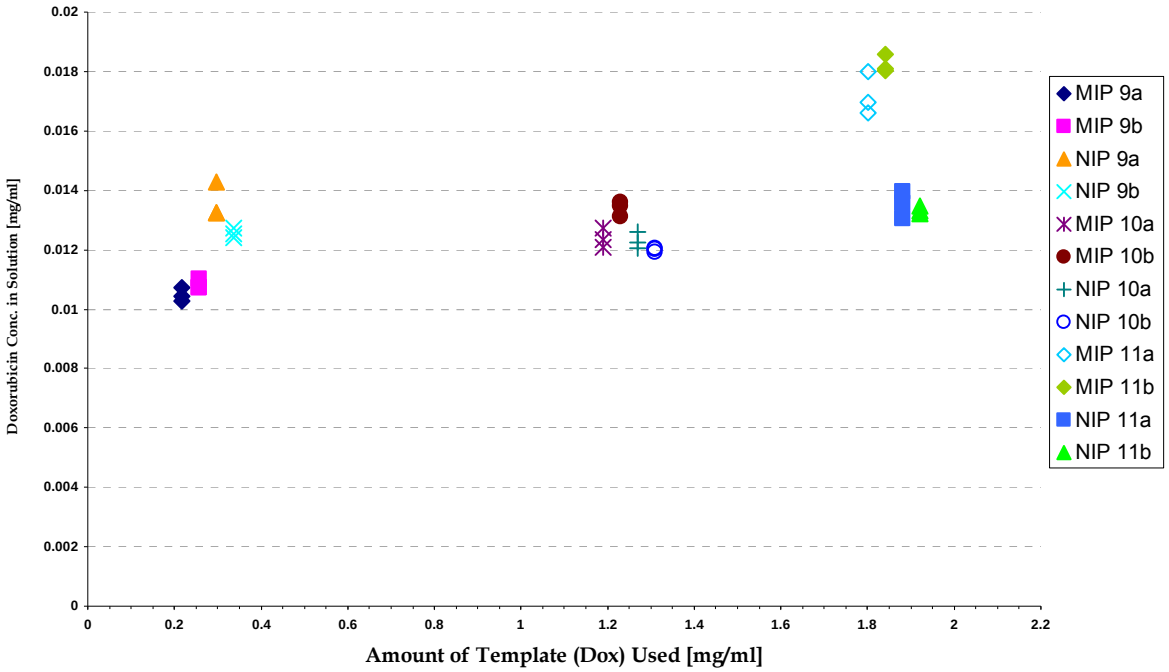


(a)

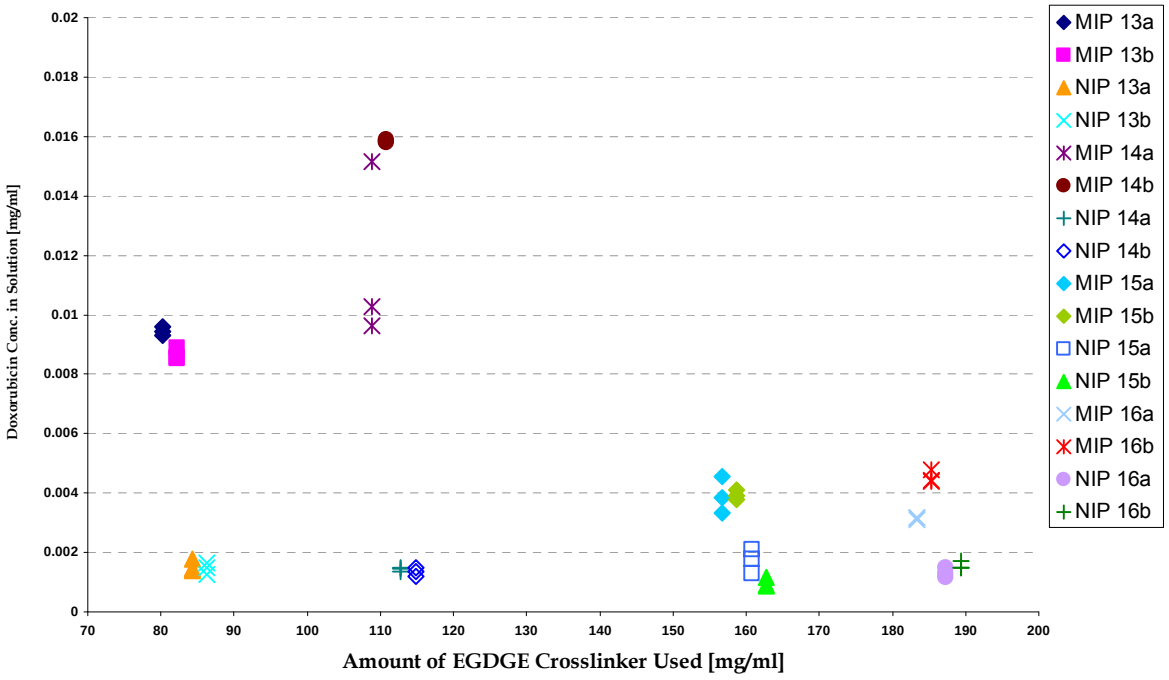


(b)

Figure 6-3. Change in solution concentration after 15 h release study of template (Dox) from gel for rebinding studies conducted with high MW chitosan. Numbers indicate particular formulation samples (refer to Table 6-3 below for specific conditions), while letters reflect replicates of the gel examined. MIP: molecularly imprinted polymer, NIP: non-imprinted control polymer.



(a)



(b)

Figure 6-4. Change in solution concentration after 15 h release study of template (Dox) from gel for rebinding studies conducted with reduced MW chitosan. Numbers indicate particular formulation samples (refer to Table 6-3 below for specific conditions), while letters reflect replicates of the gel examined. MIP: molecularly imprinted polymer, NIP: non-imprinted control polymer.

Table 6-3 - Polymerisation formulations for Dox-MIP gels used in preliminary bulk gelation study. Non-imprinted controls (NIP) were made in the same fashion, with the replacement of template solution by equivalent volumes of buffer.

Gel Sample	MIP Formulation
1	unfractionated polymer, 280 μg/ml template, no crosslinker
2	unfractionated polymer, 1.3 mg/ml template, no crosslinker
3	unfractionated polymer, 1.9 mg/ml template, no crosslinker
4	unfractionated polymer, 2.6 mg/ml template, no crosslinker
5	unfractionated polymer, 1.1 mg/ml template, 83 mg/ml crosslinker
6	unfractionated polymer, 1.0 mg/ml template, 110 mg/ml crosslinker
7	unfractionated polymer, 0.9 mg/ml template, 160 mg/ml crosslinker
8	unfractionated polymer, 0.8 mg/ml template, 190 mg/ml crosslinker
9	reduced MW polymer, 280 μg/ml template, no crosslinker
10	reduced MW polymer, 1.3 mg/ml template, no crosslinker
11	reduced MW polymer, 1.9 mg/ml template, no crosslinker
12	reduced MW polymer, 2.6 mg/ml template, no crosslinker
13	reduced MW polymer, 1.1 mg/ml template, 83 mg/ml crosslinker
14	reduced MW polymer, 1.0 mg/ml template, 110 mg/ml crosslinker
15	reduced MW polymer, 0.9 mg/ml template, 160 mg/ml crosslinker
16	reduced MW polymer, 0.8 mg/ml template, 190 mg/ml crosslinker

A general, weak imprinting effect was seen consistently for each of the samples in which change of the template concentration was being investigated. This could be explained with the presence of high amounts of residual template molecules (remaining from pre-polymerisation mixture following crosslinking) providing a higher concentration in solution as they leach from the MIP gels. This would suggest insufficient washing was performed prior to the recapture study. Although a common practice to remove template molecules from MIPs, a washing step with AcOH/SDS was not used, as it has been reported to yield false-positive readings with molecularly imprinted chitosan.[217] While no distinct imprinting effect could be observed for all of the samples, it is difficult to elucidate any effect the amount of template had on imprint recognition. It was expected that an increase in template should produce more imprint sites, which would result in higher degree of subsequent binding to the MIP; to some maximum value where the presence of the template interferes with polymerisation of the matrix or begins to self-aggregate and limit proper imprinting in set sites. When using the higher MW polymer, an increase in the template amount used during crosslinking did not result in improved recognition by either of the MIPs. For the lower MW chitosan, however, we did observe an increase in the amount of release of Dox

from MIP gels versus the control NIPs, as the amount of template used in the pre-polymerisation formula was increased. The amount of Dox released from chitosan MIPs increased with higher template levels used, while the NIP gels maintained a fairly constant level of release. At the high value of template concentration (approximately 1.8 mg/ml in pre-polymerisation solution) there was a distinct imprinting effect, where $\text{Dox Release}_{MIP} > \text{Dox Release}_{NIP}$. This suggests using higher levels of template during the polymerisation of an MIP when possible, in subsequent molecular imprinting studies with this model.

The additional use of EGDGE in the pre-polymerisation solution had a more pronounced effect on achieving molecular imprint recognition. For template release of the bulk chitosan gels made using the crosslinker, we saw a consistent higher level of doxorubicin being released after 15 h from imprinted gels than controls. This held for both MWs of polymers investigated. Using increased crosslinker amounts in the pre-polymerisation solution produced an increased in the MIP recognition, however, resulted in lower uptake and release of template during the rebinding stage for both chitosan MWs. For any samples tested which incorporated crosslinker at concentrations beyond ~ 112 mg/ml, we noted a pronounced drop off in the amount of released Dox, and a noticeable poorer response compared to the corresponding NIP controls. Although more template was released from imprinted samples, the imprinting effect was diminished with higher crosslinker concentrations. This was a not altogether unexpected result, as the tighter network imparted by an additional crosslinker should provide greater interaction with the template molecules, up to a certain value, beyond which the narrowing of the matrix mitigates proper envelopment of the template, and as well becomes too great too allow efficient diffusion into and out for target exchange to occur. This also supports the findings of other studies, in that the benefits of using additional crosslinker in the pre-polymerisation formula of an MIP are only manifested up to a certain level.[147, 149, 213]

While no distinct trend was able to be evident from the small sample size used for this investigation, it appeared from these cursory results that the lower MW

polymers faintly improved the imprinting efficacy versus their larger chain counterparts, as the proportional uptake and release amounts against the controls were slightly greater on the average across all MIPs tested. Having lower MW chains as a starting monomer would support improved imprinting effect, as a tighter chitosan network should result in greater recognition of the template during the pre-polymerisation stage. Similarly, as we observed the use of EGDGE as a crosslinker to improve the efficacy of the MIP, such tighter networks do imply a greater ability to offer a distinct binding enhancement as a result of the molecular imprinting process, suggesting confirmation of these results. The overall released amount of Dox for the lower MW chitosan, however, was lower than for the unfractionated polymer. Thus, the ultimate application of the MIP is important, as to whether a greater need for high loading amounts is desired, such as in drug delivery applications, or whether stronger differentiation over a control surface (i.e. enhanced imprinting effect) is more imperative, such as might be needed for biosensor applications, that require high sensitivity to changes or employ a reference element (e.g. NIP) as an internal control. This carries with it implications in tuneability of the imprinted polymer and varied applicability across differing platforms.

While this initial experiment provided only a general understanding of the molecular imprinting in glycol chitosan and was limited to just a few conditions and a small sample size, it did yield some important information of note. Particularly, we saw from these test gels that there was a strong imprinting effect evident when the template was held constant at approximately 1 mg/ml concentration. In each case, the MIP provided a greater release of the Dox template than the NIP polymer of the same formulation. While difficult to draw conclusive arguments from the limited number of samples involved, as a cursory study these results nonetheless validated the ability of the bulk chitosan hydrogel to be a legitimate material for molecular imprinting, and this study forms a compelling case to justify further experimentation of chitosan MIPs.

Greater study was needed as a follow-up, using more samples and with an improved and consistent gelation process, to expand this bulk investigation with an

additional and broader range of lower MW residues, keeping in mind that, with the increased rigidity of the lower-MW network, the chains are going to be less flexible and able to re-arrange, therefore potentially yielding a lower degree of interaction with the template, while also limiting the diffusivity of molecular targets to and from imprint sites. Similarly, it appeared evident that there is some optimum level at which the inclusion of a crosslinker will support enhanced molecular imprint recognition, but beyond which it actually has a negative effect on the amount of capture template. As it was difficult to derive any substantiated concrete assertions from this study, and no qualitative or statistical analysis if the admittedly limited results was possible, the next aim was towards investigating bulk imprinting in chitosan gels using more formulation conditions and with a greater number of samples. This also enabled more conclusive statistical comparisons to be drawn with regards to the recognition effects of the chitosan MIPs.

6.3.2 Examination of Experimental Parameters

Changes made to the gelation procedure, such as the use of a Teflon mould (Figure 6-2) for shaping the hydrogel during crosslinking, and using a more intense light emission source for photoinitiation, while also allowing for more thorough pre-polymerisation completion and exposure to completion, offered a significant improvement over the prior study. Resultant chitosan gels from polymerisation with these apparatuses were thicker and more consistent, and were produced in more rapid fashion. Examples of the gels resulting from this crosslinking method are shown below in Figure 6-5. The presence of the Dox template is evident in these photos from the strong red colour seen in the MIPs versus the clear/white colour in the NIPs. It is also clear that the additional use of crosslinker in the pre-polymerisation mixture yielded a translucent gel as a result of higher hydrophobic separation of immiscible phases, whereas the gels produced without additional crosslinker remained transparent.

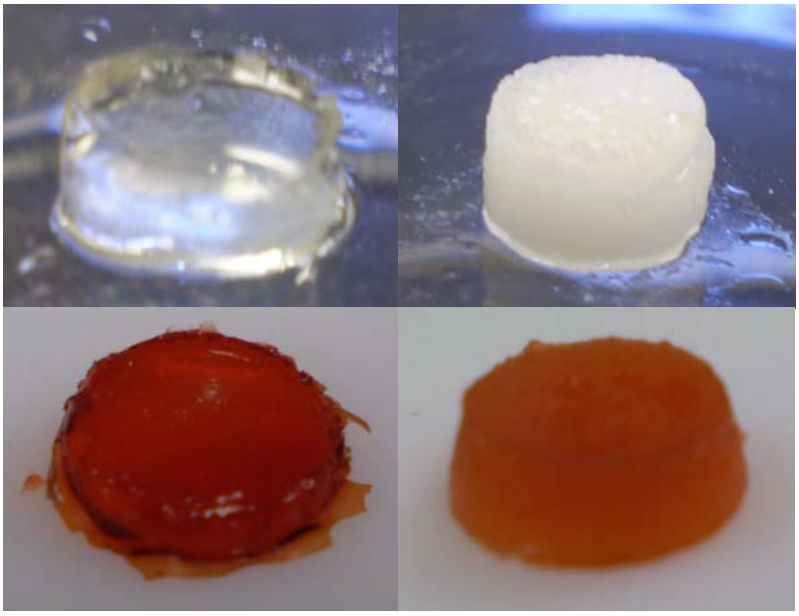


Figure 6-5. Visual appearance of sample chitosan gels following UV photocrosslinking of initiated solutions in Teflon moulds. Without Dox template molecule, top (NIP); with trapped template molecule, bottom (MIP). Gels on left hand side were made using DEDGMA as crosslinker, those on right with EGDGE.

Results from the series of experiments conducted according to the Box-Behnken design were used to gauge the amount of template captured by MIPs and their corresponding NIPs, to observe the effect of molecular imprinting in the chitosan gels, and the contributions to the imprinting effect from certain individual polymerisation parameters examined The release of captured model template (doxorubicin) from the bulk chitosan gels was evaluated by changes in UV absorbance in the surrounding media, at time periods of 70 h and 15 days. These results are summarised graphically in Figure 6-6 and Figure 6-7.

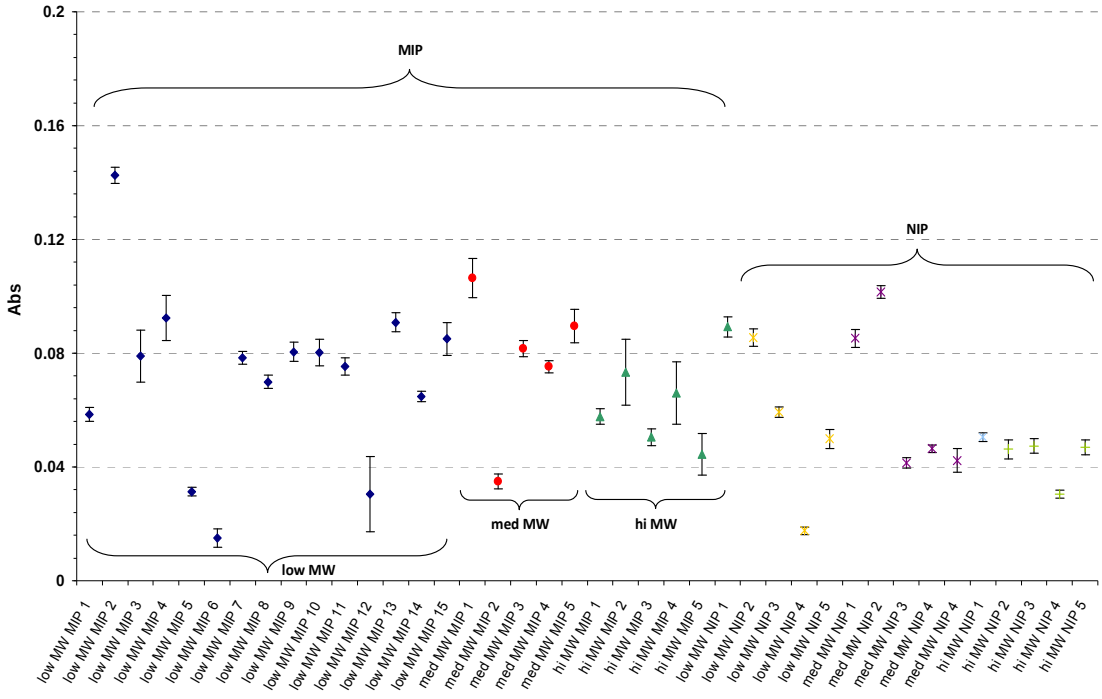


Figure 6-6. Measured absorbances indicating release of doxorubicin template from varying formulations of bulk (monolith) molecularly imprinted chitosan gels after 70 h soaking period.

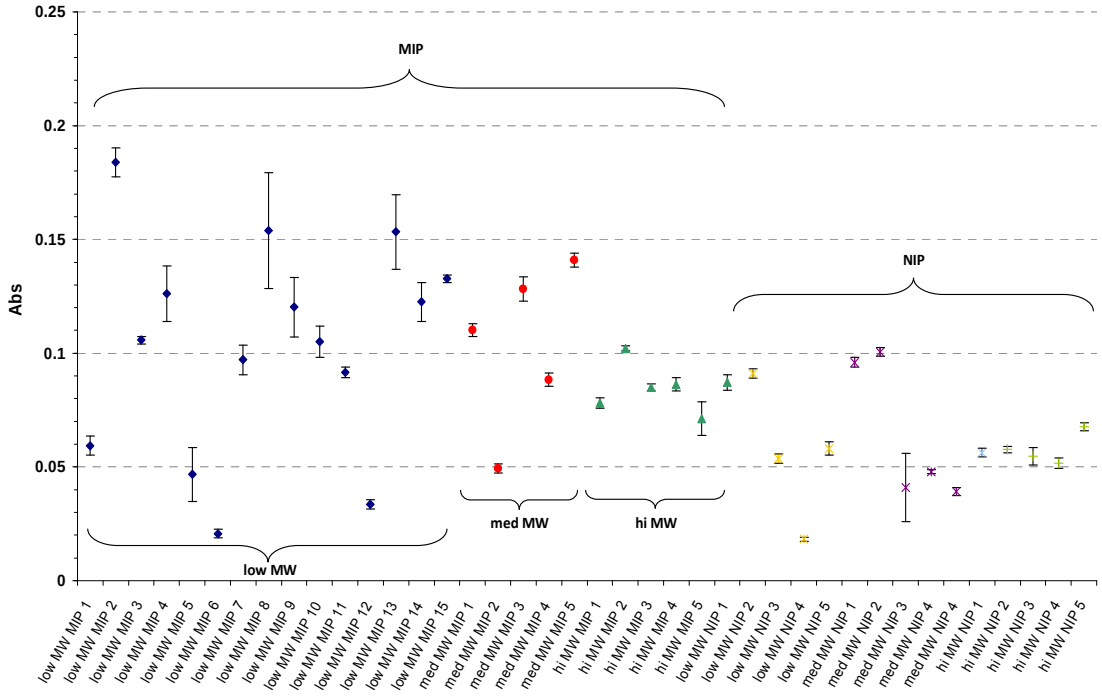
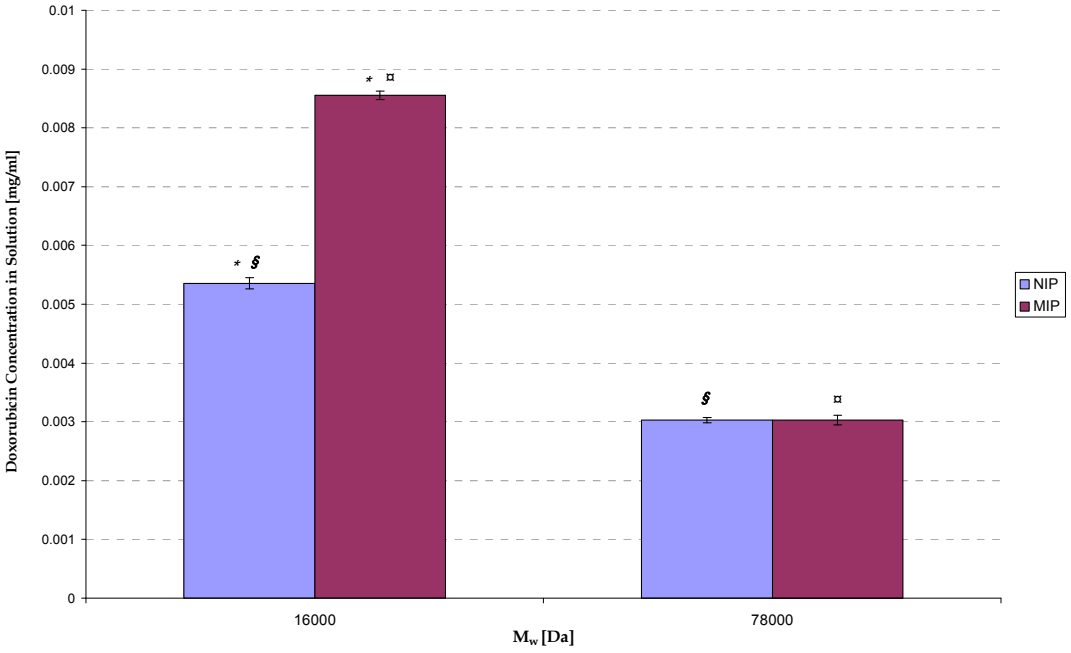
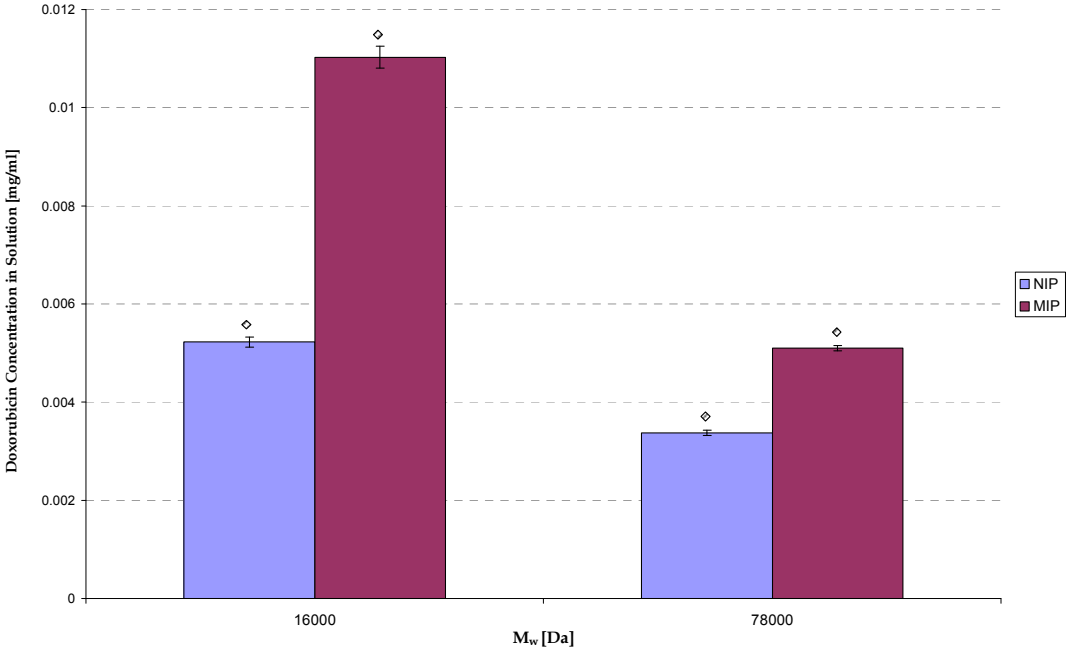


Figure 6-7. Measured absorbances indicating release of doxorubicin template from varying formulations of bulk (monolith) molecularly imprinted chitosan gels after 15 day soaking period.

While the graphical representations presented above contain considerable information, it is perhaps easier to summarise the results from the experimental design by examination of some of the individual paired samples to observe discrete effects. Figure 6-8 to Figure 6-13 display a summary of a few of the isolated parameters used for examination in the B-B experimental design. Each sample was tested for significance both in pairing with its corresponding NIP, and/or similar polymer(s) made with altered parameter set (e.g. each other sample appearing on the same plot below, and the respective non-imprinted control). Statistical significance of the data was assessed using a two-tailed, Student *t*-test with an α value of 0.05, assuming unequal variances in the data sets. Nearly all paired samples examined were found to be significant, in that we can say that means of the data sets were different from one another at 95% confidence, with the exception of a few paired cases unmarked with any notation in the figures below. Significance is depicted graphically in the respective plots by symbol pairs for each of the conditions examined. For ease of visualisation of the plots involving the 15d time point, wherein all the examined pairs of formulations were found to be statistically significant from every other sample, this has been noted individually. Notably, as an immediate observation, it is evident that the chitosan gel samples showed greater significant difference after the 15d time period, indicating the longer time allowed for release in these cases provided better assessment of the differences in captured Dox template by each formulation, and therefore also a more relevant gauge for evaluating molecular imprinting effect. This also suggests that the Dox release from the bulk chitosan polymers followed a more gradual release, as opposed to an immediate mass or burst release as the monoliths swelled in solution.

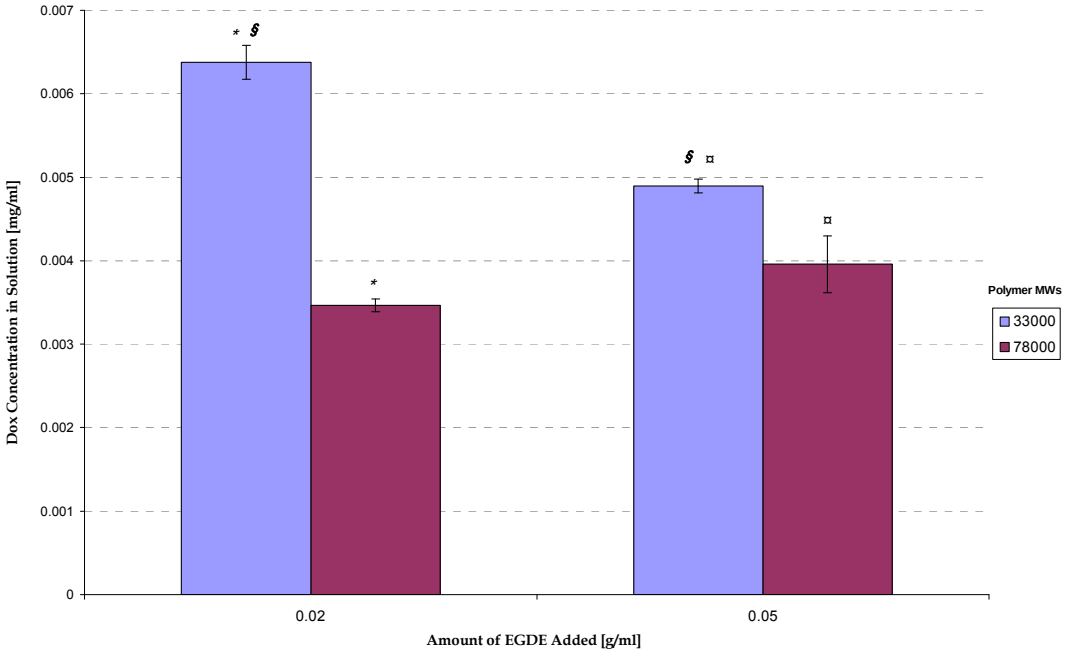


(a)

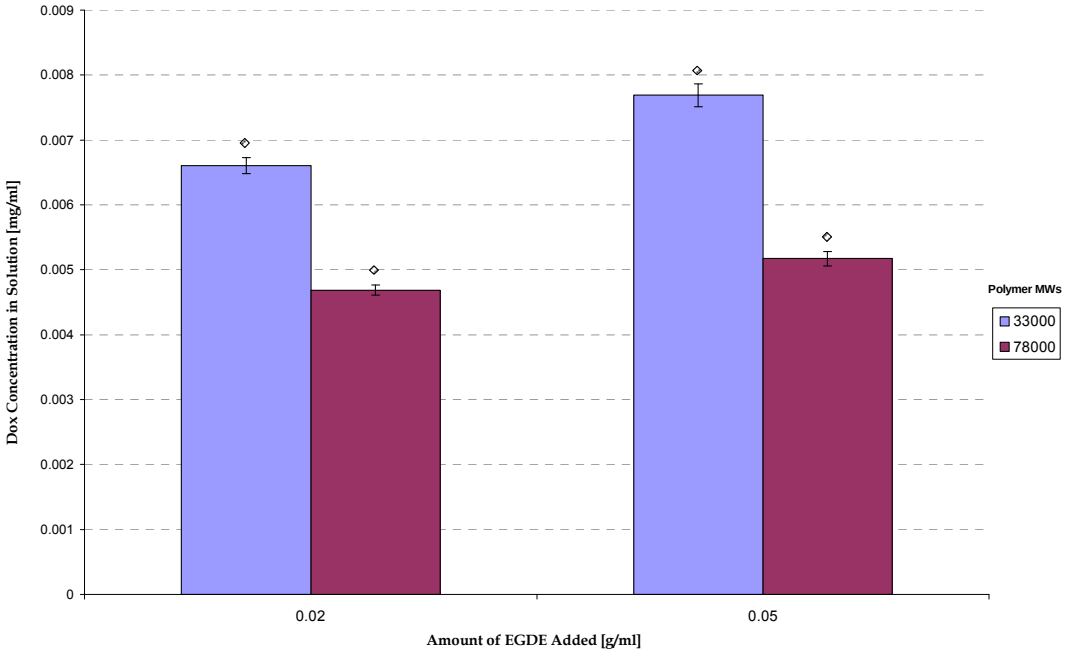


(b)

Figure 6-8. Comparison of molecular imprint recognition to template molecule with changes in chitosan polymer MW. (a) 70 h release, (b) 15 d release. Symbols (e.g. *, ¤, §) denote significant difference between formulation pairs (α = 0.05), while diamonds, ◊, indicate statistical significance with respect to all other samples shown.

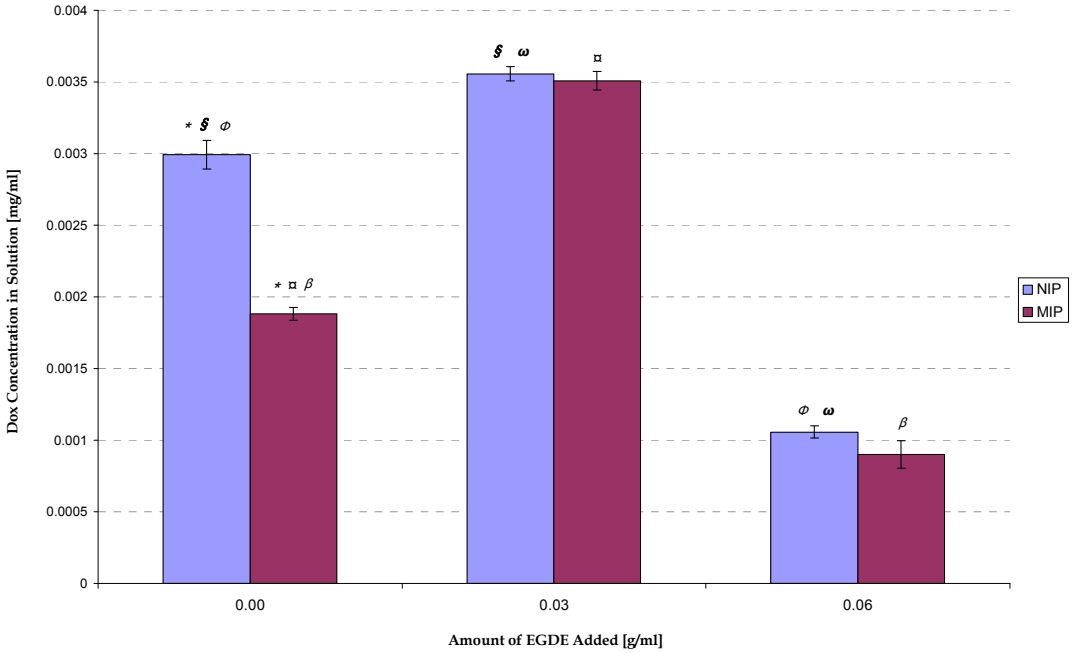


(a)

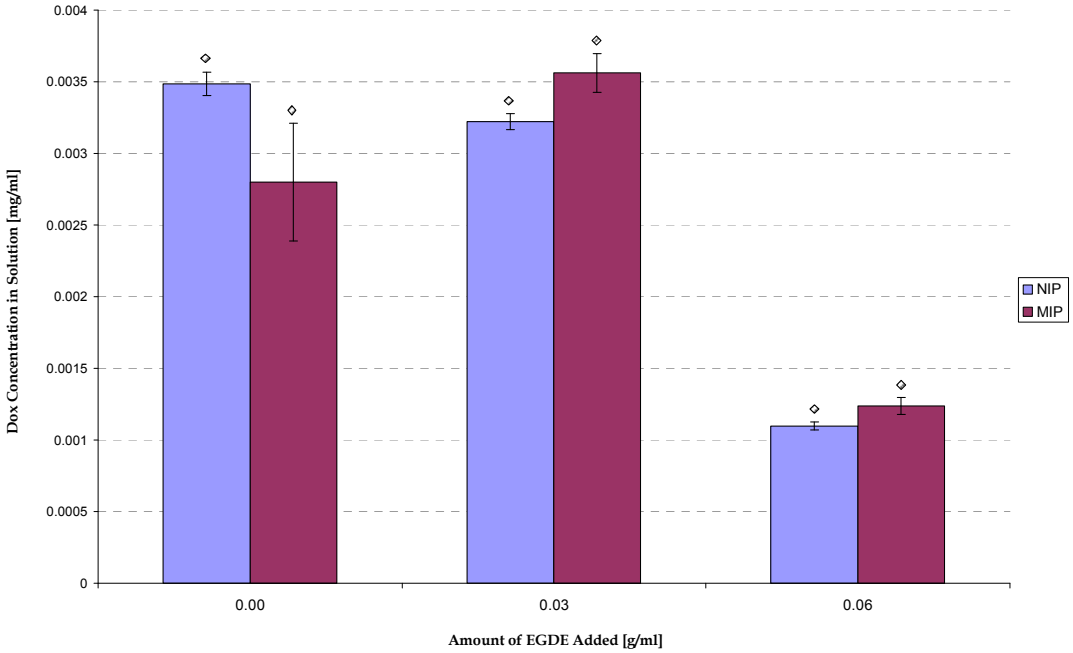


(b)

Figure 6-9. Effect of increasing EGDGE crosslinker concentration on Dox release from chitosan MIP gels (with 60ml DEGDMA used). (a) 70 h release, (b) 15 d release. Symbols (e.g. *, ¤, §) denote significant difference between formulation pairs (α = 0.05), while diamonds, ◊, indicate statistical significance with respect to all other samples shown.

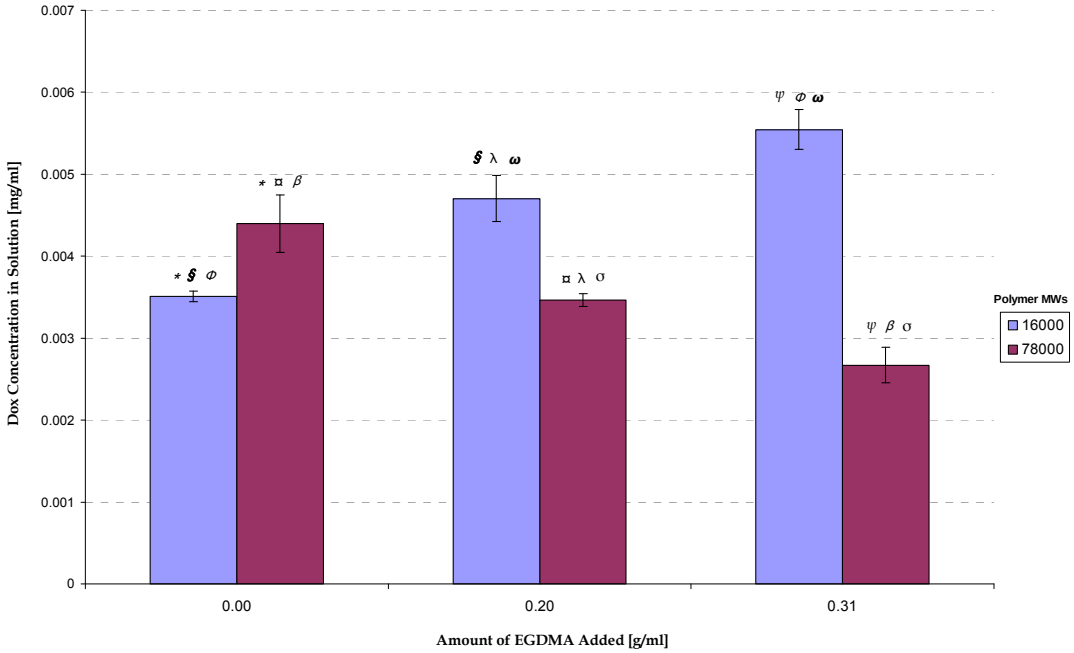


(a)

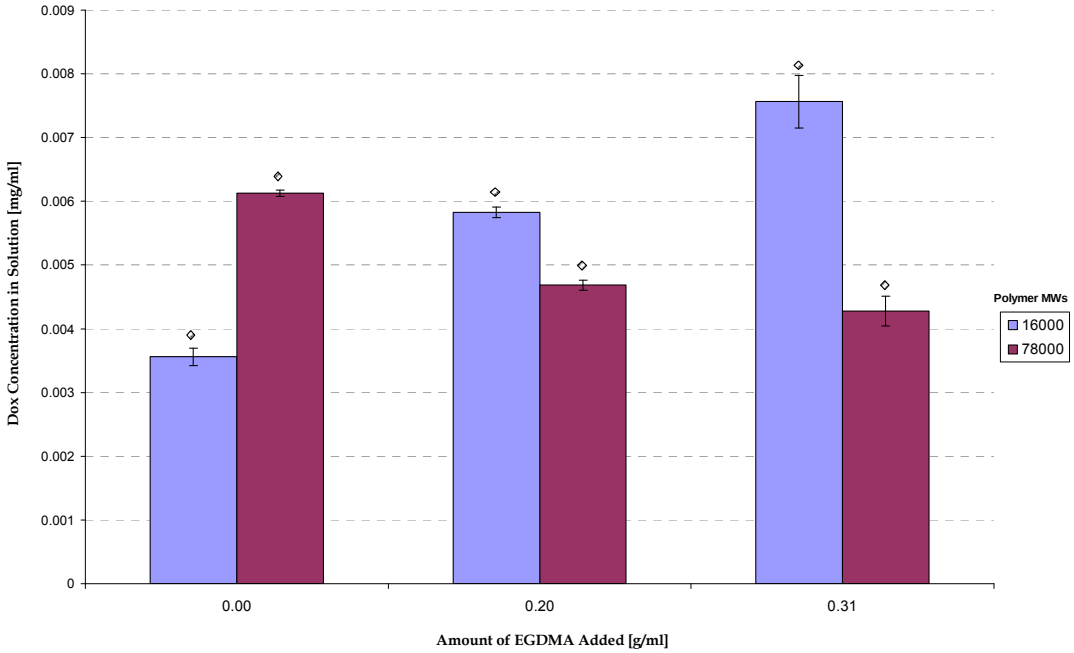


(b)

Figure 6-10. Comparison of template recognition by chitosan MIP gels and NIP controls, to changes in concentration of added EGDGE crosslinker (with no DEGDMA used). (a) 70 h release, (b) 15 d release. Symbols (e.g. *, ¤, §, ϕ, ω, β) denote significant difference between formulation pairs ($\alpha = 0.05$), while diamonds, ◊, indicate statistical significance with respect to all other samples shown.

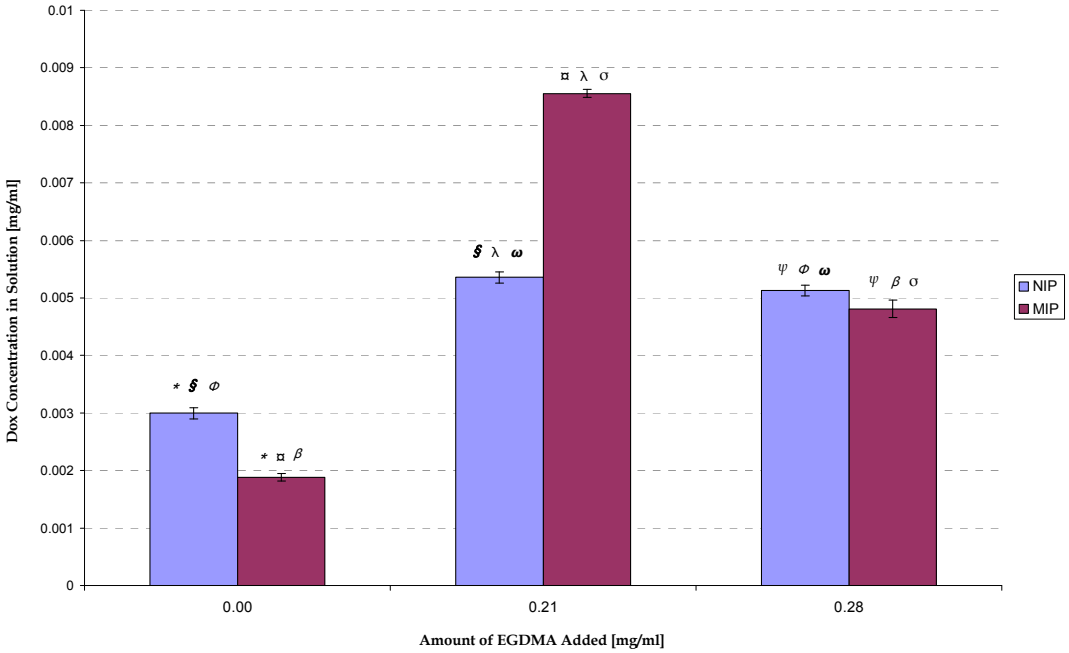


(a)

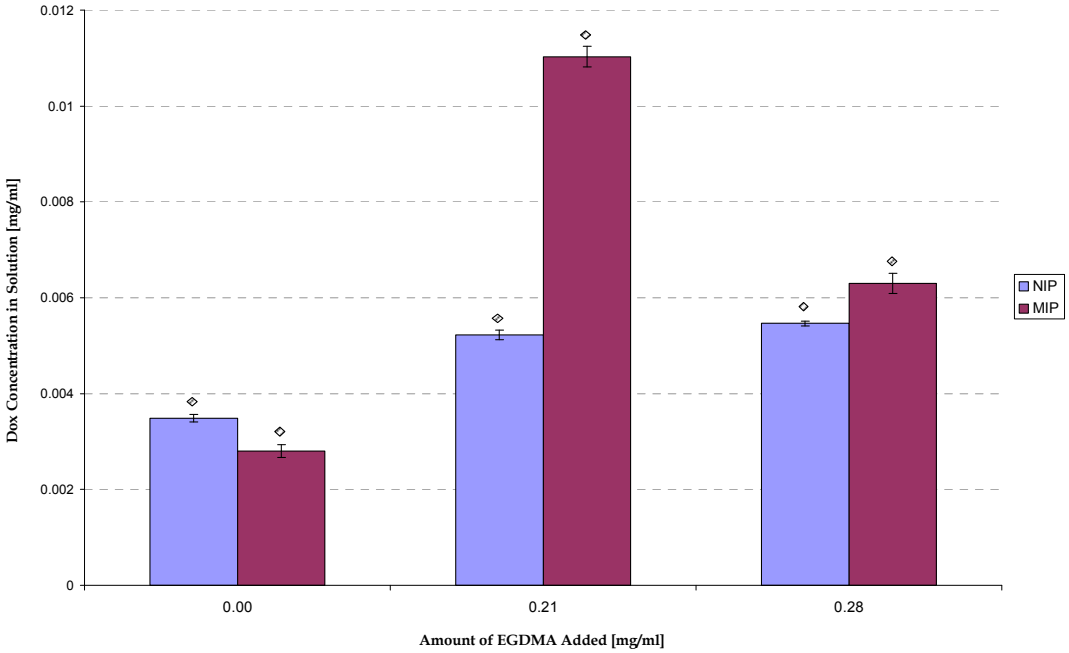


(b)

Figure 6-11. Effect of increasing DEGDMA crosslinker concentration (with 6µl EGDGE added) on released template from chitosan MIPs, using two different molecular weight polymers. (a) 70 h release, (b) 15 d release. Symbols (e.g. *, ¤, §, ϕ, ω, β, λ, σ, ψ) denote significant difference between formulation pairs (α = 0.05), while diamonds, ◊, indicate statistical significance with respect to all other samples shown.

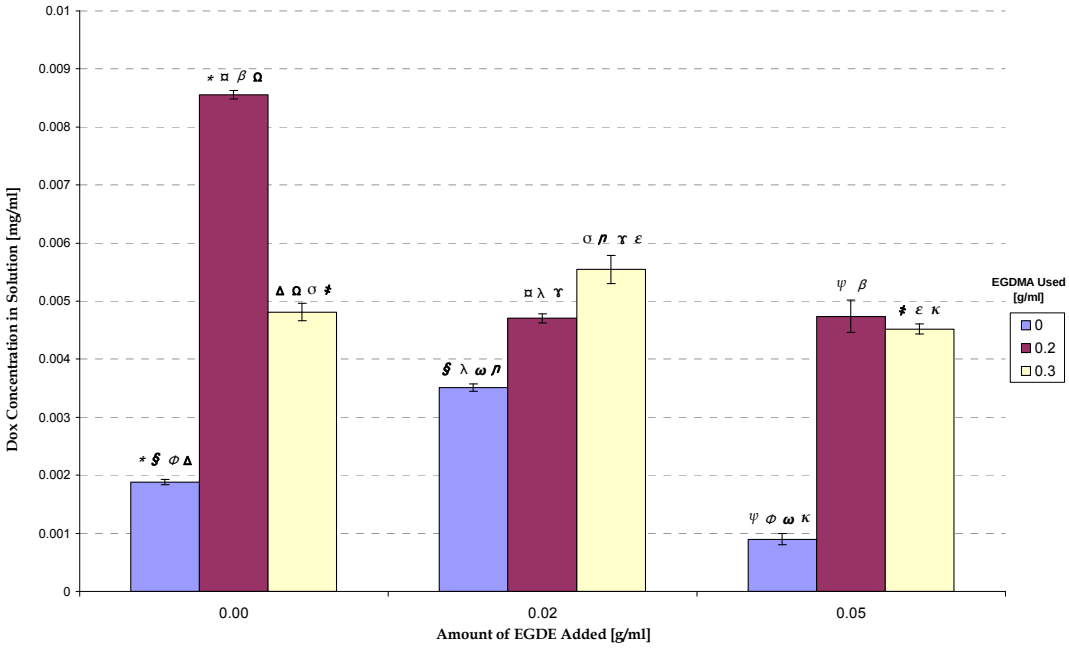


(a)

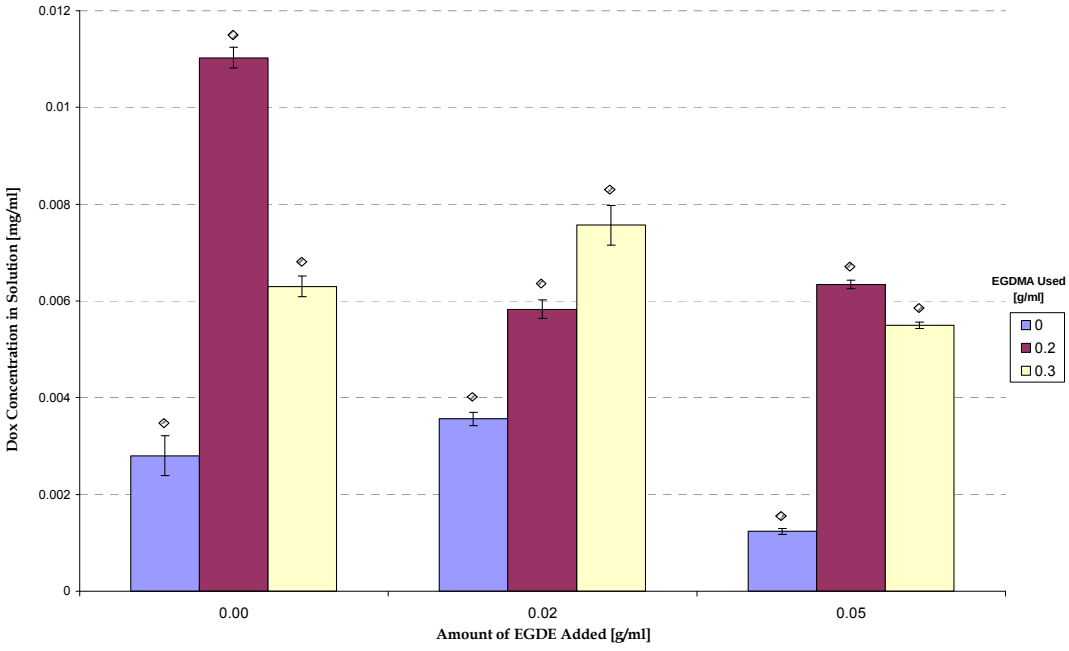


(b)

Figure 6-12. Comparison of model template release from chitosan MIPs versus NIPs for varying added DEGDMA crosslinker concentrations (with no EGDGE used). (a) 70 h release, (b) 15 d release. Symbols (e.g. *, ¤, §, ϕ, ω, β, λ, σ, ψ) denote significant difference between formulation pairs ($\alpha = 0.05$), while diamonds, ◊, indicate statistical significance with respect to all other samples shown.



(a)



(b)

Figure 6-13. Evaluation of the co-effect of incorporation of additional crosslinkers simultaneously into the pre-polymerisation mixture (using low MW chitosan). (a) 70 h release, (b) 15 d release. Symbols (e.g. *, ¤, §, ϕ, ω, β, λ, σ, ψ, Δ, Ω, ǂ, ∩, ϒ, ε, κ) denote significant difference between formulation pairs (α = 0.05), while diamonds, ◊, indicate statistical significance with respect to all other samples shown.

From the graphical above data, we can summarise a few trends about the behaviour of the chitosan MIPs. Firstly, it becomes noticeable that there was generally a greater change in template capture noted in the samples after a longer time period of examination, i.e. 15 day release study. This is a reasonable result, as likely not all of the template had been fully removed from the bulk gels at the initial sampling time point of 70 h, thus we could detect further changes in template release from chitosan resulting from the molecular imprinting process, from a result of greater recognition and capture of the target, as well as changes to the chemical properties of the matrix which slowed the release, that were altered by the model parameters explored.

Examining individually a few of the specific key parameters investigated, we note that capture and release of the template was generally higher for the lower MW chitosan starting polymer, and that the difference between MIP and the control was also greater for these samples. Once again, this confirms the situation of having a tighter network yield stronger interaction with the template molecule. The use of the EGDGE crosslinker appeared to have an improvement effect on the release of template, most noticeably at the longer time point, and that this difference was higher for lower MW chitosan. However, a strong contribution to the imprinting effect was not observed as a corroborative result. When DEGDMA was also used as a crosslinker in the pre-polymerisation mixture, increasing its amount had a negative or no effect on the release from the high MW chitosan, but did improve overall release from those gels made using lower MW polymer. When used on its own as the sole crosslinking agent, it was found that DEGMDA at an intermediate concentration offered the greatest increase in MIP recognition over the control. Examining the release behaviour from the polymer made of the two crosslinkers together, we saw the greatest level of Dox release from gels made using lower concentrations of DEGDMA and no EGDGE. This pattern follows the trends we observed for them individually. Thus, we can summarise that DEGDMA was shown to have some positive effect on the recognition and release of the template molecule from bulk chitosan gels, and that this effect was particularly evident when using lower MW polymer (more chains to crosslink), while EGDGE had little or negative effect on preferential binding of the template to the MIP.

To reach a more conclusive analysis of the effect of each of the parameters investigated in the B-B design, these results were examined quantitatively and statistically (using a two-tailed Student *t*-test) for significance, comparing release behaviour from samples of the gels based on the following parameters: MIP vs. NIP, low MW chitosan vs. high MW chitosan, with/without DEGDMA as crosslinker, and with/without EGDGE as crosslinker. This was done under the assumptions that the data follow a normal distribution, and have equal variances. These samples have been listed below in Table 6-4, along with the results of the test for statistical significance (at $\alpha = 0.05$) of each difference. The patterns of significance in the data largely confirm those seen graphically above.

Table 6-4. Examination of certain chitosan samples by paired model parameters, to evaluate imprinting effect and for significance of results (at $\alpha = 0.05$), at 70 h and 15 d time points. 'Yes' indicates paired observations of samples were found to be statistically significant at a 95% confidence level, while 'No' reflects an inability to reject the null hypothesis from the given data sets.

	Sample	Description	Paired With	Description	Significant 70d	15h
	2	MIP: med MW, med temp, no DEGDMA, hi EGDGE	**31**	NIP: med MW, no temp, no DEGDMA, hi EGDGE	**Yes**	*No*
	8	MIP: hi MW, med temp, no DEGDMA, med EGDGE	**38**	NIP: hi MW, no temp, no DEGDMA, med EGDGE	**Yes**	**Yes**
	9	MIP: hi MW, med temp, med DEGDMA, no EGDGE	**36**	NIP: hi MW, no temp, med DEGDMA, no EGDGE	*No*	**Yes**
MIP	**17**	MIP: low MW, low temp, no DEGDMA, no EGDGE	**40**	NIP: low MW, no temp, no DEGDMA, no EGDGE	**Yes**	**Yes**
vs	**22**	MIP: low MW, med temp, hi DEGDMA, no EGDGE	**33**	NIP: low MW, no temp, hi DEGDMA, no EGDGE	**Yes**	**Yes**
NIP	**24**	MIP: low MW, hi temp, no DEGDMA, med EGDGE	**34**	NIP: low MW, no temp, no DEGDMA, med EGDGE	**Yes**	**Yes**
	25	MIP: low MW, hi temp, med DEGDMA, no EGDGE	**32**	NIP: low MW, no temp, med DEGDMA, no EGDGE	*No*	**Yes**
	13	MIP: low MW, low temp, no DEGDMA, med EGDGE	**34**	NIP: low MW, no temp, no DEGDMA, med EGDGE	*No*	**Yes**
	14	MIP: low MW, low temp, med DEGDMA, no EGDGE	**32**	NIP: low MW, no temp, med DEGDMA, no EGDGE	**Yes**	**Yes**
	1	MIP: med MW, low temp, med DEGDMA, med EGDGE	**7**	MIP: hi MW, low temp, med DEGDMA, med EGDGE	**Yes**	**Yes**
MW	**4**	MIP: med MW, med temp, med DEGDMA, hi EGDGE	**10**	MIP: hi MW, med temp, med DEGDMA, hi EGDGE	**Yes**	**Yes**
	5	MIP: med MW, med temp, hi DEGDMA, med EGDGE	**11**	MIP: hi MW, med temp, hi DEGDMA, med EGDGE	**Yes**	**Yes**
	6	MIP: med MW, hi temp, med DEGDMA, med EGDGE	**12**	MIP: hi MW, hi temp, med DEGDMA, med EGDGE	**Yes**	**Yes**
	19	MIP: low MW, low temp, med DEGDMA, med EGDGE	**7**	MIP: hi MW, low temp, med DEGDMA, med EGDGE	**Yes**	**Yes**
	15	MIP: low MW, med temp, med DEGDMA, hi EGDGE	**10**	MIP: hi MW, med temp, med DEGDMA, hi EGDGE	**Yes**	**Yes**
	16	MIP: low MW, med temp, hi DEGDMA, med EGDGE	**11**	MIP: hi MW, med temp, hi DEGDMA, med EGDGE	**Yes**	**Yes**
	2	MIP: med MW, med temp, no DEGDMA, hi EGDGE	**4**	MIP: med MW, med temp, med DEGDMA, hi EGDGE	**Yes**	**Yes**
	8	MIP: hi MW, med temp, no DEGDMA, med EGDGE	**11**	MIP: hi MW, med temp, hi DEGDMA, med EGDGE	**Yes**	**Yes**
EGDMA	**13**	MIP: low MW, low temp, no DEGDMA, med EGDGE	**16**	MIP: low MW, low temp, hi DEGDMA, med EGDGE	**Yes**	**Yes**
	18	MIP: low MW, med temp, no DEGDMA, hi EGDGE	**23**	MIP: low MW, med temp, hi DEGDMA, hi EGDGE	**Yes**	**Yes**
	17	MIP: low MW, med temp, no DEGDMA, no EGDGE	**22**	MIP: low MW, med temp, hi DEGDMA, no EGDGE	**Yes**	**Yes**
	24	MIP: low MW, hi temp, no DEGDMA, med EGDGE	**27**	MIP: low MW, hi temp, hi DEGDMA, med EGDGE	**Yes**	**Yes**
	9	MIP: hi MW, med temp, med DEGDMA, no EGDGE	**10**	MIP: hi MW, med temp, med DEGDMA, hi EGDGE	**Yes**	*No*
EGDGE	**14**	MIP: low MW, low temp, med DEGDMA, no EGDGE	**15**	MIP: low MW, low temp, med DEGDMA, hi EGDGE	**Yes**	**Yes**
	17	MIP: low MW, med temp, no DEGDMA, no EGDGE	**18**	MIP: low MW, med temp, no DEGDMA, hi EGDGE	**Yes**	**Yes**
	22	MIP: low MW, med temp, hi DEGDMA, no EGDGE	**23**	MIP: low MW, med temp, hi DEGDMA, hi EGDGE	**Yes**	**Yes**
	25	MIP: low MW, hi temp, med DEGDMA, no EGDGE	**26**	MIP: low MW, hi temp, med DEGDMA, hi EGDGE	**Yes**	**Yes**
	1	MIP: med MW, low temp, med DEGDMA, med EGDGE	**6**	MIP: med MW, hi temp, med DEGDMA, med EGDGE	**Yes**	**Yes**
TEMP	**13**	MIP: low MW, low temp, no DEGDMA, med EGDGE	**24**	MIP: low MW, hi temp, no DEGDMA, med EGDGE	**Yes**	**Yes**
	14	MIP: low MW, low temp, med DEGDMA, no EGDGE	**25**	MIP: low MW, hi temp, med DEGDMA, no EGDGE	**Yes**	**Yes**
	7	MIP: hi MW, low temp, med DEGDMA, med EGDGE	**12**	MIP: hi MW, hi temp, med DEGDMA, med EGDGE	**Yes**	**Yes**

6.4 Discussion

The primary target of this research was to observe the effectiveness of the modified chitosan as a matrix for molecular imprinting. Across the various samples, an overall stronger binding of the template, i.e. imprinting effect, was observed when MIPs were contrasted with similar control non-imprinted polymers (NIPs); this was very pronounced as a high level of model template release over controls for certain formulations of the gels. Although it was not possible to fully construct a mathematical model for the MIPs from the results of the B-B design, study of these experimental

parameters did provide valuable information as to the success of molecular imprinting. The average template uptake by the MIPs was higher than NIPs taken as a whole, and in most cases this was found to be significant conclusion within a 95% confidence level. There was also seen a clear influence of multiple polymerisation parameters and interactions affecting the ultimate recognition seen by the imprinted chitosan.

Despite not using the results of the B-B experimental design to fully develop a concrete empirical model for the molecular imprinting in chitosan hydrogels, the trends elucidated from the template release study gave evidence of improved binding of the target as a result of molecular imprinting effect, which was the primary objective, and supported arguments made about the important influence of multiple polymerisation parameters on template recognition. From these results, some key generalised observations of significant effects could be made:

i. while certain MIPs did not differ greatly in all cases from their corresponding NIPs, particular formulations (the majority of cases) of imprinted polymers showed higher preferential binding levels of the target. This was proven significant in certain samples at 70 h, and generally the gap between imprinted polymer and control was increased after 15 d. Those samples which showed a significant improvement in the earlier timepoint maintained this difference later on, and others showed a greater difference over controls for the longer release period.

ii. it appeared that lower MW polymers improved the imprinting efficacy, as uptake amounts were slightly higher on the average across all bulk MIPs tested. This difference was also found to be statistically significant in most of the cases. Lower MW chains would support improved imprinting effect, as a tighter chitosan network should result in greater recognition of the template during the pre-polymerisation stage.

iii. while the amount of DEGDMA used as crosslinker had no or small effect on MIP recognition, gels without EGDGE resulted in higher uptakes of template in all cases. This independence of imprint effect with crosslinker concentration is not a wholly unexpected result, and supports the arguments proposed by other authors.[26, 322]

iv. increased amounts of template used in the pre-polymerisation formula did not translate to a greater number of imprint sites created (higher binding of target molecule). While the sample evaluated shows significant difference from one another, this did not follow any trends with changes in template concentration.

There are a few possible explanations for some of the poor results seen with this initial imprinting study, which led to a lack of a complete model from the B-B design. Firstly, a major issue was deficient gelation of the chitosan. Not all bulk hydrogel networks retained their structure during the course of the investigation, and a partial breakdown of the network severely impacted results of the study. While it is not fully clear why certain samples did not form enough crosslinks to maintain a stable gel after repeated swelling and washing cycles, it does suggest that the chemistry during crosslinking with and without additional molecules present (e.g. template) may not be the same. Further, to improve the overall crosslinking efficiency, future samples of high bulk volumes should be sparged with N_2 to eliminate oxygen which can act as a free-radical scavenger during polymerisation and limit the crosslinking reaction. For better template recognition, it may be necessary to allow a longer equilibration time, maintaining the components in solution prior to UV exposure to stabilise the pre-polymerisation complex. Gelling can also be done in a transparent mould, to increase the amount of UV light entering the solution, rather than just at the exposed surface, for homogeneous reaction and polyermisation kinetics to attain more uniform crosslinked networks. Ideally, a more concentrated solution of chitosan would be desired, to improve the interactions with the template, and have a tighter overall network. There is a limiting value to this, however, as at highly concentrated values, chitosan solutions

sometimes suffer a tendency to form a pre-gelled mixture through Michael-addition type reactions between the functionalised methacrylate groups added to the polymer and the amines of chitosan. To improve on the uniformity of crosslinking, along with the aforementioned methods, the light intensity (and heat) should be reduced either by filtering or using larger distances away from the source. While this would lengthen the overall polymerisation time, it will allow radicals to distribute throughout the chitosan solution, and result in a more continuous exposure to the UV light (e.g. without forming a crosslinked film at the surface).

6.5 Summary

Examination of molecular imprinting with the modified chitosan polymer in a bulk capture and release model provided some invaluable information about the system and allowed us to further our hypotheses. We were able to confirm that, indeed, there was some potential for molecular imprinting in the GC matrix, demonstrated through preferential capture of the template, proven to be statistically significant in certain formulations. While the study itself did not yield a great extent of quantitative data, the knowledge it shed on the overall approach to polymerisation; and molecular imprinting in general with chitosan, was extremely valuable. The poor results seen from the gelation studies allowed us to improve the polymerisation methods and augment the experimental design. We were able to verify that, in spite of the predicted difficulty in trying to achieve specific recognition of a molecular target with this loose hydrogel in water, it was possible to see some degree of molecular imprinting effect. Furthermore, the studies using bulk chitosan polymers established the key importance of some of the pre-polymerisation parameters investigated, such as starting polymer molecular weight and the use of crosslinkers. These factors then allowed us to further develop the chitosan-MIP model, to improve on the technology to eventually reach a stage where an imprint effect could be measured. The initially poor results seen also brought to light the import of detection methodologies and instrument sensitivities, which were a sizeable weakness in the manner of this study, in order to rethink new approaches for assessment of molecular imprints over control in a reproducible and sensitive fashion.

7 Molecular Imprinting of Proteins in a 2D Gel

As was discussed in Chapter 2, the challenges involved in the molecular imprinting of proteins are numerous. Their complex structures and sensitivity to environmental conditions and storage, coupled with the difficulty of aqueous-based MIP-recognition, create a significant encumbrance to the ease of production and application of any such devices. Nevertheless, protein recognition plays a major role in the biomedical sciences; as reference biomarkers in sensors, adherent moieties for tissue scaffolds, capturing ability in delivery devices, etc., and remains therefore a foremost area of research. While the use of antibodies, bifunctional crosslinkers, and multiplex surface chemistries have all enabled the incorporation of proteins into biomaterials and detection designs, these designs are also not without their respective drawbacks. Thus, there remains a strong impetus to overcome these limitations to develop a relatively simple, low-cost, and stable alternative strategy that might enable similar protein recognition. Molecular imprinting can provide us with such a method. Because of this, the molecular imprinting of proteins in a simple fashion, which could be ultimately expanded to biorelevant applications, remained a principal goal of this thesis research. We sought to investigate a means to create a rapid and facile detection system for protein targets. For ease and purposes of miniaturisation, a two-dimensional model was envisioned as a fitting exemplar for preliminary investigation.

The initial results from bulk gelation studies suggested potential for the chitosan matrix to act as the foundation for an imprinted unit. Whereas the recognition was improved upon with lower molecular weight polymer, by extrapolation it was surmised that using a larger molecule, such as a protein, might likewise offer augmented interaction with the matrix, possibly even without necessitating any reduction in the MW of the polymer. As a demonstration for the applicability of a chitosan MIP to the molecular imprinting of proteins, a simple proof-of-concept was developed. A qualitative examination of imprinting effect was desired that could simply and rapidly demonstrate the binding differential between a MIP and a corresponding NIP. A micropatterned chitosan hydrogel imprinted with model proteins was designed, utilising

fluorescence as an indicator of target capture. This scheme could be modified in future work to observe the effects of imprinting conditions on specific recognition with little effort, and enable eventual quantitative assessment when desired. It would also allow for the localisation of target binding sites and miniaturisation of the binding surface towards the development of a small-scale sensing and detection platform.

Surface patterning, in both two and three dimensions, can be used to observe and guide cell attachment and migration,[323, 324, 325, 326] as well as cell sorting and screening.[327] This can be achieved through localised binding areas[328] (contact printing) or photolithography.[329] The former often makes use of patterned stamps of polydimethylsiloxane (PDMS), in which micrometer-sized features can be created, which can then be used to apply a thin layer of molecules to a flat surface on the points of contact.[330, 331, 332] In the case of the latter, a photomask of a stenciled design is used to screen light from reaching some areas, often to create UV-initiated crosslinked surfaces.[333, 334, 335]

Soft lithography, as a form of microfabrication, has become very popular with scientists and engineers for its relative ease and precision, since being first introduced by Unger, Whitesides, and others.[336, 337] Soft lithography generally refers to a family of techniques for the production of replicating features or surface structures using elastomeric stamps, moulds, and photomasks.[338] As opposed to other common forms of lithography, these techniques are differentiated by the use of 'soft' elastomers, typically poly(dimethylsiloxane), as the key element used in patterning.

Soft lithography offers several advantages over the more traditional photolithography, the primary of which is a significant reduction in equipment needs and cost.[339] Generally, the patterns produced using soft lithography are limited to the 10^2 μm scale, due to limitations of both the stamping polymer (low elastic modulus) and inking material (diffusion during printing); however with fine control of the stamping conditions and higher MWs it is possible to bring the resolution to below the 50 μm level.[340] Co *et al*. have demonstrated that such patterning can be used with

multiple substrates, including chitosan, to achieve microarrays of different cell types in co-culture.[341] Elloumi Hannachi *et al*. applied microstamped co-culturing to produce cell sheets.[342] Chitosan membranes have also been functionalised using this system to support spatial arrangement of cell growth.[343] In some cases, multiple functionalisations can be added to a surface, though in such situations precise alignment of the stamps is vital.[344] Proper stamping technique is a critical factor in achieving controlled printing.[345, 346] Several strategies now exist for improving stamp inking, and using tailored target affinity to increase selective transfer of 'heavier' inks such as biomolecules.[347]

An extension of the surface patterning system is to use similar PDMS stamps to act as moulds for crosslinking in bulk.[348, 349] For fairly rigid polymer systems, micropatterned surfaces can produce very well-defined structures, with micrometer-scale features.[350] Proper tuning of the stamping conditions can reduce pattern definition to the sub-micron range.[351] With hydrogels, the soft nature of the material and contrast against a rigid surface[352] can lead to surface disruption during removal of the stamp, resulting in topographical distortion of the pattern site.[353] This is understandably sensitive to feature size and spacing.[354] One method to correct for this is to partially crosslink the gel before stamping,[355] while other authors have used surface treatments to limit adherence to the PDMS.[356] Whilst it is established that using a micromoulding process can create with great versatility patterned substrates,[357] stamping of bio- and biofunctional materials is now of increased relevance.[358]

Few studies have been thus far reported on molecular imprinting strategies combined with soft lithography. The benefit towards molecular imprinting is clear, however: a thin layer of MIP offers high binding area to limited volume, is rapid to produce, provides an enhanced degree of transport exchange for access of large molecules, and can create selective capture sites for targets on a thin surface. Huang *et al*. have used a combination of UV polymerisation with a photoresist to create a chemical sensor towards albuterol.[359] Voicu *et al*. showed that specifically-oriented imprinting of template could be achieved by binding the molecule to the PDMS stamp.

After coating with monomer and transferring the recognition matrix to a surface, a 2D array of selective MIP was created.[360] Forchheimer *et al*. created a stamped imprint array of submicron MIP pattern, however the sensor device suffered from substantial residual resist, and only a cursory study of imprinting effect was undertaken.[361] Molecular imprinting in a layer-by-layer construct has also been used to "ink" micropatterned surfaces, to transfer the template selectively to a substrate.[362] An extension of this technique is to use a substrate to adhere or bind a template, and then stamp this into the solution of pre-polymer mix. As already suggested, this can allow specific orientation of the template, and is very effective at creating a thin layer of MIP, where all the imprint sites are located at the surface of the polymer. It also has the benefit of working very well for large molecules that cannot easily be imprinted due to their size, even reaching the scale of cell/bacteria imprints.[363, 364] Lin *et al*. used this method to achieve strong imprinting effect for some model proteins, effectively creating a surface of artificial receptors that can act as antibody mimics.[365] With proper design this approach can yield a highly selective surface.[366]

The work in this research builds upon the principles of molecular imprinting, soft lithography and polymer gelation to demonstrate a facile method by which a functionalised surface can be created to re-capture a protein of choice, selectively. This could be applied to a wide array of uses, such as protein separations, antibody refinement, antigen removal, and in sensing and biodetection. The glycol chitosan used as base material was chosen for its water solubility, available and abundant functional groups, and previously reported properties as a strong candidate for a biomaterial. As it has not been established in the field as a polymer for molecular imprinting, this research work lays the foundation towards many possible uses for an imprintable chitosan matrix. Using *in situ* polymerisation via UV light adds an additional degree of freedom to the materials that can be made from the MIP gels; these can include thin films, microspheres, membranes, or bulk monoliths. UV-initiated crosslinking has shown to be more effective in terms of resultant MIP selectivity compared with thermally-cured polymers.[302] A simple qualitative assessment was planned using fluorescence detection

of a labelled counterpart to the template molecule. This type of analysis has been validated by Lalo *et al*. for MIP nanopatterned surfaces.[367]

7.1 Gel formation

7.1.1 PDMS Stamp

The mould used for stamp production was obtained via photolithographic etching of a silicon substrate using an SU-8 photoresist. The lithographic pattern was designed with commercial software tools (AutoCAD 2008, Autodesk Inc., San Rafael, CA). The photoresist was spincoated onto a 5" silicon wafer and selectively exposed to UV light using the photomask. Polydimethlysiloxane (Sylgard 184, Dow Corning) was mixed in a 10:1 ratio of elastomer precursor to curing agent, added to the surface of the silicon plate, degassed, and cured at 60^{o}C for 16 hours. The cured polymer was separated from the mould, and stamps were cut out into their desired size using a scalpel. Mean thickness of the PDMS stamps used was roughly 3 mm.

7.1.2 Polymerisation

Surfaces of glass slides were prepared by cleaning with Type II water followed by ethanol wash, dried under a stream of N_2, and subsequently treated with oxygen plasma for two minutes. Printing locations were isolated on each slide (two for each, MIP and NIP) using an hydrophobic barrier pen. Modified chitosan (40 mg/ml) was mixed with template solutions, crosslinker, and photoinitiator (Irgacure 2959; 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone). The templates used for study were human serum albumin (HSA) and avidin (AV), while for binding studies the recaptured proteins added were the fluorescently labelled analogues of these proteins, cyanine-3 labelled albumin (HSA-Cy3) and fluorescein tagged avidin (Av-Fl). In the case of non-imprinted polymers (NIPs) template solution was replaced by an equivalent volume of 1x phosphate buffered saline. When additional crosslinkers (i.e. MBAm and EGDMA) were used to form the polymers, they were added to the pre-polymer solution in 0.5 ml amber microcentrifuge tubes and thoroughly vortexed to dissolve the additional

constituents. The pre-polymerisation complex was allowed to form *in situ* in sealed tubes for 1 h. 60 μl of initiated pre-polymer solution was added to the surface of the glass slides, and the PDMS stamp gently pressed down to the surface. The solution was then crosslinked inside a light box equipped with high intensity UV flood lamp (Dymax 5000-EC curing system). After 20 min the gels were removed and allowed to cool to room temperature. PDMS stamps were carefully lifted vertically off of the slides, leaving the imprinted pattern.

7.2 Assessment of Imprinting

7.2.1 Protein recapture

Following polymerisation of the chitosan, removal of entrapped template from the gel surfaces was done by soaking in solutions of MilliQ water. As the crosslinked network is water-swellable, it allows for ease of solvent access to the imprint sites, and should be sufficient in this case to disrupt the weak binding between template and receptor site, and thus no further treatment of the polymer surfaces was required. Certain template removal steps for MIPs, such as a common AcOH/SDS wash have been shown to result in 'artificial' recognition in imprinted chitosan.[368] Water was changed at regular intervals to maintain the 'infinite sink' condition.

Fluorescently labelled analogues of the template protein (HSA-Cy3) and a competitor molecule (Av-Fl) were dissolved in PBS (200 μg/ml). Solutions of fluorescently tagged proteins (100 μl) were added to the gelled areas. The slides were placed in humidified chambers, wrapped in foil to protect from light exposure, and incubated at 4^{o}C for 24 h. Bound areas were gently rinsed with water to remove surface adsorbed proteins, and the slides were immersed in a water bath for 30 min. After another light surface rinse, gels were coated with ~ 80 μl of antifade fixative, and gently covered and sealed with a glass coverslip.

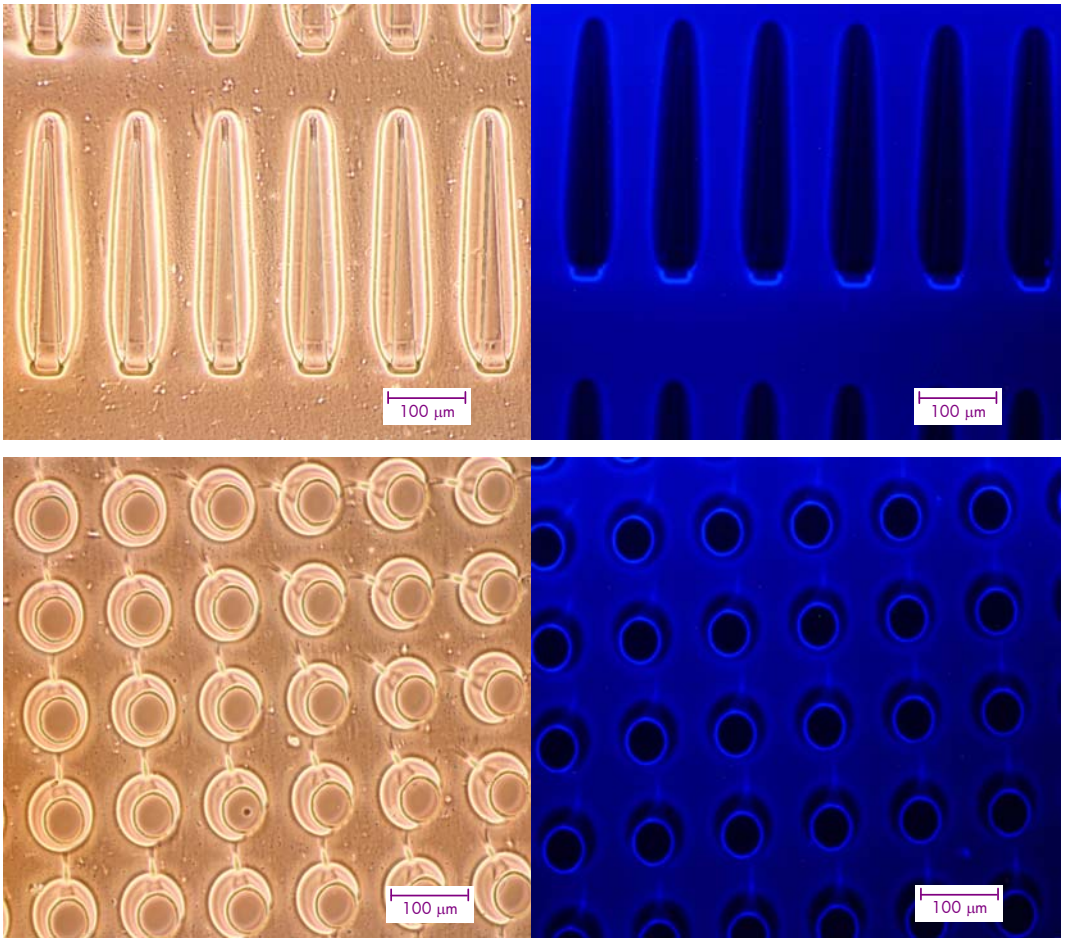
7.2.2 Determination of binding

Sections of patterned gels with recaptured fluorescent proteins were visualised using confocal laser scanning microscopy (Zeiss LSM 510 microscope; Jena, Germany). Care was taken to use like slides for test areas of molecular imprints and controls, and to examine each under the same experimental conditions (e.g. timing, magnification, exposure). Multiple images were taken to verify reproducibility among test gel samples.

7.3 Results of 2D Molecular Imprinting

7.3.1 Chitosan Patterning

Initial studies of patterned chitosan gels demonstrated a high degree of success for the polymerisation and stamping methods employed. The resultant polymer surfaces exhibited clearly defined patterns, and were visualised under light microscopy for homogeneity and shape distinction and for autofluorescence (Figure 7-1). It was observed that upon excitation and visualisation under blue wavelengths, the crosslinked chitosan gel itself was found to fluoresce to some degree, as is visible in Figure 7-1*(b)*. This feature was used as a control reference for localisations of the overall gel patterns on the stamped slides (e.g. when the 3D depth of the gelled surfaces was small enough to limit profile distinction from above, or should there be no fluorescently-labelled protein remaining bound on the surfaces during microscopy) and as an estimate of the background fluorescence levels in analysis channels.



(a) *(b)*

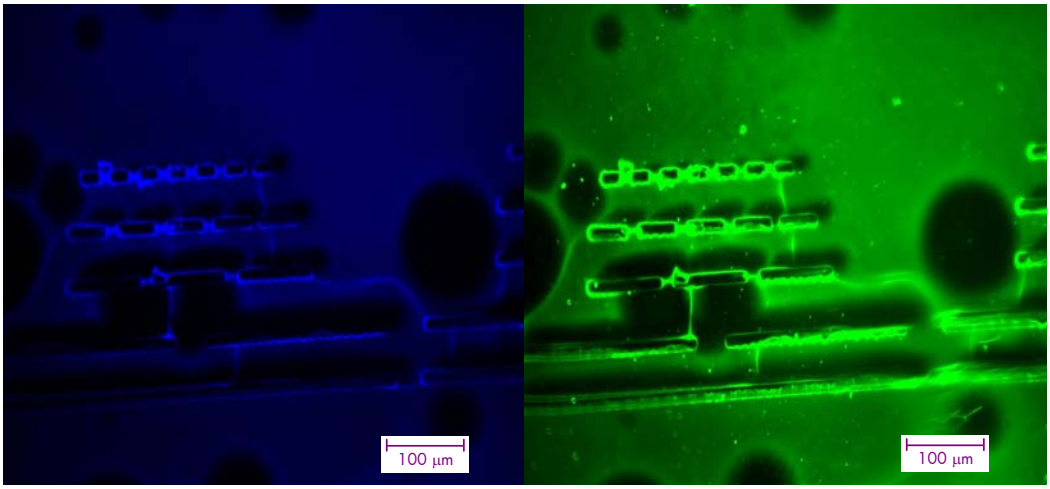
Figure 7-1. Surface patterning of thin layer chitosan gels showing the relative depth and definition possible, accomplished through polymerisation with PDMS moulds made through photolithographic plates. (a) light microscopy, (b) fluorescence. [100x magnification]

7.3.2 Comparison of MIPs with NIPs

7.3.2.1 Preliminary study with IgG

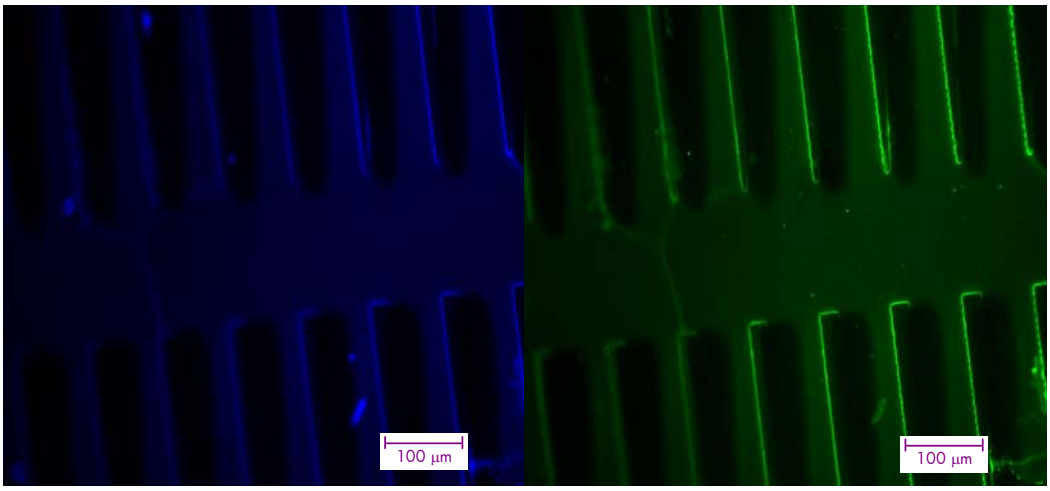
An initial trial of the ability to imprint proteins in the modified chitosan matrix was investigated using FITC-conjugated IgG protein. As a considerably larger template molecule, IgG should offer a significant degree of physical interaction with the polymeric matrix, allowing the recognition effect to be more easily determined. Following an overnight incubation at 4°C of fluorescently labelled target on patterned IgG-MIP surfaces, the gels were lightly washed with water to remove solution and non-specifically adhered template. Rebound IgG captured by the chitosan gels (MIP and

NIP control) was then observed under fluorescent light. Sample images of the surfaces can be seen in Figure 7-2 and Figure 7-3. Blue fluorescence (as show in images below) was used as visual reference guide of the patterned gel location (i.e. overall binding surface area of the gel for analyses), while the green acted as indicator of FITC-conjugated protein capture and to localise binding regions in the chitosan gel.



(a) ***(b)***

Figure 7-2. Fluorescent imaging of MIP surface showing (a) patterned areas of chitosan gel (autofluorescence in blue) and (b) bound protein (via green filters). [100x magnification]

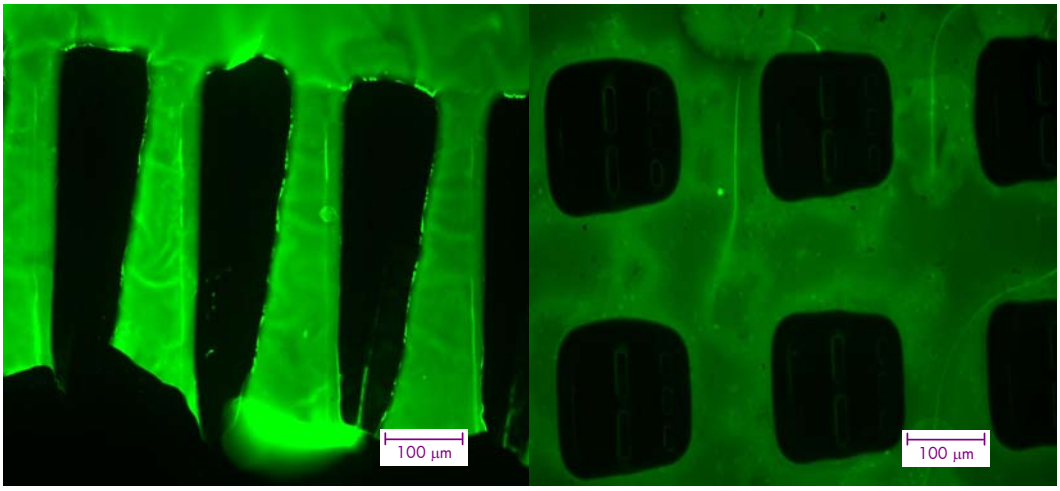


(a) ***(b)***

Figure 7-3. Fluorescent imaging of NIP gel surface after protein exposure, with blue (a) for overall chitosan patterning on the surface, and green (b) for captured IgG filters. [100x magnification]

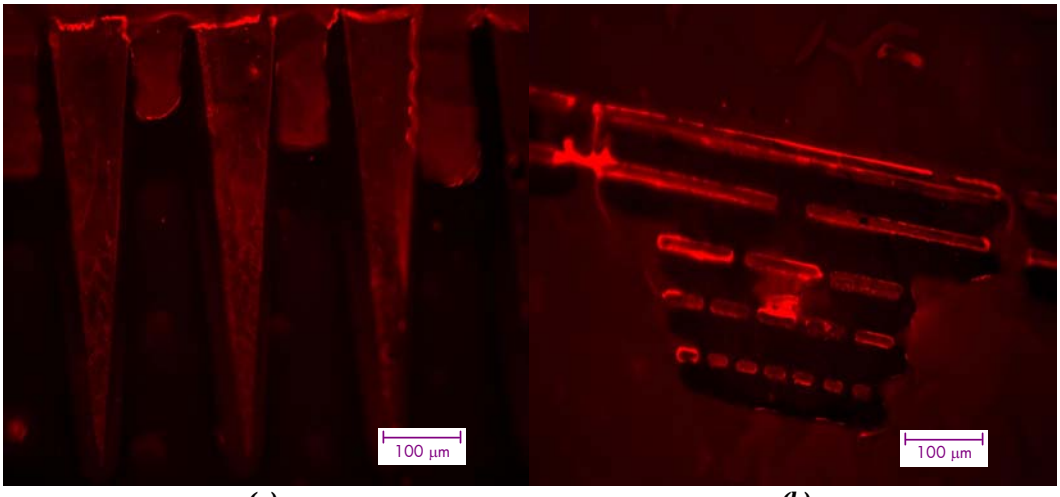
7.3.2.2 Examination using model templates

As the intended targets for this chitosan imprinting model would likely be smaller biomolecules or peptide chains, as contrasted with the comparatively large size of IgG, the experiment was repeated using BSA-Cy5 and heparin-FITC. Gels were produced in a like manner as above, using BSA and heparin as template molecules. A comparison of the fluorescent target binding in NIPs and MIPs for each of these proteins is shown in Figure 7-4 and Figure 7-5, respectively.



(a) ***(b)***

Figure 7-4. Captured fluorescent images of chitosan gels following exposure to rebinding solutions of FITC-labelled heparin, for NIP (a) and MIP (b) surfaces. [100x magnification]



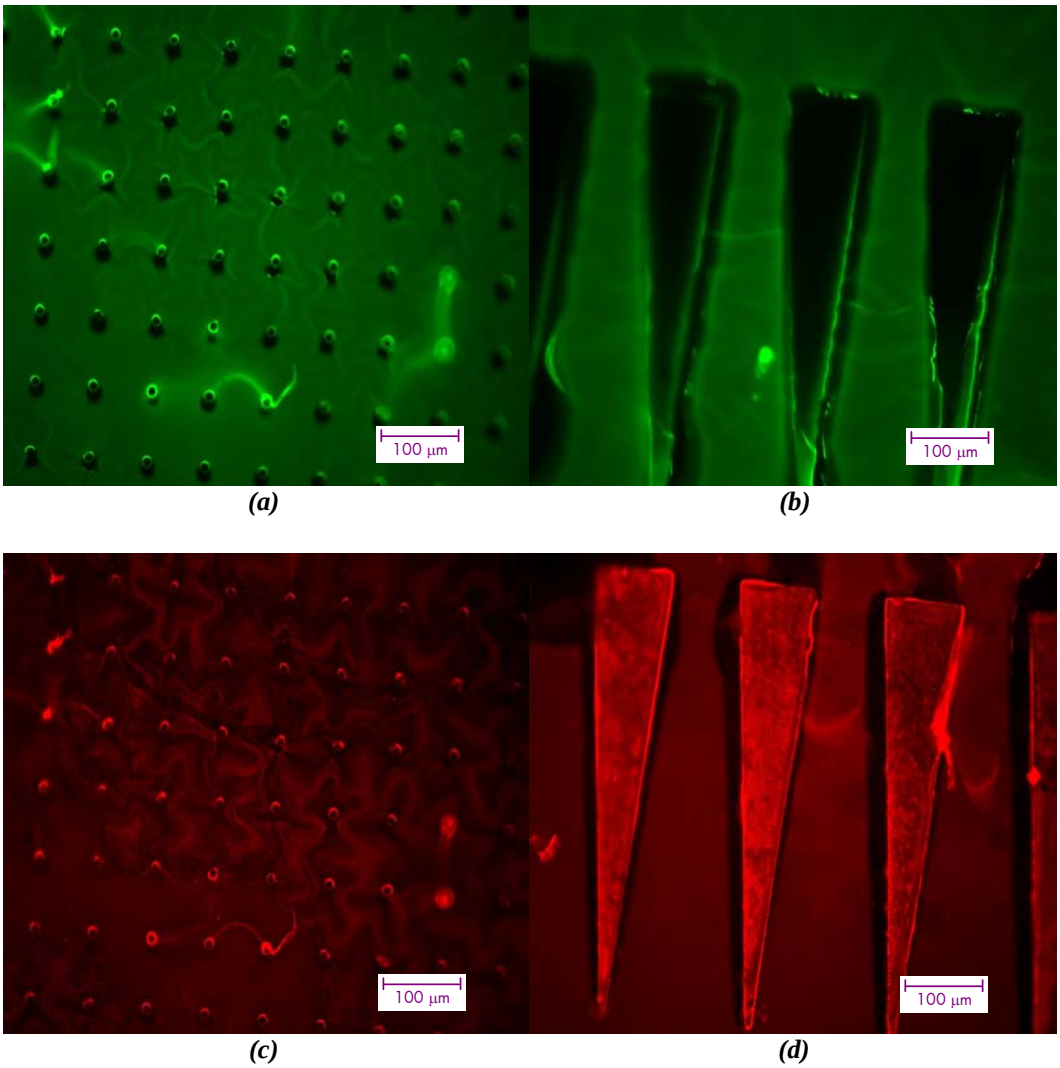
(a) ***(b)***

Figure 7-5. Captured fluorescent images of chitosan gels following exposure to rebinding solutions of Cy5-labelled BSA, for NIP (a) and MIP (b) surfaces. [100x magnification]

From the above images, it was observed that there was a noticeable improvement in the amount of rebound albumin template for the corresponding imprinted gels (Figure 7-5). While there still occurred some non-specific general absorption of protein to the chitosan, the increase as a result of molecular imprint effect is especially noticeable at the edges of the gels, where more surface area (from a top-down perspective) is available for binding. The heparin-MIPs showed no noticeable improvement over the NIP controls (Figure 7-4), and it is not immediately clear if this lack of a discernable imprint effect is a result of the template being much smaller in size versus the albumin, and thus being below the particular threshold of interaction with the crosslinked matrix, as formed under the given conditions, a weaker degree of interaction with the chitosan network, or from an overall higher degree of autofluorescence under the green wavelengths.

7.3.3 Competitive Recognition Study

The selectivity of an molecularly imprinted matrix is a key component in measuring imprint success, as a rebinding situation involving a mixture of competing (and, possibly, blocking) molecules in solution together with the imprint sites more accurately reflects the conditions one expects in physiological samples. To evaluate the MIP recognition under such conditions, mixed solutions of the two fluorescently labelled proteins were added to the moulded gels specifically imprinted with only one of the targets.



(a) *(b)*

(c) *(d)*

Figure 7-6. Images of BSA-imprinted chitosan gel surfaces following exposure to equal concentrations of heparin-FITC as competitor molecule (visualised in green, (a) and (b)) and BSA-Cy5 as molecular imprinting template analogue (visualised in red, (c) and (d)) [NIPs left, MIPs right]. [100x magnification]

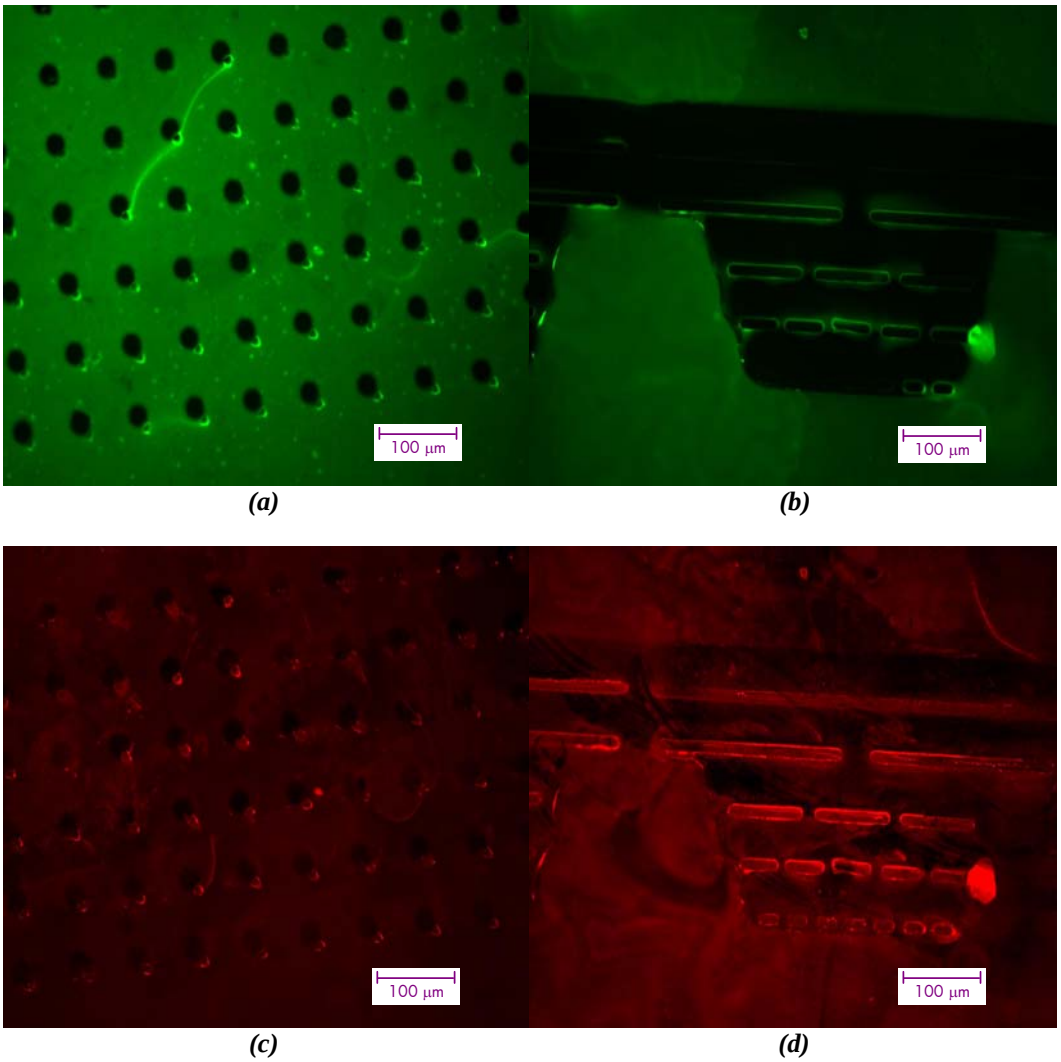


Figure 7-7. Images of Hep-imprinted chitosan gel surfaces following exposure to equal concentrations of heparin-FITC as target molecule (visualised in green, (a) and (b)) and BSA-Cy5 as binding competitor (visualised in red, (c) and (d)) [NIPs left, MIPs right]. [100x magnification]

Figure 7-6 demonstrates that rebinding of heparin to the HSA-MIP is more or less equal to that of the NIP, in other words indicating that residual protein present following washing was only generally adsorbed to the polymer and was not as a result of non-specific binding to the imprint sites in the chitosan MIP. Imaging under red wavelengths showed an increase in the amount of bound albumin in the MIP, pointing to a preferential capture/recognition of the template molecule.

Conversely, when heparin-imprinted gels were examined with solutions of both fluorescent targets (see Figure 7-7), there was not an appreciable increase in bound protein levels for the template to the corresponding imprint. Both MIP and NIP gels showed similar levels of fluorescence for captured heparin. When observing the amount of albumin present in the patterned chitosan, it appeared to be increased in the MIP sample. It is possible that this arose partly as a result of the imprinting procedure in the gel, which increased the interchain porosity/available binding sites in the chitosan matrix, and that there was a higher degree of non-specific interaction between the albumin molecule and the polymer chain.

7.3.4 Examining the Effect of Crosslinking Agents

Micropatterned gels of modified chitosan were subsequently made using a series of additional crosslinkers to determine what effect this had on imprint recognition of the matrix. In a series of experiments, three conditions were examined: using no crosslinker, and with the addition of either 1% v/v EGDMA, or 1% v/v MBAm into the pre-polymerisation solution. In each situation, the gel formulations were examined in the presence of an incorporated template molecule (MIP), in this case using human serum albumin as a model protein, or having the equivalent volume of PBS added as a substituting element, to account for slight changes in concentration (NIP). Following gelation, all chitosan surfaces were washed gently with water to leach out soluble template molecules, and subsequently subjected to rebinding soaks with Cy5-labelled human serum albumin (HSA) in solution (overnight, 4^{o}C), to allow protein recognition and capture into the gels. A light surface rinse was used to remove off any loosely bound or gravitationally adhered proteins. The patterned surfaces were then visualised with confocal fluorescence microscope under identical parameters (e.g. time, gain, exposure). Imprinted samples and their respective non-imprinted controls were made at the same time and on the same glass slide, to limit variation between preparation as much as possible. Each sample of chitosan gel was repeated in duplicate or triplicate, produced under the same conditions. Example fluorescent images of these patterned gels under the varying crosslinking situations are shown in Figure 7-8.

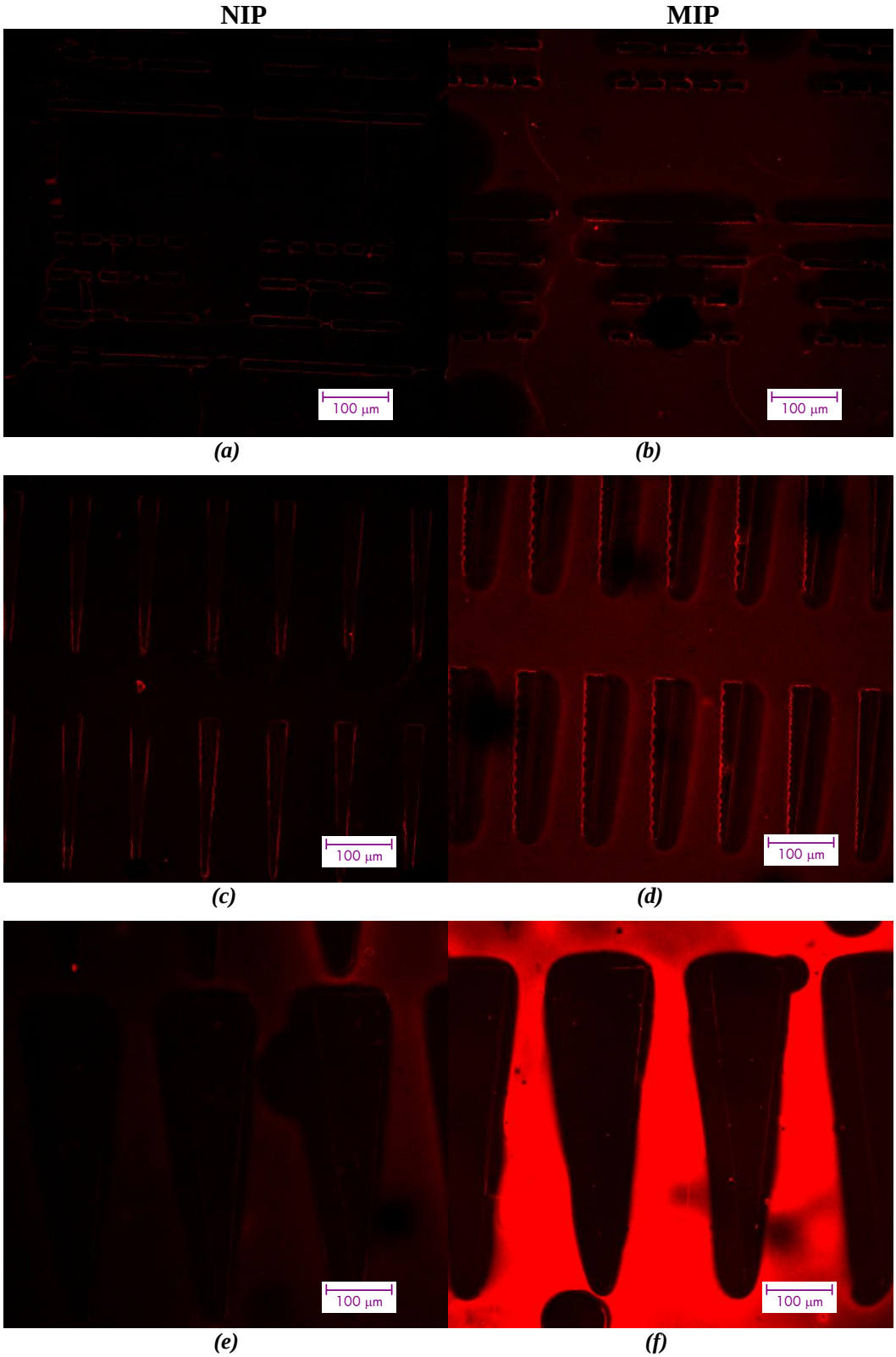
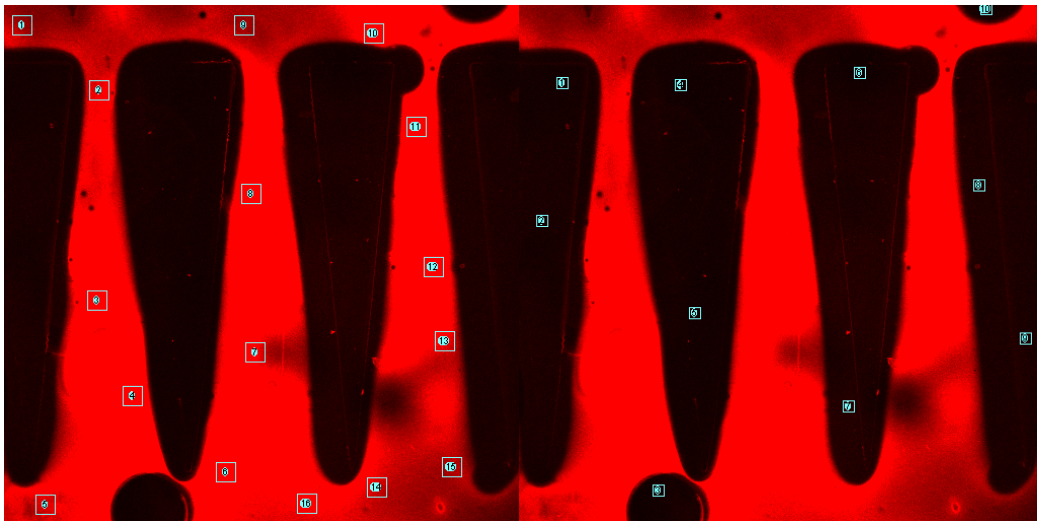


Figure 7-8. Effect of varying the type of crosslinker used in chitosan polymerisation on MIP protein recognition, visualised by fluorescence of rebound Cy5-labelled HSA. No crosslinker (a-b), MBAm (c-d), EGDMA (e-f). NIPs shown on left, MIPs on right. [100x magnification]

7.3.5 Quantitative Evaluation of Molecular Imprinting Effect

To examine further whether this simple assay design of using patterned two dimensional gels could be used as a model for relatively easy and rapid measurement of target recognition in a molecularly imprinted polymer, we sought out to apply quantitative measurements of the changes in fluorescence intensity for the sample as a measure of bound protein levels, to empirically assess any change between NIP and MIPs. This also allowed us to get a better understanding of the true effects of using additional crosslinkers incorporated into the polymerisation formulas. For this purpose, we re-examined the image results produced from the study of patterned chitosan surfaces, both imprinted and non-imprinted, polymerised using formulations without any additional crosslinking reagent, or with either MBAm or EGDMA (see preceding section 7.3.4).

By means of the ImageJ software (EMBL distribution package, *v*1.0f with associated plugins - European Molecular Biology Laboratory, Heidelberg, Germany), the appropriate colour channels of the confocal images, through the Leica software produced .LSM files originating from the confocal microscope, could be analysed. Using the 8-bit red filter images, selected regions of the patterned chitosan gels were chosen which adequately reflected the average condition (i.e. homogeneity) and coverage of the surface. These regions of interest (ROIs) were chosen as arbitrarily as possible to minimise operator judgment and provide the best approximation of the actual gel surface, for consistency. At least 15 ROIs were chosen for each sample evaluated, and each was set at an area of 30 μm x 30 μm. For the measure of a control background level, ten non-fluorescent areas of the stamped pattern of 10 μm x 10 μm were selected for each sample image for blank subtraction. A representative example of this selection procedure is demonstrated graphically with the image pair shown in Figure 7-9. Additional image examples of this sample type and those of the other formulations were carried out in the same fashion described.



(a) *(b)*

Figure 7-9. Example images depicting unbiased regional selection of ROIs on a patterned chitosan gel surface, in this particular case representative of a surface of EGDMA-crosslinked MIP. (a) Specifying areas of measurement readings for average intensity estimate. (b) Non-fluorescent regions used for background correction.

Through the ImageJ software, the integrated density (i.e. surface area intensity) was calculated for each ROI selected. From these, a mean fluorescence was calculated for each sample, from which was subtracted the mean background reading for the same surface. This provided an average fluorescent intensity. This procedure was repeated for all samples of chitosan MIPs and their corresponding NIPs. For each of the patterned chitosan samples, the collected data points were used to represent a mean value of intensity (from the selected regions of the gel surface) and these were used to compare against other surfaces samples (comparable data sets). Statistical significance of the values was evaluated to using a two-tailed Student-*t* test, assuming unequal variances. Each individual sample was examined against both of its controls in statistical pairing, for example, in a given crosslinker formulation, MIP 1 against NIP 1, MIP1 against NIP 2, MIP 2 against NIP 1, and MIP 2 against NIP 2, and so forth. A summary of the results of the 2D image intensity measurements is shown in Figure 7-10. In every case, the molecularly imprinted samples were statistically different from their corresponding controls; the higher fluorescence values being indicative of an improved binding of the target protein imparted by the imprint effect. The difference between the samples was found to be statistically significant at an α value of 0.05.

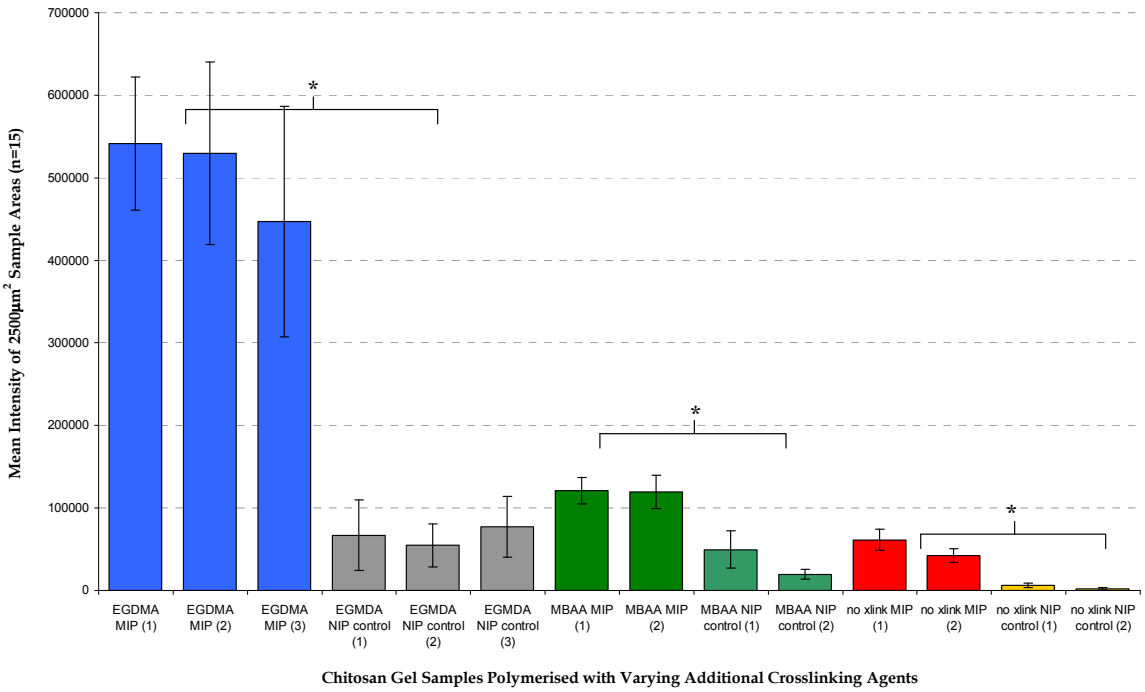


Figure 7-10. Summary of fluorescent intensities measured for each of the patterned chitosan samples following protein capture. Each of the points is expressed as a mean from indiscriminately selected ROIs (n = 15) with standard deviation. Statistical relevance of the paired complementary samples is indicated by * ($\alpha = 0.05$).

These results show that there was a consistently stronger recognition towards the model protein for the molecularly imprinted polymers in each of the formulations examined (normal gels, and using two separate additional crosslinkers in the formula). In each case, regardless of crosslinker or sample chosen, we observed a higher level of recaptured protein (higher measured fluorescence intensity) for the molecular imprinted polymers (MIPs) over the control non-imprinted polymers (NIPs). These were verified to be statistically significant results at 95% confidence, indicating that there was successful molecular imprinting achieved for the thin layers of chitosan gel.

From the average intensity readings (i.e. bound protein levels) for each of the samples examined we are able to calculate the imprint factor, α, as a ratio of the amount of template bound to the MIP versus the amount captured non-specifically in the NIP of similar formulation. This was done according to the relation,

$$\alpha = \frac{\bar{I}_{MIP,x}}{\bar{I}_{NIP,x}} \qquad \textbf{[7-1]}$$

where $\bar{I}_{MIP,x}$ is the average fluorescence intensity for chitosan MIP gel formulation made using crosslinker x, for the mean across the samples examined (n = 3), and $\bar{I}_{NIP,x}$ represents the average fluorescence intensity determined for the NIP made under the same conditions (control gel surface).

The results of tabulated imprint factors calculated across the different crosslinker formulations are summarised below in Table 7-1 for MIP gel samples of each condition and their respective controls.

Table 7-1. Summary of imprinting factors (α) for 2D chitosan MIP surfaces based on qualitative assessment of fluorescent images in albumin rebinding studies, for varying gel formulations.

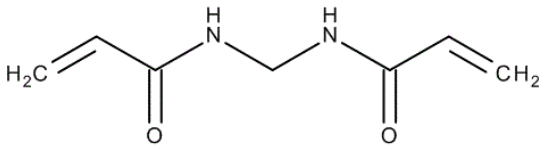
Comparison of Bound Protein Intensities	α
No crosslinker MIP v. No crosslinker NIP	12.6
MBAm MIP v. MBAm NIP	3.6
EGDMA MIP v. EGDMA NIP	8.1

From the above values, we can clearly see that in each case there was an improvement in the recognition (capture) of the target protein by the chitosan surfaces as a direct result of molecular imprinting. All other conditions being held constant, the increase in the amount of average fluorescence seen in the patterned gels, as a result of a higher level of bound tagged protein, can be linked to a higher degree of recognition towards the template molecule due to the imprint sites being created in the MIP during polymerisation. This confirms the successful molecular imprinting of a protein with this chitosan model, as well as validating the simple flat stamping method for forming and assessing imprinted polymers.

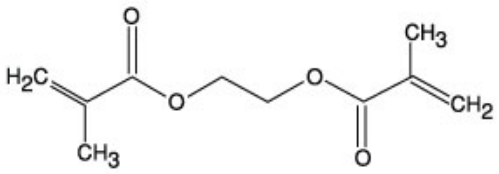
These data also allow us to compare the polymerisation conditions to each other quantitatively. Looking further at the effects of the crosslinkers on the ultimate recognition of the MIP, we can observe that the addition of either crosslinker to the

chitosan matrix has an effect of reducing the recognition of the protein. Comparing the effect of the varying crosslinkers added into the chitosan formulation, we observed that, despite apparent increase in the visual brightness of the images (as seen in Figure 7-8), when examining the results quantitatively using representative measurement of the fluorescent intensities, the imprinting effect was not improved with the addition of crosslinkers to the gel formulations. The MIPs constructed using additional EGDMA crosslinker provided a stronger degree of recognition towards the template molecule than those made using MBAm, however each was found to have bound a lower ratio of template than the chitosan gels alone. It could be that this is linked to a stronger tightening of the polymeric matrix that prevents the necessary arrangement of the network around the template to successfully form the imprint site, as well as narrowing the interconnectivity and porosity, impeding the diffusion out of, and subsequently in to, the gel post-crosslinking. While the additional crosslinkers act to produce a stable gel, because there is a reduction in the number of imprint sites available for binding (as a consequence of the suggested network changes), the MIP and NIP become more alike, and the imprinting factor is reduced.

The imprinting factors shown in Table 7-1 also indicate that the incorporation of EGDMA as a crosslinker into the pro-polymerisation solution had less significant an impact on the reduction in template recognition than did the use of MBAm. This could be due to the differing chemical structure of the two crosslinker molecules as well as the chain length. These two crosslinking agents, as seen below in Figure 7-11, possess different functional groups and in different positions from one another. This will affect the manner and degree in which the polymer matrix interacts with the template molecule. By the reasoning applied above to the discussion of template recognition and mobility in relation to matrix density, because the molecular length of the EGDMA crosslinker is also longer, it should result in a greater space between crosslink points after polymerisation, which implies that there would be greater availability to entrap a larger molecule such as a protein, than would occur with a very closely packed network (e.g. from a short crosslinking chain). Thus, in such a situation, one can expect a higher degree of binding of the target to transpire .



(a)



(b)

Figure 7-11. Chemical structure of crosslinkers used in 2D gelation study with the modified chitosan. (a) *N,N′*-methylenebisacrylamide, and (b) ethylene glycol dimethacrylate.

While it appeared visually from the images presented in Figure 7-8 that the addition of crosslinkers into the hydrogel resulted in an increased amount of captured protein, we are able to confirm via the quantitative analysis that this trend was indeed balanced by a matching increase in overall fluorescence with the control (NIP) as well. Looking at the numbers for the specific images previously presented in Figure 7-8, which can be seen calculated below in Table 7-2, we observe that the trend for these particular samples precisely parallels that seen overall among the other chitosan MIP gels examined. This provides further confirmation that the imprinting effect measured via this method remained consistent through the samples tested, and supports the increased capture of template molecule through recognition by the MIP which was assessed through the visual observations via confocal microscopy.

Table 7-2. Imprint factors (α) calculated based on the evaluated fluorescence intensities of the specific series of patterned chitosan gels for Cy5-Hep recapture depicted in the images from Figure 7-8.

Comparison of Bound Protein Intensities	α
No crosslinker MIP *(b)* v. No crosslinker NIP *(b)*	9.7
MBAm MIP *(d)* v. MBAm NIP *(c)*	6.4
EGDMA MIP *(f)* v. EGDMA NIP *(e)*	8.3

7.4 Discussion

Demonstrating imprinting effect empirically remains a problematic issue in the molecular imprinting field. Because imprint recognition is usually measured as only a slight improvement in binding/recognition of the template over a non-imprinted control, particularly when dealing with hydrogels, a difference of just a slight increase in selectivity of the MIP is often considered a success.[152] As a result, precise measurement of this small magnitude in difference becomes very important. Such control is raised to greater importance when MIPs are integrated as a part of biosensors or clinical diagnostic tools, wherein the precise determination of the absence or presence of just minute amount of targets is vital. Most techniques allowing for such precision are often accompanied by a significant cost of expense and expertise. In this study we have developed a simple, rapid method to visually observe recognition through imprinting effect on chitosan hydrogels.

The initial observations demonstrated that the modified chitosan polymer could be quite effectively patterned using a combination of PDMS moulds prepared through photolithography and photocrosslinking. This holds promise in the application of the MIP biomaterial in high surface area coatings for biomedical implants and sensors. The next stage of evaluating the imprinting with IgG protein as a model template further elevated the expectations of achieving molecular imprinting in this matrix. While the chitosan gel possessed a fairly high degree of autofluorescence as seen in the blue wavelengths, using a green filter there was a noticeable increase in brightness between the non-imprinted control and the imprinted surface. More fluorescently-labelled protein was bound to the gels which had been modified by crosslinking in the presence

of IgG than those which remained unchanged (NIP). Immunoglobulin *G* proteins are very large molecules (~ 150 kDa), and possess a notable *Y* shape. It could be argued that because of these two factors, the protein remained entrapped in the gel through washing stages, and that this physical hindrance may contribute to any preferential binding effect observed. It is also surmiseable that the larger molecular size could increase the overall porosity of the matrix, leading to more subsequent entrapment, though this might not be indicative of a selective imprinting effect. Thus, the system was analysed further with biorelevant model templates of a smaller relative size.

By considering the rebinding of smaller template molecules, here represented by heparin and albumin, it should be possible to discriminate the effects of recognition as a result of molecular imprinting during polymerisation, without any obfuscation due to size effects. It seems evident from Figure 7-4 and Figure 7-5 that there was no particular improvement in the amount of labelled heparin that was recaptured using the imprinted chitosan hydrogel. In the case of albumin, however, the results do seem to show increased protein binding towards the MIP. While there was still some overall fluorescence visible in the negative control, it may be attributable to additional factors such as autofluorescence of the gel, some non-specific surface adsorption, or a combination thereof. However, target protein binding was nevertheless considerably elevated over the NIP, particularly at the periphery of the stamped patterns, where one can assume there exists a greater number of surface available binding sites – as seen through the 3D depth of the gel. Calculation of the imprint factor from fluorescence intensity in sample images confirmed a positive imprint effect was achieved for certain formulations of chitosan gel imprinted with albumin. The quantitative determination of fluorescence intensities reflecting capture target molecules supported the qualitative assessment of the thin layer patterned chitosan observed visually. This demonstrates preferential binding of the target in the imprinted polymer, which argues for success of the chitosan gel as an MIP matrix and validation of the fluorescent stamping assay.

Because the nature of the interactions between the polymeric matrix and the template is fundamental to the success of imprinting, side groups along the polymer

chain, network spacing, and the molecular weight between crosslinks will all affect the degree of target binding. These properties will, in turn, be directly influenced by the type of crosslinker used during polymerisation. We examined three conditions within this experimental protocol to observe these effects: without any additional crosslinker (simple chain-to-chain binding), or using either methylene bisacrylamide or ethylene glycol dimethacrylate as supplementary polymerisation crosslinking agents. The results in Figure 7-8 show comparative binding in each of these cases, respectively. Using no crosslinker, there was a slight improvement in the level of captured protein, evidenced by the fluorescence change in *(a)* to *(b)*. With the addition of MBAm, there was a slight overall increase in non-specific adsorption of protein, but the overall binding was greatly improved with the MIP (Figure 7-8 *(d)*). Changing the crosslinker to EGDMA improved the protein rebinding significantly. As can been seen in *(f)*, overall the imprinted gel fluoresced intensely, suggesting a high level of bound albumin throughout. In this case though, there was a sacrifice of some solubility, as the EGDMA is a more hydrophobic molecule, and it was evident there were regions within the polymerised gel which remained immiscible with the aqueous polymer solution during crosslinking. The intense brightness of the image might also suggest that there was an additional contribution to the fluorescence other than simple protein capturing (e.g. auto- or background fluorescence). When quantifying the effect of the crosslinkers through the measured intensity of the fluorescence signal, we verified that the best imprint factor for the chitosan surfaces among the three conditions examined was achieved with no additional crosslinking agent. The MIPs made using EGDMA had an improved recognition of the template versus those crosslinked with MBAm, however it is difficult to apply too much weight to this result, as the agents themselves might contribute some effect, and the individual samples did not show as large a gap in the calculated imprint factor.

Lastly, as we have demonstrated and quantified some specificity of the MIP versus the NIP, it is important to examine its selectivity for the template against competitor molecules. In this case, equal part mixed solutions of two fluorescent proteins that are vastly different from one another were each tested against both of the

corresponding MIP/NIP pairs. By using two proteins labelled with differing fluorophore types, and observing the emission for each at discrete wavelengths, we can isolate the selective protein binding of the template (imprinting effect) vs. general capture of a non-target protein. This would confirm the selectivity of the imprint sites created, verify binding capacity of the MIP in a competitive system, and establish a measure of preferential and general protein capture in imprinted chitosan gels versus controls.

Comparison of the samples with the two emission wavelengths confirmed that a stronger indicator of albumin was present in the albumin-MIP than heparin. The general adsorption we can see in Figure 7-6 and Figure 7-7 does indicate that the non-template molecule was present in the MIP following a rebinding stage with the mixed solution, however, using the NIP as a control reference surface to judge from, the levels of non-target capture during this phase are consistent between the two gel types, hence one can presume that the imprinting procedure does not cause an increased amount of binding for non-target proteins to the chitosan surface. The heparin-imprinted gels showed less of an effect, with almost no signs of imprint recognition between MIP and NIP. When considering the albumin binding to these same gels, it was possible to note an increased amount present in the heparin-MIP. What this would suggest is that the template sites remaining from heparin imprinting are occupied by albumin instead, conceivably preventing higher levels of heparin capture by the imprinted chitosan gel, despite its larger size. This points towards a much higher interaction ability between the polymer and albumin protein, though this is not directly reflected in non-specific adsorption to unmodified gels. Taking these results into account, the indications are of successful imprinting of albumin as a template molecule with the patterned chitosan, even in the presence of a smaller molecule that could compete for interaction at the binding sites, which once again proved its selectivity as a MIP. Though heparin did not display the same recognition in a selective binding assay, this could be due to interactive differences with the chitosan matrix under these conditions, and is supported by our other observations.

While primarily only examined in a qualitative sense for studying the binding comparison of MIPs and NIPs to judge imprinting factor, this model of moulded chitosan gels proposes a simple and rapid assay for detection of analytes of interest. The quantitative analysis done on the series of chitosan gels made with varying crosslinker validated the successful imprinting results seen in the other sections. There was a consistent trend towards higher re-capture of the target molecule in the imprint polymer than control. This supports our previous arguments and the assay results. The modified chitosan hydrogel was successfully used as a molecular imprint matrix, and was capable of specifically and selectively recognising proteins. The ability to use the fluorescence observations for the eventuality of sensitive quantitative measurement of the imprinted polymers suggests future possibilities towards improved assessment of such MIPs in thin, pattern-controlled surfaces. The ease and flexibility of being able to design any desired particular pattern for a binding surface signifies the multitude of functions and multi-capture systems it could be applied to. Furthermore, the miniaturisation of the design and ease of manufacture of the surfaces lends itself well to the fabrication of specialised detection surfaces. As reported by Guillon *et al*., these types of micropatterned MIPs can be used to form arrays of target binding sites on microbiochips that could be spatially resolved and allow for multiplex detection.[369] This is a key advantage in the development of rapid production mass-target biosensors, which represent a growing field in the future of bioengineering research.

As a cursory study, the results presented here show great promise for the application of chitosan as a MIP matrix, a field which has hitherto remained largely untested. By using a material with known benefits towards biological incorporation and with strong physical versatility in an imprinted gel, we are combining knowledge across two fields to achieve an adaptable recognition matrix. Whereas chitosan is a well studied and documented material, and offers many points of potential chemical modification, this imprinted gel can easily be fine tuned to target specific desired molecules. The ability to form the chitosan gel via photocrosslinking establishes the ease by which any number of shapes, surfaces, or patterns can be obtained. We can also report on achieving the recognition of proteins in aqueous media, a state more

representative of the native environment that is relevant in working with biomaterials, which has been a subject of limited reporting to date. With further study, the selectivity and specificity of the imprinted chitosan matrix can be quantified, and the strength of binding between template and MIP examined to better understand and tailor the polymer towards specific applications.

7.5 Summary

We have demonstrated a new technique to pattern modified chitosan hydrogel surfaces, and use these surfaces for facile observation of molecular imprinting recognition. Using the biocompatible polymer chitosan, we have shown that it is possible to attain some increased recognition towards biomolecules, assessed through recapturing of the target/template from a buffer solution, via the process of molecular imprinting. This effect was increased with larger size model proteins, here exemplified by *IgG* and albumin. Smaller sized molecules such as heparin demonstrated less of an ability to be successfully imprinted, suggesting that for the specifically given system and binding conditions examined, there exists an optimum molecular size for a template to interact sufficiently with the chitosan matrix to a degree required to allow imprinting and recognition to take place. This effect can be further supplemented with the additional inclusion of crosslinking agents into the pre-polymerisation mixture, which reduces the overall spacing between polymer chains and results in a tighter network, at the sacrifice of diffusability. Additionally, the ability for the chitosan MIPs to preferentially bind the target molecule has been validated through a competitive adsorption assay in the presence of another protein. Thus, the molecularly imprinted chitosan polymer not only verified the capacity for specificity but also provided evidence for selectivity towards the target. This substantiates the investigation of chitosan as a foundation for molecular imprinting and attests towards the value of further study in this regard. Of note in addition, particularly, is that the recognition was attained under aqueous conditions and toward larger model biomolecules. This new imprintable matrix could be developed into many applications, including biosensor devices and ‘smart’ biomaterials.

8 Evaluation of Molecular Imprinting by Dielectric Spectroscopy

It remains of great interest to scientists and engineers to be able to achieve MIP recognition in water-based solvents, in particular for applications involving biofunctional/biomaterial substrates. The imprinting of proteins, especially, is a field of rapidly growing study.[370] A principal component of this research work is to apply MIPs to monitor binding events for a complex biomacromolecule target. As potential biosensors, imprinted polymers show low detection limits[43] and rapid response times.[371, 372] Furthermore, numerous strategies are available for customised MIP generation;[373] this synthetic adaptability provides seemingly inexhaustive potential towards development of modern therapeutics.[235] As previously discussed, a key principle in molecular imprinting studies is the correct observation and evaluation of imprint effect. A discrete method is necessary that can sensitively and accurately examine the capture and/or release of a target molecule such that the difference – often slight – in this behaviour between an imprinted polymer and a non-imprinted control can be precisely determined.

Achieving a method of accurately detecting and discriminating the difference between the template and other molecules is not a trivial concern, as the measurable binding within the polymer will be masked by many non-specific interactions. This is especially true of large molecules with many potential sites of interaction, such as proteins. One of the great difficulties in quantifying any imprinting effect in MIPs is that often the difference over controls is not easily measurable, as one is dealing with detections on a molecular scale. For quantitative measurements to be reliable, for use as a gauge of imprint effect, the precision and accuracy of instrumentation and conditions must be able to discern the minute changes in target concentrations that arise from interactions with the MIP.

To overcome this challenge in detection, the most common approaches are to create large MIPs in bulk, which are crushed and packed into chromatography columns.

The larger volumes involved help to amplify the differences in signal from imprinted polymer and control. Unfortunately, this scale-up also means more template is required and the changes in concentration from binding events are more easily masked by the overall amount present. Often, separation methods such as HPLC are used with such MIP-packed columns, wherein the increased interaction of the template allows for temporal separation and detection of the analyte. This remains a cumbersome, costly, and time-consuming process. It also necessitates having large quantity of material, both MIP and template; for formation of the bulk polymer - which is not necessarily always available. Using a simpler method, such as to detect the presence of the template *in situ* optically, as by UV absorbance or fluorescence tagging, is yet another option. In such cases, however, one must sacrifice considerable analytical capability, and if the difference between MIP and NIP recognition is small (such as with low concentrations of analyte, as is typically the case in biosensors) then any signal change that arises may be lost within the inherent noise of the system. To improve upon this, MIP technologies are continually developing that make use of new detection methodologies and high levels of sensitivity. Innovation is also leading to advanced methods for real-time monitoring of systems, which allows for immediate observations, and responses which are more realistic in terms of forming a model of representative system behaviour.

The field of electrochemical sensors is rapidly growing, with ever improving levels in the limits of detection (LOD) and response times. Substrates based on molecular imprinting have become a natural branch of this, owing to their selective ability as recognition elements.[118] Generally, conductometric-based sensors are on the lower end of selectivity, an aspect whose weakness is balanced out by their sensitivity, low cost, and simplicity. Combining the properties of a selection recognition element (e.g. MIP) with such a sensor considerably improves the capabilities of the system.

Dielectric spectroscopy (DS) is a highly sensitive method of electroconductivity measurement that can allow real-time analysis of minute electrical property changes in macroscopic systems. Upon application of an electric field to the medium, the resulting polarisation can be measured, in terms of frequency-dependent complex permittivity

and conductivity, or as an impedance spectrum expressed as a relation of amplitude and relaxation time of charge density fluctuations in the sample. DS is especially valuable as it provides information on the intermolecular interactions of samples to bridge the link between molecular spectroscopy and bulk sample properties. Because of its direct, *in situ*, and real-time capabilities, DS has become a valued instrument in the analytical toolbox. It has been shown to be a reliable means for measuring drug release from chitosan polymers.[374]

By applying a frequency range and monitoring the impedance and capacitance in a measurement cell containing an imprinted polymer, it is possible to derive permittivity and conductivity information for the MIP, and their relation to changes in the interspatial medium as an analyte binds to a polymer matrix. Applying capacitive transducers to measure changes in template binding has provided improvements in detection to other approaches.[375, 376] Generally, impedance analysers have been incorporated with MIPs for the development of chemical sensors.[371, 377] In their study, Zhang *et al*. demonstrated how electrochemical impedance can detect very low analyte concentrations ($1e^{-6}$ mg/ml) in thin layers of a thioglycolic acid imprinted polymer.[378] They also observed in this case that more polar solvents yielded better detection, and that the best signal was seen close to physiologic conditions. This demonstrates promise for detection with aqueous solutions of biomolecules under native conditions. The capacity for impedimetric-based MIP chemosensors to be stable with time and reusable has been reported by Panasyuk-Delaney *et al*.[379] Taken in conjunction, these reports corroborate the potential benefit towards developing a reliable, robust, marketable biosensor using MIP technology. Sensors based on MIP and dielectric measurement technologies may combine the precise nature of the two approaches, and are thereby very capable of sensitive detection of target molecules. Recently, BelBruno *et al*. created imprinted sensors capable of detecting amino acids of lower than 1 μg analyte levels with DS.[380] Likewise, work with histamine-imprinted sensors has been shown to be effective at concentrations approaching 1 nM,[381] with rapid and consistent responses.[382]

Given the obvious advantages in cost/mass production and portability, miniaturisation of the so-called lab-on-a-chip field is regarded as the next frontier of biosensors. Suedee *et al*. have similarly taken advantage of microscale MIPs for in-line sample detection. Using interdigitated electrodes allowed the authors to obtain the differential measurement between MIP and NIP, using a true coupling between the recognition element and the transducer.[383] The ability to measure analyte-MIP binding events non-invasively is a massive benefit, which allows for its incorporation into complex detection systems. One example of this is as a component in a microfluidic platform, such as that proposed by Birnbaumer *et al*., wherein viruses can be detected in feed streams using an imprinted matrix, and the corresponding binding demonstrated specificity over other viral strains.[384] Another example is a miniaturised version of a biosensor, designed as a handheld diagnostic tool.[385] Dielectric measurements have also been used to directly monitor the release of model drugs from chitosan microspheres.[386] An understanding of these properties and determination of particle and drug conductivities can lead to quantification of release kinetics and valuable data on the dependence to carrier traits.[374]

Because of its high sensitivity and ability to monitor the real-time changes in a system non-invasively, dielectric spectroscopy was chosen as an evaluation method for measuring the template binding to the molecularly imprinted chitosan. By using a measurement cell, the polymer could be contained between the electrodes of an impedance analyser, for direct on-line measurements of electrical changes corresponding to analyte concentration, with imprinted and non-imprinted samples. As model templates for this experiment, we selected for target molecules heparin and cytochrome *C*. Both are of similar size, with a molecular weight of approximately 12 kDa, but have notably different chemical structures and functionalities. Heparin is a highly sulfated glycosaminoglycan, widely used for its protein-binding ability. It is a common anticoagulant applied in medicine. In biomedical applications, heparin has shown to promote angiogenesis, has been demonstrated to reduce inflammation, and has high affinity for fibroblast growth factors. It has also been shown to strongly interact with chitosan.[387] Cytochrome *C* is a common molecular imprinting model

protein. One of the first studies with protein imprinting made use of Cyt *C* as a template in a polyacrylamide gel.[388] It is often used in competitive studies with lysosyme, due to a similarity in both molecular weight (14.4 and 12.3 kDa, respectively) and isoelectric points (10.5-11 for lysozyme and 10.2 for cytochrome *C*).[389]

8.1 MIP Microspheres

The use of small imprinted polymeric spheres with very high surface areas and surface-to-volume ratios shows great promise from both analytical (detection) and drug delivery standpoints. Such MIPs could maximise the potential binding capacity, while also allowing flexibility with being injectable, packable, shapeable, etc. Although a range of differing techniques are available to form imprinted particles and gels,[390] microsphere and nanosphere MIPs are in general often produced by emulsion polymerisation; for example, the use of an emulsion system of chitosan in an organic solvent together with a surfactant. Espinosa-Garcia *et al*. have shown that such a polymerising approach can be used with chitosan MIPs.[162] Emulsion imprinting has the advantage of greatly increasing the surface area on the polymer for target molecule binding, while also enabling larger template molecules, typically restricted in diffusion due to their size, to be arranged and imprinted at the surface of the microspheres.

Emulsion polymerisation has been used in multiple molecular imprinting designs,[27, 162] most notably with acrylic acid/methacrylates[391, 392] or polystyrene particles.[393, 394] It has also been employed by Aherne *et al*. for cell-imprint systems.[222, 223] One concern is that the use of surfactants in emulsion polymerisation does bring with it a danger of denaturing the structure of sensitive templates like proteins.[103] If designed accurately, it can, however, allow for oriented organisation of the template, in order to imprint a specific recognition site,[395] a factor which may become important when considering the imprinting of epitopes of macromolecular bodies.[108] We selected to investigate microgels produced in an emulsion by further developing the polymerisation strategy for the chitosan MIPs. It was envisioned that such an approach

would allow for the benefits of a reduced-scale system, while simultaneously providing for the high binding capacity of biomolecular targets such a mode affords.

Gels were formed by a similar procedure as in bulk, through free-radical initiated polymerisation. Template solutions (20 mg/ml) were added to dissolved, functionalised chitosan, along with photoinitiator (150 μl). This pre-polymerisation mixture (aq) was then concentrated to remove excess water using a rotary evaporator, maintained under foil wrap to prevent premature initiation by native light exposure. Concentrated methacrylated chitosan solution was added dropwise to an oleaginous phase (mineral oil) with approximately 5% v/v Tween 80 as surfactant during emulsification. Droplet formation was achieved using an Ika T18 UltraTurrax homogeniser (Ika Works Inc., Wilmington, NC) at a discrete mixing speed of approximately 11000 rpm. A thin layer (petri dish) of the homogenised mixture was then photo-crosslinked *in situ* suspended within the oil phase. Ten minute UV bursts were used for photoinitiation of the reaction and the activated chitosan was allowed to sit and continue to reach complete gelation before removal from the organic phase. The gelled spheres were isolated in a separatory funnel, washed with excess water, and collected. Particles were then centrifuged at 3400 rpm for 3 x 10 min. Following separation of the microspheres, the supernatant solution was removed and replaced with fresh water, and the imprinted gels were allowed to soak with gentle rocking. Soaking media was changed repeatedly (in six hour interludes) as the template was leeched out from the imprinted polymer over the course of 3 days. Completion of template removal from chitosan gels was verified by measuring UV absorbance of the supernatant at 280 nm (μQuant microplate reader, data not shown) until the observed readings for solute concentration dropped to blank values.

Imprinted and non-imprinted microspheres were then soaked in sample template solutions, dissolved at concentrations of 10 mg/ml in 1x PBS. Each MIP sample type was exposed to its corresponding imprint template as well as to the analogous competitor molecules. NIPs were immersed in samples of all templates used. Following a period of 3 days, gel/template solutions were passed over 0.45 μm polyvinylidene

fluoride filters (Millipore Durapore HVLP) under light vacuum (solvent and unabsorbed template passing through), washed with an excess of 10 ml water to remove gently adsorbed template from gel surfaces, and collected. To standardise test quantities for each trial, a 150 μl volume of non-packed chitosan particles was isolated for impedance testing per batch.

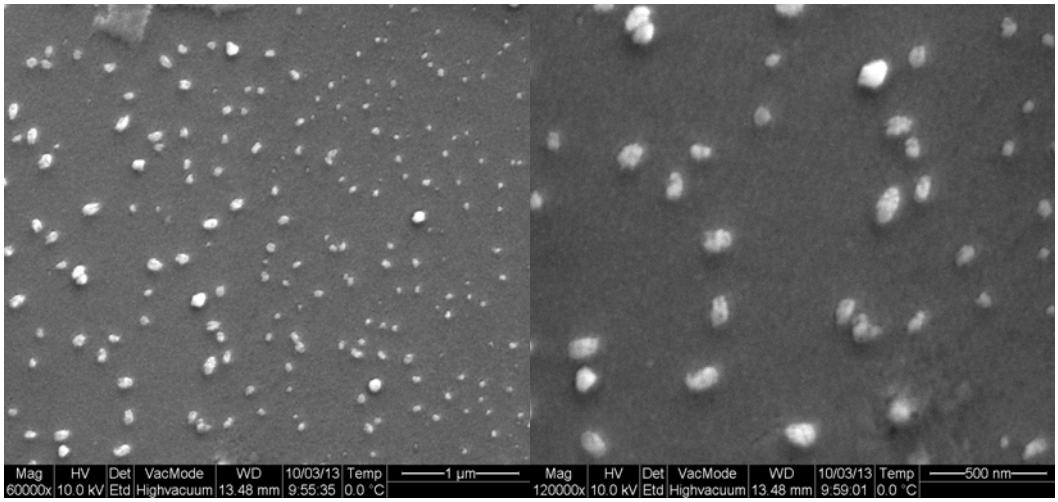
8.1.1 Controlled Nanogelation

One field where further investigation was done on this topic involved improvements to the gelation procedure used to form the chitosan spheres. As having a high surface area available for binding would increase the amount and density of molecular imprint sites in a polymer, as well as augmenting the imprinting and capture of larger macromolecules, it is desirable to create polymers that would have a high surface-to-volume ratio. One method of easily achieving this is to create nanoparticles of MIP. We addressed this by expanding our approach of using the reverse-emulsion polymerisation with the chitosan. In order to create the aimed-for polymeric beads, we switched to a chemically-initiated crosslinking system. In addition to yielding much smaller sized particles, this method allows for greater control over the polymerisation reaction (e.g. incremental addition of reactants, initiation into pre-polymerisation solution, rate adjustment with temperature and mixing speed during the process, etc.), provides more homogeneity throughout the solution and reaction, and results in an improved uniformity of size for the produced gels.

The polymerisation reaction was based upon a procedure described by Heris *et al.*[396], modified for the gelation of chitosan. Briefly, an emulsion was created starting from an oil phase of 0.25 M dioctyl sulphosuccinate sodium salt (AOT) and 0.05 M heptan-1-ol in isooctane. This was thoroughly mixed to dissolve reactants and placed in a narrow reaction flask, immersing the vessel in an ice bath to control the temperature. The organic components were stirred at an elevated speed (~ 15.5k RPM) using the Ika T18 UltraTurrax homogeniser. To this was then added the aqueous phase, namely concentrated methacrylated glycol chitosan in PBS (with or without the addition of

template solutions, as necessary), and the entire mixture was homogenised for 20 min. The reaction was initiated with the addition of 300 μl of freshly prepared potassium persulphate and 2 μl of TEMED. The speed of the mixer was reduced to ~ 12k RPM, and the emulsion allowed to react fully (under temperature-mitigated conditions) for 3 h. Following this, the solution was removed and decanted into a large separatory funnel, to which was added 5x the volume of acetone to precipitate the gelled particles. Collected nanoparticles were washed, sonnicated, and centrifuged repeatedly (2x) to fully rinse organic residues and supernatant. This procedure was then repeated to rehydrate the solution, firstly with resuspension in isopropyl alcohol (two washes), followed by 100% ethanol (two washes), 70% ethanol, 50% ethanol, and finally, water.

Chitosan nanospheres produced by this technique are shown in Figure 8-1. From the SEM images, we can estimate the size distribution of the gels to be approximately 80-200 nm. They are roughly spherical in shape, and have ample distribution among the sample, possibly due to some gel fracturing taking place at the high speeds of emulsification involved. To narrow down the size range of the collected nanoparticles, filtration was attempted using 100 nm (lower limit to remove fractured particulates) and 200 nm (upper limit to cutoff coalesced gels and aggregates) pore size membranes. Unfortunately, due to the high volumes involved with producing and washing these nanoparticles in each step, and the very small sizes required for filtration and isolation of the resultant gels, this system proved exceedingly inefficient due to the volume and loss of nanogels, and hence was not pursued further as a course for forming MIPs of chitosan. While the gelation achieved from this method was more consistent and reliable, the poor efficiency in separation and high loss of material at each stage reduced any benefit gained from it. Thus, it was decided to follow the course for production of the photocrosslinked microspheres primarily in the subsequent investigations, and leave pursuit of the production of chitosan nanospheres by this method until a future study.



(a) ***(b)***

Figure 8-1. SEM images of chitosan nanospheres produced through chemical crosslinking of a high-speed emulsion at (a) 60 Kx, and (b) 120 Kx magnification.

8.2 Impedance Analysis

The evaluation for imprinting effect in the gelled chitosan microspheres was conducted by dielectric spectroscopy. To accomplish this, a measurement cell was constructed as shown in Figure 8-2. It was comprised of two machined polyacrylic halves, fixed with two parallel plate platinum electrodes electroplated with platinum black to reduced electrode polarisation effect. The cell was maintained watertight with a rubber gasket between the plates and secured together with aluminum screws.

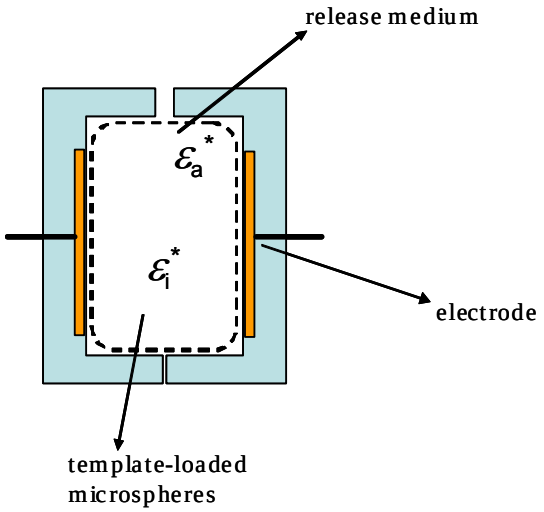


Figure 8-2. Schematic depiction of constructed polyacrylic flow cell used for complex permittivity measurements of chitosan-MIP microspheres (adapted from Chen *et al*. [374])

For release studies, chitosan microparticles were first incubated with an appropriate volume of template solutions, e.g. Hep/Cyt C at a concentration of 20 mg/ml, for 48 hours. Following the soaking interval with target analyte, the particles were then filtered to remove the surrounding solution and subsequently resuspended in a fixed volume of PBS, ~ 2 ml, as a final surface wash of adsorbed protein. The chamber was loaded with either imprinted or non-imprinted microspheres, securely sealed, connected to the unit electrical input, and filled with 400 μl of water just prior to analysis. Impedance measurements were conducted in a controlled non-flow cell, using an Agilent 4294A Impedance Analyzer equipped with a 16092A *Spring Clip Fixture* accessory, within a frequency range of 40 Hz – 110 MHz. Data was collected at ten minute intervals, for a total measurement period lasting six hours. This was evaluated to be sufficient time for the total releasable amount to be leached, based on initial experimentation (wherein measurements of observed behaviour attained a plateau equilibrium).

In order to determine change in the overall capacitance, the accumulated spectra were fitted to the Cole-Cole equation in order to extract dielectric parameters and

correct for electrode polarisation using MATLAB. The complex capacitance was firstly calculated by:

$$C^* = C + \frac{G}{j2\pi f\varepsilon_o} \qquad \textbf{[8-1]}$$

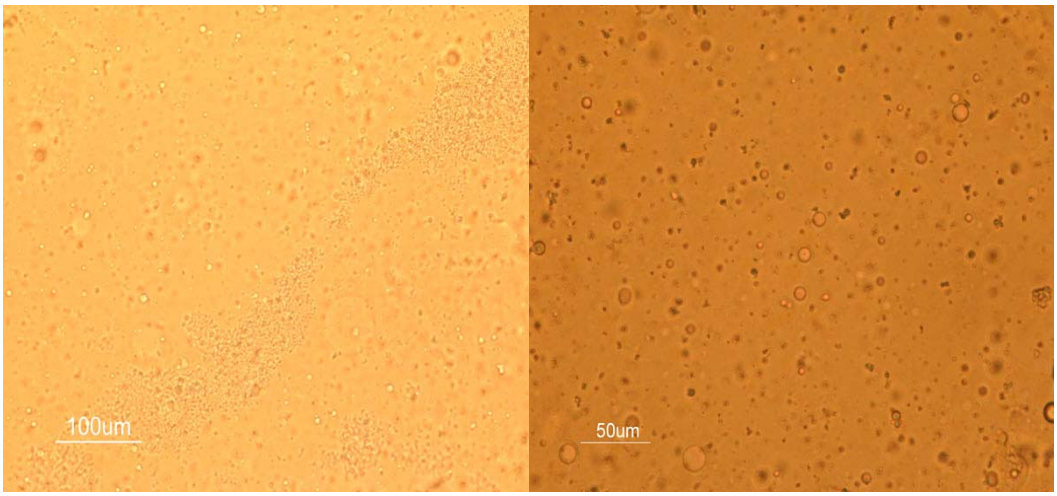
where C and G are the collected capacitance and conductance, respectively, and ε is the permittivity of vacuum (8.854e-12). Since the characteristic frequency, f_c, of our data set was ~ 100 kHz, the relative capacitance was extracted from the fitted curves for each time point. The increase in relative capacitance during microparticle drug release was then plotted.

8.3 Sphere Characterisation

Immediately following crosslinking, chitosan gels were isolated from the oleaginous phase via separatory funnel and collected. Subsequently, gels were not fully dried, i.e. removed from solution, to minimise particle aggregation (data not shown). It has been shown that for some acrylamide MIP particles, a drying stage can also permanently disrupt any chemical recognition.[8] Initial light microscopy observation indicated that the general size of the particles to range from 10-25 μm (see Figure 8-3). To further elucidate the size distribution, samples were run under scanning electron microscope (SEM). These results are shown in Figure 8-4. From the SEM images, it was possible to get a more clear idea of the particle size; again one can see a fairly large range of size, with individual spheres ranging from 3 up to 10 μm (Figure 8-4*(b)*). Figure 8-4*(a)* does show the presence of a few much larger artifacts having a distribution of roughly 20-40 μm in diameter. It is unclear why this unusual assortment arises, as it seems clearly distinct from the general array of spheres, but could possibly come from fusion of a range of particles into one larger mass. As the general shape remains spherical, this diversity likely occurs prior to gelation but after homogenisation, when emulsified droplets come together in union to form a larger 'macro' bead. It is expected that this occurrence would arise on a regular basis, however, and be a natural contributor to the overall size distribution; the sparse and irregular nature of these large particles suggest

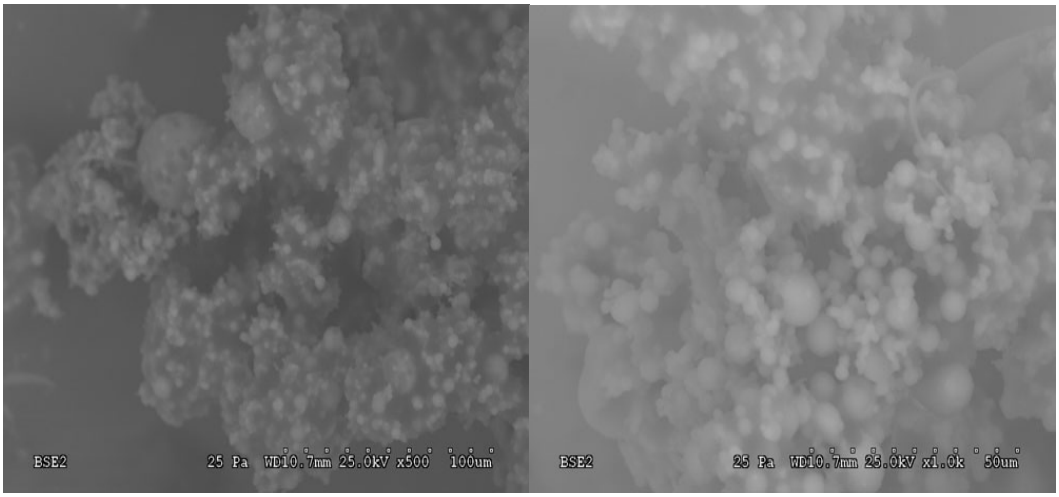
that this is not the case. A secondary explanation for their presence is that these larger beads formed as a result of an inner core of oleaginous phase within a micelle, leading to a core-shell microsphere from a double emulsion before polymerisation. Because the 'ideal' conditions for this to occur are less likely to arise randomly in the single emulsion system, one would not expect many of these type of spheres. Indeed, their size does suggest a duplication effect over that observed for the single chitosan microspheres. Accordingly, this rationale best explains the abnormalities and is thought to be the cause of the larger particles seen.

(a)



(b) ***(c)***

Figure 8-3. Chitosan microspheres produced via polymerisation in reverse emulsion. (a) Dry gelled microspheres, and light microscopy contrast images of microspheres in solution following crosslinking shown at (b) 200x and (c) 400x magnification.



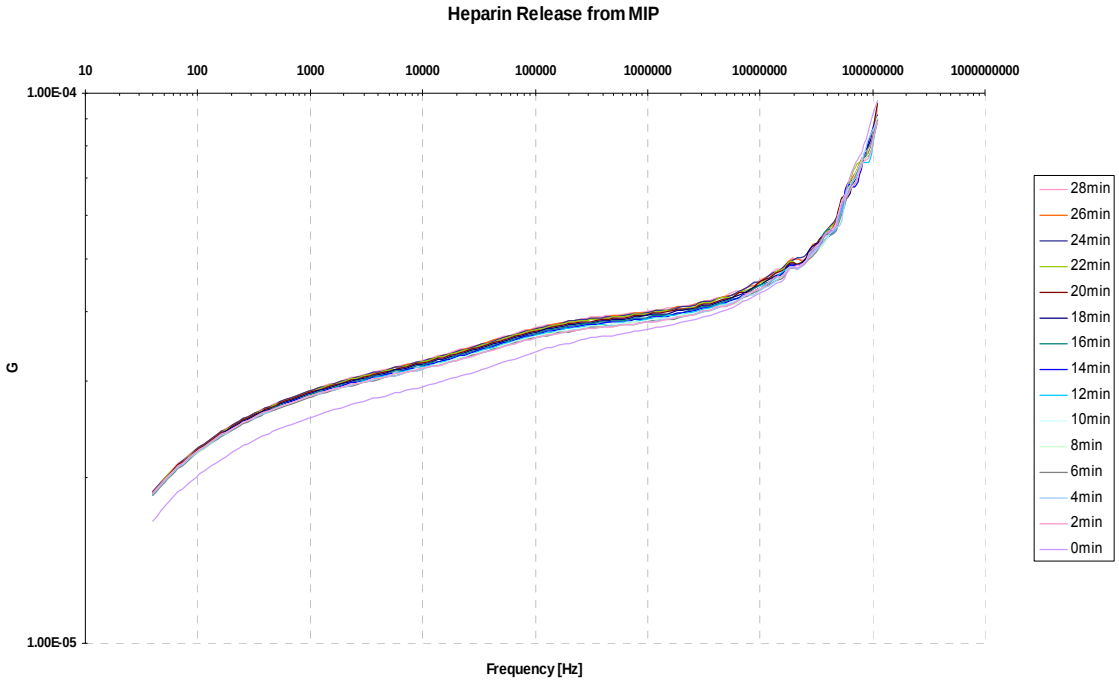
(a) ***(b)***

Figure 8-4. SEM images of polymerised chitosan microspheres at (a) 500 x, and (b) 1 Kx.

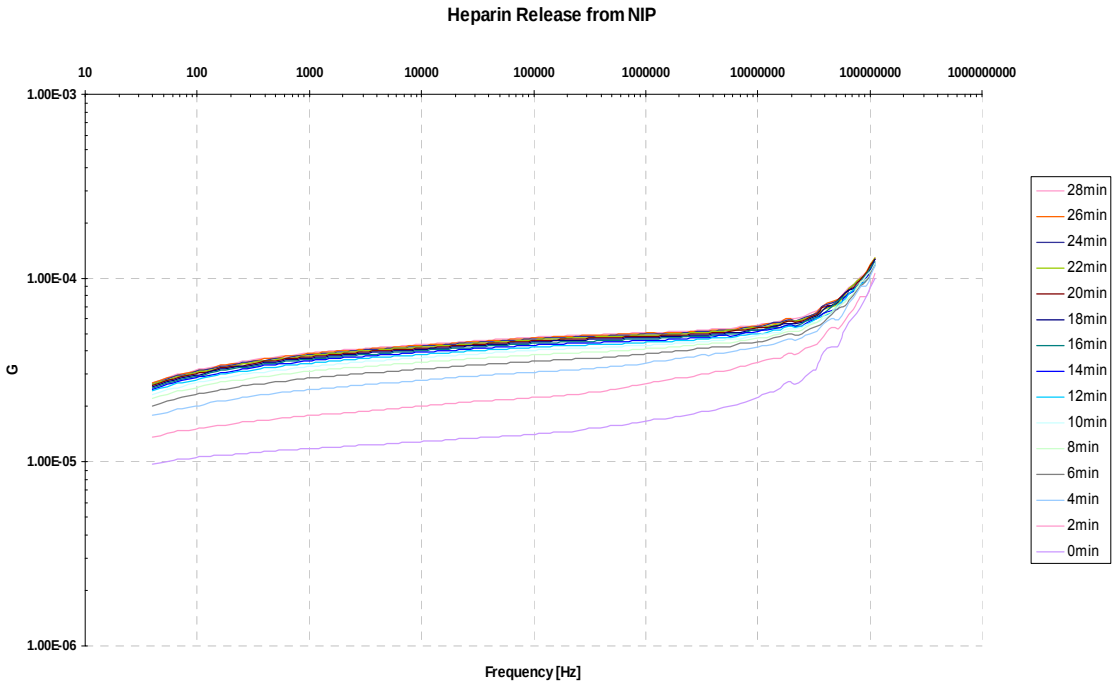
8.4 Binding and Release Results

8.4.1 Comparison of MIPs with NIPs

The changes in conductance of the media surrounding the chitosan microspheres seen from the samples of molecularly imprinted gels pointed towards a slower, gradual release of template molecule when compared against non-imprinted chitosan. Figure 8-5 shows the dielectric behaviour of a model sample (n = 3) with frequency scans over time, for both an imprinted gel formulation and a non-imprinted control sample. This pattern would indicate that there was an effect due to the imprinting process that causes higher uptake of template molecules into the microspheres and delayed release kinetics due to multiple preferential binding areas within the chitosan polymer, distinguishing this from simple absorption and dissolution.



(a)



(b)

Figure 8-5. Sample dielectric spectrum showing temporal changes of conductance (for heparin release) in solution with frequency shifts, following release from heparin-imprinted MIP (a) and NIP (b) microspheres.

Examining the release profiles of each of the proteins tested against the three types of chitosan gels (i.e. the corresponding imprinted polymer, the oppositely imprinted polymer, and the non-imprinted control) is useful in that it allows one to observe the response behaviour of the target to the various polymers, and determine whether any interaction, specific or non-specific, selective versus general, is present. Figure 8-6 and Figure 8-7 show the plots of changes in solution conductance detected within the measurement cell by DS as the proteins leached out from the polymers over the course of three hours. These data sets represent an n of 3, with each sample made up of an equivalent test volume of chitosan microspheres, using sweep frequency averages.

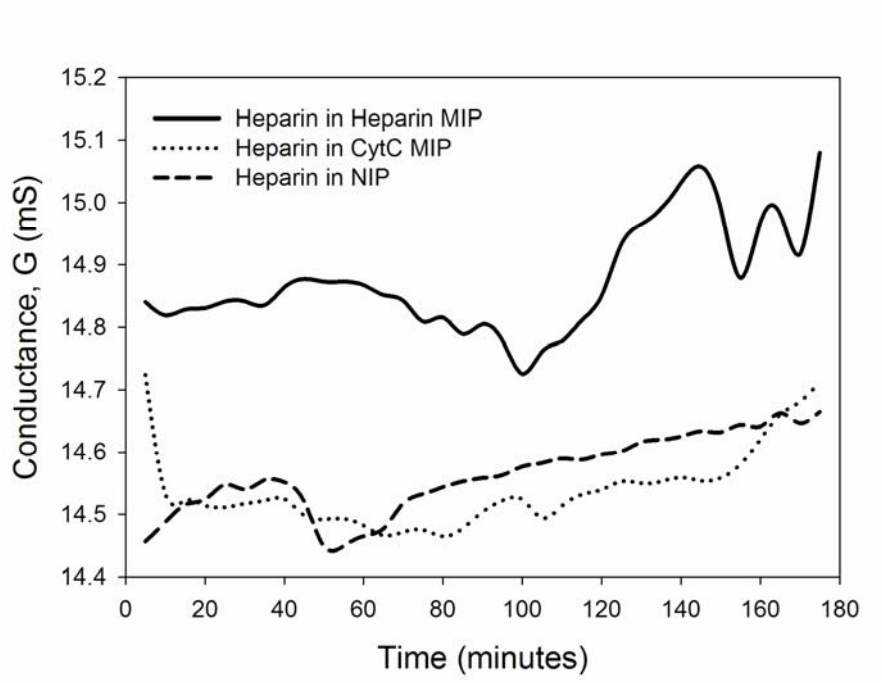


Figure 8-6. Heparin release from chitosan microspheres plotted as changes in solution conductance of the surrounding medium, measured by DS. Solid line = heparin imprinted microspheres, dotted line = cytochrome C imprinted microspheres, dashed line = non-imprinted microspheres (control).

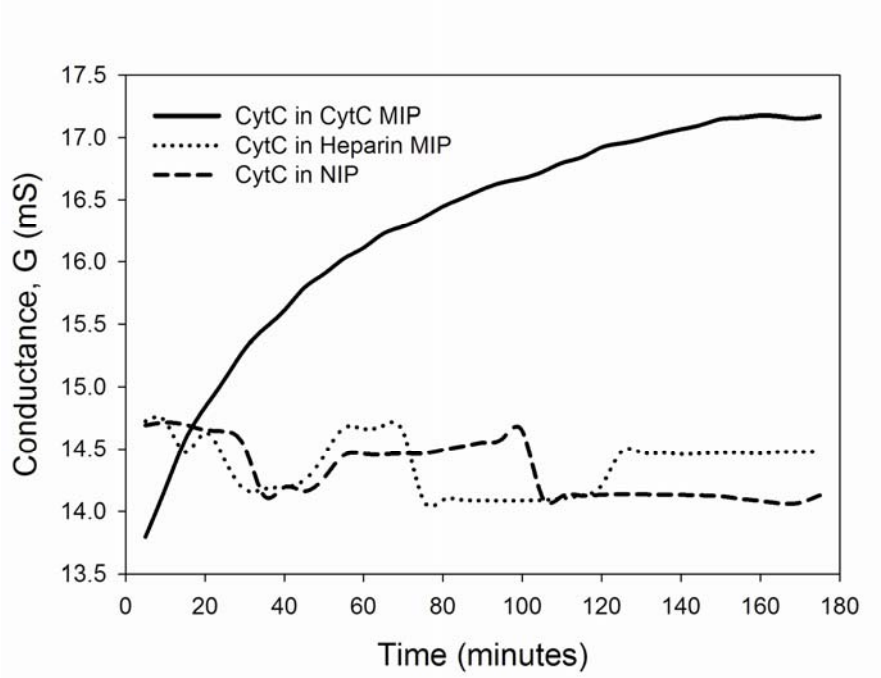


Figure 8-7. Cytochrome *C* release from chitosan microspheres plotted as changes in solution conductance of the surrounding medium, measured by DS. Solid line = cytochrome *C* imprinted microspheres, dotted line = heparin imprinted microspheres, dashed line = non-imprinted microspheres (control).

It seems evident from these graphs that there was a distinct change of target protein release from the matching MIP, which arose as a result of the molecular imprinting procedure. The conductance changes in protein release are very low by comparison for the opposing imprinted polymer and the NIP controls. These results suggest that not only has the physical nature of the chitosan gel been altered during the polymerisation in the presence of the template molecule (i.e. imprint sites created for binding), the sites produced are of recognition specific to the template molecule as a target, as the subsequent binding of an imprinted gel to the competitor protein does not show the same behaviour. Notable as well, the releases of template protein from both of the matching MIPs not only differs significantly from the control, but that the control and non-matching MIP in each case show very similar behaviour to one another, suggesting that any protein observed as being released from those gels [in which it was not the template molecule] results from only general adsorption common to the chitosan polymer, and is not impacted by physical gel changes during the imprinting procedure. Thus, we can state that the imprinted chitosan microspheres showed both degrees of

specificity and selectivity to the targets, as judged from the DS releases. This is an indication of successful molecular imprinting of proteins via this platform.

It is possible to observe these effects more predominantly and in a relevant fashion when the values are shifted from their raw forms and converted to examine the changes in relative capacitance values of the system, and plotted logarithmically with time. The release of heparin from chitosan gels over the initial period of 175 min is shown in Figure 8-8.

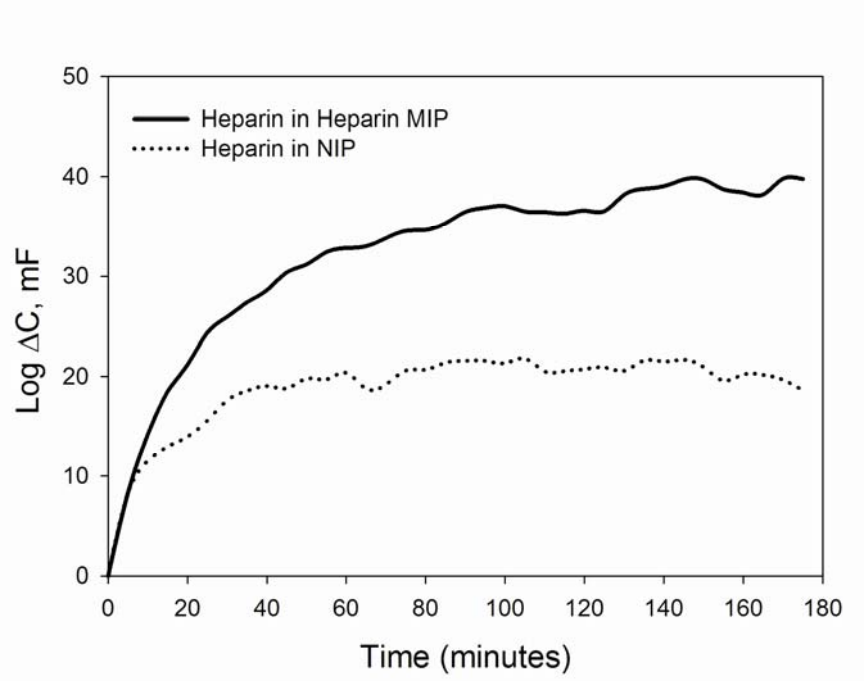


Figure 8-8. Release profile, in terms of calculated change in relative capacitance, from chitosan microspheres, MIP (top, solid) and NIP (bottom, dotted).

A similar change in the release profile (via relative capacitance) was seen with the Cyt *C* imprinted gels. These results are depicted in Figure 8-9 for a 175 min release period.

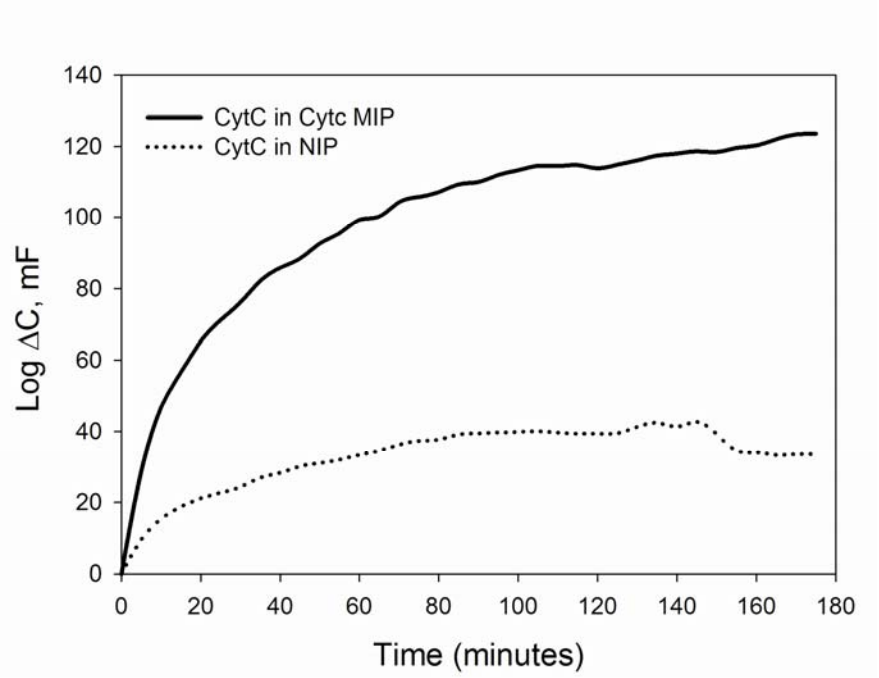


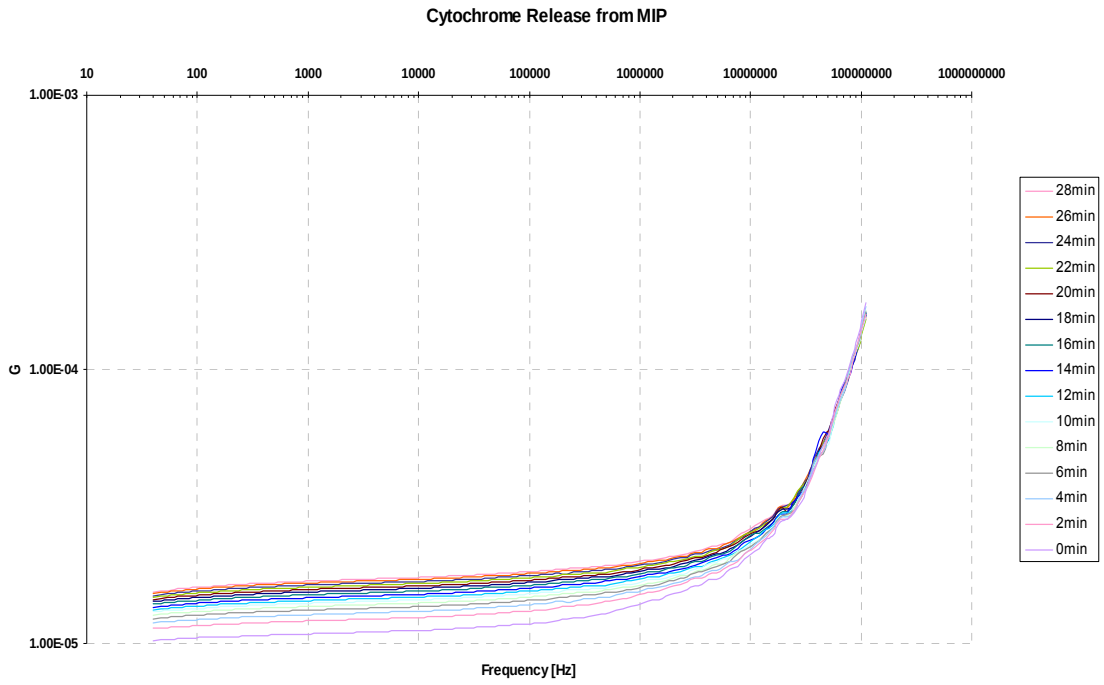
Figure 8-9. Release profile of cytochrome *C*, in terms of calculated change in relative capacitance, from chitosan microspheres, MIP (top, solid) and NIP (bottom, dotted).

In each of these cases, what is observed is a longer sustained release of a greater amount of the corresponding template protein from the chitosan microparticles that were molecularly imprinted than in the gels made in a standard fashion. Because the formulations did not show cross-reactivity to the other protein examined, this release is not merely an artifact of increased porosity within the polymer matrix and can only be attributed to selectivity of the matrix coming from successful molecular imprinting. Such release profiles of model proteins from chitosan suggest strong potential to adapt the developed technique to drug delivery methodologies.

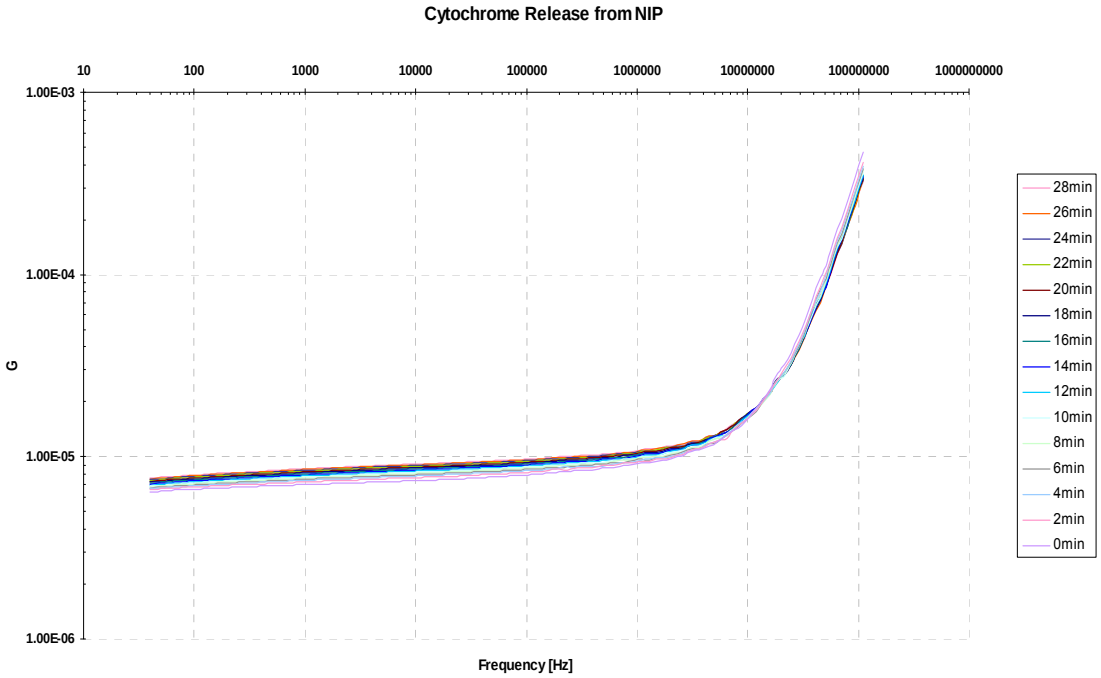
8.4.2 Comparative Recognition of MIP towards a Competitor Molecule

When each of the MIPs was exposed to non-template molecules of a similar molecular size, the release seen was merely general, following a typical profile curve, leading to the conclusion that any uptake of the competitor molecules is non-specific, and distinct from the imprinting effect of the target analyte. Figure 8-10 shows the release of cytochrome *C* from both a heparin-imprinted sample as well as a NIP. Once again, the

release profile indicates a uniform, consistent release, as would be expected without any preferred binding areas. The cumulative release was also less than that seen with heparin, indicating that less competitor molecule (Cyt *C*) is taken up by the gels than the template. The preferential absorption suggests a degree of recognition toward the target protein. This again points towards specificity of the imprint sites created in the chitosan microgels from the molecular imprinting procedure.



(a)



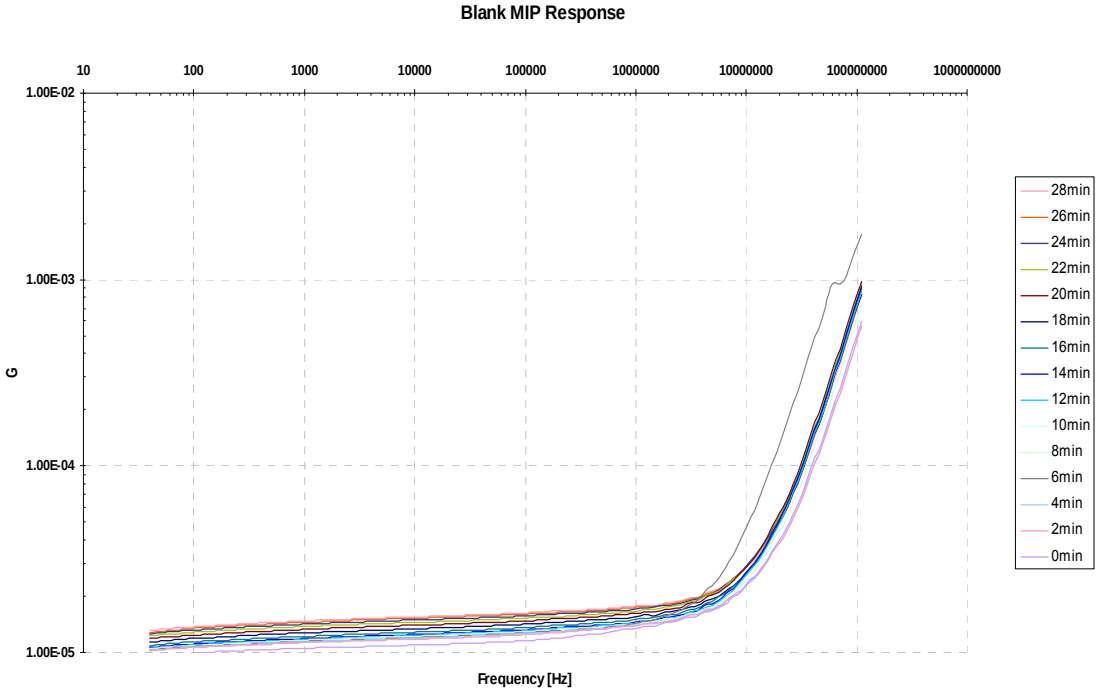
(b)

Figure 8-10. Conductance of cytochrome *C* in solution, following release from heparin-imprinted chitosan microspheres. MIP (a) and NIP (b).

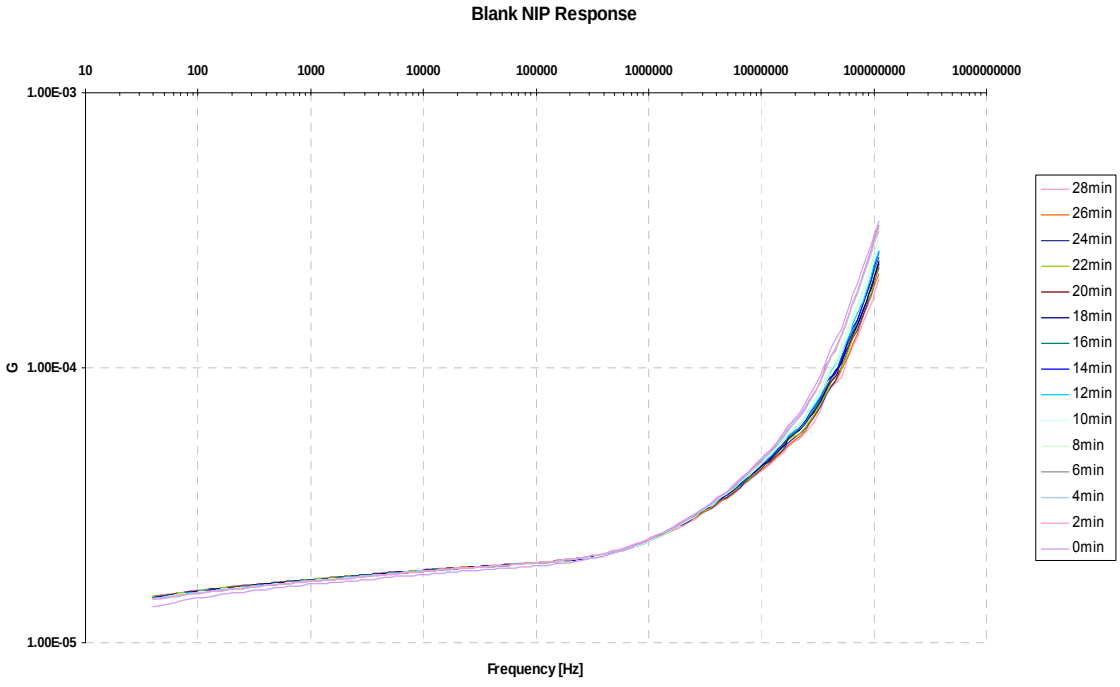
8.4.3 Measurement of Chitosan Gels Alone

An interesting result was observed when control and MIP gels were tested via dielectric spectroscopy in the same DS measurement cell, without the presence of either any additional target or competitor molecules. As can be seen in Figure 8-11, these 'blank' readings showed a significant difference between microparticles that had been imprinted and those that had not. Thus, there seems to be some physical change of the electrical properties imparted on the chitosan gels during the imprinting process, likely as a result of the rearrangement of the polymer matrix, and therefore the electrochemical regions. This confirms a structural difference in the chitosan MIP microspheres over the NIP controls, which supports the argument that the imprinting procedure had a measureable effect on the physiochemical structure of the gelled chitosan network. This is important in that it confirms the particular basis of the successful molecular imprinting procedure, and, when combined with the selectivity and specificity observed in the protein capture and release results, shows that these new

sites in chitosan have been created as a direct result of interactions with the template molecules.



(a)



(b)

Figure 8-11. Permittivity measurements conducted on modified chitosan gels alone in solution.

8.5 Discussion

The DS results examining the binding of relatively small biomacromolecules show great promise towards achieving selective recognition in chitosan-based MIPs. It is evident that when contrasting the binding and release of two molecules of similar size (in terms of MW) but different functionalities, there is some difference between the behaviour of the imprinted matrix towards its template versus an analogue. Using molecular imprinting of a model target in a chitosan matrix during polymerisation allowed us to change the pattern of interaction of the gel with a desired biomolecule.

For both the smaller range and larger molecules, there was a consistent delayed release of the template, and higher cumulative delivered amounts. NIPs, by contrast, demonstrated similar release behaviour to both template and competitor molecules. These results suggest that the imprinted gels show specificity towards their template molecule, in the form of higher initial binding sites as a result of the imprint formation, and slower release, as the template undergoes various rebinding events during its gradual diffusion from the polymer.[28]

When comparing the imprinting effect of each of the templates with their respective polymers against one another, it appears that the heparin-MIP may have provided a slightly higher degree of interaction with the imprinted matrix as the release was more gradual than with Cyt *C*. However, the Cyt *C*-MIP provided a greater degree of improvement from the imprinting process, and the difference between release for MIP and NIP was more substantial for this template. In results seen by Bossi *et al*., there was not a significant imprinting effect achieved when Cyt *C* was used with a polymer bearing a net positive charge.[124] It may be that the chitosan used here provided a stronger degree of selective recognition towards Cyt *C*, or that the interactions were caused by, or supplemented with, non-covalent forces beyond electrostatic charges. While the observations made might not directly reflect the binding of a true competitive assay - wherein multiple analytes and the target are present simultaneously - it can be surmised that if any recognition effect results from the imprinting process, that the imprint sites should have a specific binding interaction preferential to the template, and

this higher affinity would eventually lead to a displacement of competing molecules, though a multi-step adsorption event or 'recognition of the fittest.'[164]

The higher uptake and improved delivery of template imparted through the imprinting process provides a strong advantage to drug delivery applications. Because release from a MIP does not rely solely on physical entrapment with a porous matrix, aqueous polymers such as chitosan could be used for higher drug loading models. The delayed temporal release as a template undergoes continuous re-binding/liberation mechanisms at imprint cavities also reaches towards the archetypal ideal of zero-order long-term delivery for many treatments. The release kinetics can be tailored to the application, by altering properties such as monomer composition and concentration.[153, 154, 155]

By polymerising the chitosan within an emulsion to form microspheres, in the fashion described herein, the scheme bestows an ability to surmount many of the difficulties experienced with large molecule imprinting in bulk. Binding is considerably increased in this case, as are release events, since access to imprint locales is not dependent on typical diffusion through the polymer matrix (avoiding steric impediments). Imprint patterning on a surface also proffers the potential recognition of multiple domains of much larger macromolecules, or possibly even cellular domains/membrane epitopes. These multi-imprint sites will result in orders of improved selectivity and specificity of a MIP. This partial or epitope-imprinting has been applied to recognise various peptide sequences,[210, 397] and can be used to functionalise support materials to increase cellular adhesion and growth.[114] Using such microgels also provides a means of attaining very high binding locales (through surface area and amount) for a comparatively low volume of MIP. This is a vital factor when having to create imprinted polymers using pharmaceuticals or biomolecules that are not easily obtained or costly. One can maintain a strong recognition effectiveness with less starting material, and achieve an improved degree efficiency by allowing for high binding in capture of the target, compared with typical bulk MIP approaches.

Provided these results could lead to the development of a robust, specific system capable of inveterate detection, the indiscriminate nature of the polymerisation procedure employed allows for the incorporation of multiple target molecules, such that a resultant MIP formed in this fashion would possess polyactive binding motifs. This would enable the creation of a matrix capable of recognising several proteins or drugs simultaneously.[150] Meanwhile, current research into protein imprinting continues to advance. New tools are now available for the modelling of these systems on a molecular level, to further study the complicated interactions involved, and design a specialised MIP matrix specific for each application.[314, 398, 399]

It is worthwhile noting that several limitations of the current system remain to be fully elucidated. For one, the mechanism of intermolecular interaction between template and chitosan remains unknown. While hydrogen bonding in the system is necessarily limited given the polarity of the aqueous media, it is equally unlikely that there exist any strong ionic force interactions between the presented molecules. Hence, the association between template and MIP is a probable combination of weak interactive forces (H, ionic, van der Waals). This is further obfuscated by the lack of tangible insight into how the imprint sites within the crosslinked polymer are maintained or lost as the matrix swells and contracts.

Based upon the fundamentals of molecular imprinting theory, one can hypothesise how the optimum rebinding of a template would occur under the same conditions as during polymerisation. This includes, but is not limited to, effects of pH, temperature, ionic strength, water content, and matrix alignment. As many of these conditions are easily altered, may also be interconnected with one another as changes occur, and are made further indistinct by the fact that conditions shift during polymerisation, one can begin to grasp the difficulty in reaching the ideal equilibrium. It has already been shown that the physical conditions during imprinting have been shown to affect the template recognition, in that the best results are seen at or near the temperature[205] and pH[214] of the polymerisation. However, the existence of such a drawback can be also made into an advantage. This reliance on achieving highly

specific imprint conditions for recognition can be manipulated to achieve tailored release of the template molecule at desired pH ranges[160] or control release profiles.[27] In such a way, once can exploit an apparent 'weakness' in developing a stable MIP matrix to create a tuned/'smart' recognition material.

Related to this concern over polymerisation conditions, one must exercise due diligence in examining the applicability of the MIPs produced by these methods to accurately reflect binding of the target proteins' native structures. As a biosensor, template-loaded matrix, or biospecific scaffold, any imprinted device should necessarily be sensitive towards recognition of a protein in its natural environment. The target molecule must be in its native state during interaction with the polymer both during polymerisation when the imprint sites are formed, as well as when the recognition with the MIP matrix is meant to occur. Maintaining such delicate conditions throughout the process is not a trivial concern. While a similar study showed little to no effect of using high shear rates for homogenisation on protein denaturation, this same report reflected the fact that both intense ultraviolet light and certain surfactants can have this effect.[103] The difficulties associated with successful imprinting of proteins are nonetheless offset by the considerable potential protein MIPs encompass.[102] If scientists are to achieve successful protein recognition with artificial matrices, means of production must be balanced with appropriate considerations made towards target and binding site state.

In a three dimensional system, as even spatially-controlled polymerised MIPs conceptually amount to, it is prudent to also recognise that, not only do the environmental conditions affect the polymerisation, but there is also a contribution from any crosslinker that may be used, the template itself, and the solvent – which forms a large constituent of the total volume; these effects are not easy to deconvolute. This becomes especially important in comparisons done between any MIP and a NIP. To be an accurate control, the non-imprinted matrix should be more or less identical to the 'normal' MIP, with the exception of imprint sites being present. However, as has been shown by Oral and Peppas,[400] there is some role played by the template itself during polymerisation, and this will unavoidably change the resultant network. This has been

reflected here by the changes in electrical properties of the two polymers seen in DS measurements of the 'blanks.' Clearly, adding additional components into a mixture will have multitude effects on the resultant polymer, that are not simply limited to the interactive site. One cannot merely regard the contributions of an extra molecule during polymerisation as being restricted to formation of an imprint site. It is possible the presence of the template affects the overall chemical reactions (e.g. speed, path, etc.) that take place. The properties and alignment at distal locales of the polymer can also be changed during polymerisation. Thus, one must be cautious in comparing too closely the two materials. Nonetheless, a NIP produced in the same manner as the MIP (or as close to it) comprises the best achievable control to estimate as a reference in most situations.

Whereas this study reports on the selectivity of MIPs as measured by the capture and release of target molecules, it is noteworthy that many authors use an expansive variety of forms to assess this value. Oftentimes, imprinting success can be reported as binding capacity or imprinting factor (selectivity). Indeed, the 'affinity' measurement of imprinted polymers is an important property that should be fully elucidated, as MIP capacity really reflects an average from a distribution of high-to-low affinity sites. It is strongly suggested that the more heterogeneous a MIP, the greater the potential for high-affinity sites (though the increase in low-affinity sites has a net result).[215] Furthermore, when considering a site-specific affinity measurement, this property becomes further convoluted by the localised swelling of the matrix. A collapsed gel would lead to multi-point adsorption, for example, whereas a swollen gel might only undergo single point adsorption.[147] Due to the desired homogeneity of this system, the estimate of overall capture and release is taken to best reflect the ultimate aims of the polymer recognition desired.

It is also important to discuss that, while the current study reports release from chitosan microspheres for a period of up to six hours, the nature of the measurement setup is limiting in that it is extremely difficult to control the uniformity of release, as the particles do not remain in a homogeneous suspension, but, rather, tend to settle

relatively quickly in water due to the density differential between gel and solution. This of course greatly alters the diffusion rate of template into the media. It also creates asymmetric non-uniformity across the measurement cell. Possible solutions to overcome this, for example creating a fluidised bed of the gelled spheres to maintain equal distribution throughout the chamber, would also potentially impart a variety of undesired electrical properties (e.g. addition of non-conducting gaseous phase) that would significantly impede accurate measurement.

Lastly, although the results would suggest that the differences seen are on account of an 'imprinting effect,' one must also consider the case that, though approximate sizes of the template and competitors used were similar, the differences in chemical structure could contribute to generalised interactions (or lack thereof) with the MIP matrix and/or solvent, that could be contributing to the observed recognition effect. One cannot fully discount that the molecular behaviour plays any role, as it is difficult to apply an analogue target alike to the template molecule in all but one aspect, to fully isolate each effect. This is especially true of biomolecules like proteins; it is much more difficult to obtain (or examine) simple stereocentres and isomer groups, such as the organic analytes commonly used in molecular imprinting studies. We must therefore acknowledge the existence of a limit in the observation of binding with competitive non-template molecules. These facts notwithstanding, this design for chitosan MIPs opens a pathway for development of non-traditional imprinted polymers, that can have a vast array of applications towards biomaterial and biomedical scientists.

8.6 Summary

A new form of imprinted chitosan microspheres was created via free-radical gelation using a water-in-oil emulsion method. In addition to a novel polymerisation scheme, this represents an original imprinting concept, in that it is one of the few reported models of aqueous MIPs using chitosan alone for the constituent polymer. At the same time it demonstrated imprint recognition properties for biomolecular targets. Through dielectric spectroscopy, it has been demonstrated that changes in the matrix properties

were attained during the imprinting process, as a result of the template's presence during the polymerisation stage. These changes resulted in specific interactions with the target, as evidenced by the change in uptake and release behaviour, that did not occur with a control polymer. Further, chitosan MIPs realised a preferential recognition towards the template molecule over a competing analogue of similar size, and this effect could be monitored in real-time using an impedance analyser. The developed model represents a novel system with great potential towards future biomacromolecule imprinting.

9 General Discussion

The individual results from the varying imprinting studies conducted as part of this thesis work should be regarded as a whole to envision the greater potential of the modified chitosan as a matrix for molecular imprinting. While each study on its own demonstrated some improvement in template binding which is related to a result of imprinting recognition, of great import is that in the studies shown overall the data supports the original hypothesis that the modified chitosan polymer might be a strong candidate for targeted MIP construction. The cumulative message from each investigation was a positive one for achieving recognition by this approach. There is compelling argument to warrant the study of this chitosan as the basis for molecular imprinting.

The results from characterisation studies done on the chitosan polymer for this research confirmed earlier data regarding manipulation of its chemistry. By adjusting factors such as the molecular weight and degree of substitution, through the methods presented in this thesis, it is possible to tailor the properties to desired values. Adding in the ability to adjust the nature and degree of crosslinks by the use of additional crosslinkers and choice of polymerisation method, we establish that hydrogels of a wide variety of forms and consistencies can be created from the starting material. In this way it is possible to customise the chitosan polymer to fit any desired biomaterial applications; for example to meet particular requirements of mechanical strength, swelling ratio, porosity, equilibrium water content, etc. It also importantly allows for the many physical forms (films, beads, fibres, nanogels, etc.) of the polymerised structure to be made. Depending on the target use, this versatility enables the same base material to be slightly modified as needed to meet a range of aims. Particularly for applications that would involve molecular imprinting, wherein multiple targets of varying functionalities and sizes might be used, this flexible tuning ability is extremely beneficial. As a polymer for molecular imprinting, the modified chitosan has been proven to be finely tunable in all of chemical structure, size, physical attributes, and form, thus yielding a robust basis for a MIP.

One of the properties that is expected to play a principle role in the way the template and polymer matrix interact is the molecular weight of the polymer. From the results of investigating the effect of polymerisation conditions on template recognition, it was shown that there was a significant improvement to the selective binding of a small model molecule when the MW of the chitosan was lower. This is explained by the chitosan used in these experiments as having a fairly long molecular chain; compared against standard MIP monomer molecules it is substantially larger. When the MW is reduced it results in a network of shorter chains, that is more free to rearrange itself, and this organisation, when crosslinked, brings about a denser interconnected network. As a result, the close polymer chains (and their freer movement in the pre-polymerisation solution) allow for closer bonding and a higher degree of 'surrounding' of the template molecules, and hence an augmented degree of interaction. This then produces a MIP with enhanced recognition towards the target.

It is important to note that, as this condition was evaluated here at only three levels, and was particular to the template applied in this study, the specific MWs used are not as important as the trend elucidated by resultant recognition behaviour. The observations made herein indicate a pattern of interaction towards the template with respect to the polymer length. When considering the molecular imprinting of other molecules, especially those of larger size, there would eventually be an impediment to having a very tight polymer network, as steric hindrances are increased. This would ultimately limit the binding capabilities for any high MW targets. At what point where sacrificing a level of interaction to increase diffusivity is acceptable depends on achieving a fine balance between the two, and will be predicated by the specific polymer system and template pair, as well as the targeted application.

Applying similar reasoning, this cursory study of imprinting effects with bulk chitosan gels pointed towards little contribution of the crosslinkers used. However, the influence of the crosslinker in forming the MIP network is not to be understated. In these individual cases they did not follow a significant trend in demonstrating a specific impact on the template recognition for the levels used; however, this can be attributed

to sufficient binding taking place between the crosslinking of side chain groups from the functionalised chitosan. The chitosan polymer in its chemistry and length is a fairly flexible molecule, and as the structure of the side groups at the locales of functionalisation also possess an additional branch length, relatively, it may not be necessary to have additional crosslinkers to form a tight network of interaction about the template. The direct crosslinking of the methacrylate groups from one chitosan chain to the paired moiety on another may be sufficient to form the branched network; in fact, extending this linkage through the inclusion of an additional spacer molecule may be unfavourable if the template molecule is of small size, as the extra crosslink length opens up the space between the polymer chains and might act to reduce the degree of interaction. Thus, the use of, type, length, and amount of crosslinker present still more additional parameters for this imprinting system that can be optimised for further study based on the target molecule chosen.

The recognition capability of imprinted chitosan gels was further probed by examination with larger biomolecular templates. The MIP samples with fluorescently tagged models of albumin and heparin indicated an improvement (i.e. enhanced specific binding) towards the former, but little change to the latter. One cannot make conclusive statements with regards to the recognition based solely on these qualitative results from the fluorescent imaging, but they do suggest some patterns in the imprinting recognition. Heparin, being a relatively small molecule, did not demonstrate noticeably improved binding to its MIP than observed in the control polymer. This implies that the spacing of the molecular chains for this particular MW of chitosan and degree of substitution, as used for the microcontact stamped gels, was too far apart to provide a sufficient degree of bonding interactions with the template to allow for specific recognition of it in the imprint to occur. Albumin, on the other hand, has a MW of around 67 kDa, nearly five times larger than heparin (12-15 kDa). The larger size of the molecule means it would occupy more space within a surrounding polymer matrix. The higher binding observed in the HSA-MIP suggests that there is a degree of recognition towards the template molecule as a result of interactions with the chitosan polymer. Thus, we can conclude that, when the gelation parameters and polymer are kept

constant, there will be some minimum value for molecular size of the template, below which the chitosan chains are either too far apart, or the mobility is such that the template cannot be 'fixed' in a sufficient enough manner within the intermolecular forces to create successful MIP recognition. By these arguments, albumin lies in the window of recognitive interaction with the chitosan polymer, while heparin was below this threshold limit.

These presumptions were further supported by the results of competitive binding assays. While there was significant fluorescence resultant from captured heparin, it was found that it was nearly equal in MIP and NIP, and also of a similar level to the bound amount seen with the HSA-MIP. As the binding reaction attained is largely non-specific, we would expect to see the same results from the imprinted polymer and control, and likewise in the presence of another imprinted polymer. A slight increase in binding was observed to the albumin imprint, which is explainable by the general non-specific binding being supplemented by a degree of binding to the imprint sites created by the template during polymerisation. As the heparin molecule is small relative to albumin, it can easily penetrate into the pre-arranged sites of interaction (vacated by leached albumin). This would explain the minor increase in captured amount we see in the oppositely imprinted gel during the competitive recognition study.

To expand on the results from study of the polymerisation conditions on imprinting, we again considered the addition of crosslinkers to the chitosan matrix. Only gels imprinted with human serum albumin were examined for this end, due to the poor recognition seen with Hep-MIPs in earlier stages. We found a substantial change in the amount of re-bound fluorescently-labelled template with the use of both *N,N′*-methylenebisacrylamide and ethylene glycol dimethacrylate. It was observed that when MBAm was added to the polymerisation solution, the resultant gel captured more of the template molecule (brighter fluorescence) than the normal MIP. The intensity was even greater when an equal amount of EGDMA was used, indicating a very strong degree of captured target. These results showed a much stronger effect of using a crosslinker in

increasing the overall reaction between the MIP matrix and the template molecule than previously indicated.

While these results are qualitative, they provide important insight into the chitosan MIP system that supports the previous arguments. Firstly, we can make the general inference that, as expected, increasing the crosslink density of the MIP (by the use of the additional crosslinking agents) has the effect of improving overall recognition of the template. We can also surmise that the increased molecular size of the templates in this case provided a greater degree of interaction with the polymer in both quantity (more areas in contact with the MIP) and degree (higher functionality). Unlike in the previous examination of the crosslinker effects, in which no significant improvement was noted, here the proteins are much larger molecules and would separate the polymer chains and cause greater intermolecular interaction more so than the smaller model templates. They also possess multiple areas of functionality across their structure of varying chemical groups that allow for interaction with the surrounding polymer. Thus, the additional inclusion of the smaller crosslinker units acts to provide a more complete and surrounding environment for the template molecule in the 'gaps' between the chitosan. This improves the specific recognition of templates in the MIPs for these gels, and yields a much clearer effect of crosslinker.

Although the target binding was heightened even more for EGDMA-crosslinked gels than their MBAm counterparts, generally the gels made with the addition of acrylamide were more consistent (i.e. stable and homogeneous), owing to the higher degree of water solubility of this crosslinker. The addition of EGDMA also renders the gel more hydrophobic, leading to lower swelling ratios and more restricted access to imprint sites. This may account for the stronger recognition seen with small molecules, as the network is maintained to a greater extent in its 'imprinted' state, whereas this factor acts as an impediment to the diffusion and imprinting of larger proteins. These results are supported by a report from Korytkowska-Wałach, who showed that the increased crosslinking of hydrogels imparted by MBAm improved selectivity and effectiveness of hydrogel recognition, and was better than comparable MIPs made used

EGDMA.[401] Overall, this evidence supports the strong potential for the modified chitosan to be used together with acrylamide monomers/crosslinkers in a bi-polymer MIP. Such a construction would offer the combined benefits of both materials; for example the strong network and biomaterial properties of chitosan, with the consistent polymerisation and tight recognition ability of acrylamides.

When the samples were examined qualitatively, a direct measure of the template recognition could be determined and a distinct comparison could be drawn between them. For this, we used the imprint factor as a measurement of the improved binding for the MIP to its control sample. This evaluation confirmed the previously made qualitative results. Just as in the prior study, the chitosan samples made with EGDMA as crosslinker showed an increase in the amount of captured template over the gels made using MBAm. This held true for both heparin and albumin templates, although a greater difference was evident for the former. This could be explained by a stronger degree of interactive contribution made by the electronegative groups in EGDMA leading to higher binding towards the template. Importantly, however, the imprint factors were highest for the chitosan polymerisation system without any additional crosslinker, bearing out our choice of initial system and validating the hypothesis for chitosan as a MIP matrix for protein targets. Ultimately the data confirms successful molecular imprinting of macromolecules was accomplished, proves the recognition of the MIPs was still effective in an aqueous media, and thus substantiates the ultimate hypotheses of this research.

Once a recognitive capacity was demonstrated with the chitosan MIP, a practical study of binding and release behaviour was desired. Using the dielectric flow cell provided an excellent opportunity for this, as it allowed not only quantitative study of the imprinting effect and binding kinetics, it additionally provided the ability to observe responses in real-time to system changes. It also presented a method of validating dielectric spectroscopy as a means to corroborate molecular imprinting effect.

Observations of the release profiles of template molecule from the imprinted microspheres showed a sustained release over the course of the investigation. This was in contrast to the NIPs, from which was observed a fairly typical and expected rapid release profile. We can attribute this difference to the specific capture of the template at the imprint sites inside the chitosan MIP. Whereas the templates experience no particular interactive forces with the normal polymer matrix, the adherence to and diffusion seen from them follows simple release kinetics of plain entrapment in the matrix. Some burst effect is expected, as the initial amounts of analyte adsorbed to the surface and close to it are immediately washed out of the matrix. When the polymer has selective recognition sites present, however, as imparted though the process of molecular imprinting, this causes the template to interact with the matrix along its normal path of Brownian motion inside the bulk polymer. Hence, its diffusion out of the MIP is hindered by repeated binding and release events as it passes individual imprint sites. On top of this retardation is a naturally slower release of the molecule from the polymer resultant from the interactive forces which cause the template to be immobilised at the imprint site. This produces the slower, steady delivery of target from the polymer. While these results are important in that they allow us to precisely characterise the imprinting effect in the chitosan polymer, they are equally noteworthy because they demonstrate that the principle of molecular imprinting can be applied with these hydrogels to develop sustained release profiles. Through a very simple modification during production, one can greatly improve the amount of captured target and slow its release; a tremendous benefit in the development of drug delivery applications. This imprint effect could be further augmented by selective polymer parameters that could easily be incorporated with the versatility of this chitosan gelation system. Ultimately one would aim to achieve near constant zero-order release of the entrapped target molecule, through the use of molecular imprinting.

The gelled MIPs showed an improvement in both specificity and selectivity as a result of the imprinting process. The model templates used, heparin and cytochrome *C*, are fairly small molecules from a biological standpoint. As we discerned a notable improvement in the recognition using this polymer system with larger macromolecules

in the previous studies, using similar protein templates for these imprinted chitosan microspheres might further enrich the recognition and detection via impedance measurements.

Beyond showing the improved recognition of the matrix to the target and selectivity over competitor analogues, the results from the dielectric spectroscopy studies hold import for the establishment of the methodology and design itself as a means to sensitively detect the small changes of molecular imprinting in real-time. Despite its strong capabilities, DS is not a well-known analytical technique, and has been little explored in the examination of MIPs. The potential advantages to using DS with imprinting studies offer enhancement to the standard analytical techniques used to determine imprint effect. The sensitivity of the instrument is very high for one, and does not require further modifications or signal augmentation that may be necessary in other methods such as SPR. It is capable of measuring the binding of the original template *in situ*, and not a slightly modified binding analogue (e.g. fluorescent tag).

As discussed, a key property with the use of DS is the real-time measurements of binding and release possible, a considerable advancement over indirect assays that rely on determination in changes of solute concentrations. As such, DS allows for the resolution of time-dependent changes, such as calculation of release kinetics, and provides the ability for the user to monitor changes to the behaviour directly as changes are made to the environmental system. Furthermore, because the setup is incredibly versatile, relying only on a simple system of paired electrodes, a number of different design arrangements can be used to determine the imprinting effect based on need. The example presented herein, a flowcell microreactor, is useful as it (*i*) allows MIPs of varying size and shape to be examined inside, (*ii*) permits continuous fluid flow into and out of the chamber to add reagents/change solvent/maintain infinite sink conditions/etc., and (*iii*) makes possible continuous and repeatable measurements in a simple module system. In addition to these benefits, while the single measurement cell in itself is highly practical for MIP assessment, it is easily possible to expand the design to encompass multiple individual measurement cells. Having multiple independent or

connected cells allows simultaneous determination of replicate samples, measurement of responses of MIPs and NIPs for real-time comparisons, and immediate calibration control to selected environments. Thus the design developed for this study provides an excellent basis for future work in MIP examinations.

There remains strong debate in the scientific community over an appropriate method to describe the selectivity of MIP substrates.[96] In an idealised imprinted polymer, all recognition sites would be equal and homogeneously distributed throughout the material. These would be modelled by a simple Langmuir isotherm with a single binding constant. However, in practical theory we know this to be an highly improbable situation. As discussed in section 2.1.4, the binding sites in MIPs are often modeled as a series of high affinity sites and low affinity sites. Many investigators report their binding isotherms to a bi-Langmuir fit, which is assumed to model these two distributions, and this has classically been the accepted school of thought. Recently, however, other authors have suggested that a continuous distribution of binding site strengths is more probable, and have presented results supporting this theory.[54] The choice of isotherm model can produce significantly different affinity constants and binding capacities for MIPs, though an analysis regression may indicate a good fit for either.[402] In an extensive study of isotherms, Sajonz *et al*. found that a close fit to experimental data can be obtained through either a bi-Langmuir or Freundlich model, whereas a single isotherm did not provide good approximations, while still others closely resembled tri-Langmuir models.[403] This presents a very important question in the discussion and evaluation of MIPs: how to accurately model the results from binding studies, and how to compare the recognition from one reported system to another.

One of the core concepts inspiring the creation of MIPs was the early development of so-called 'artificial antibodies.' If we are to extend this analogy to the multiple types of bonding sites apparently present in imprinted polymers which account for the variation in binding isotherms, then the appropriate model analogue might be described as that of polyclonal antibodies. Thus, the calibration curve for interpreting

binding results would apply over a broader concentration and account for the existence of heterogeneity in binding strengths. It is therefore important to discuss whether the competitive assays used to judge MIPs through adsorption isotherms are possible independent of affinity site distributions. Pap *et al*. have argued that in homologous binding assays it is possible, provided the isotherm is not linear.[96] So, while applicable and still useful for comparison, these simplified studies may only represent a narrow definition of the imprinting effect.

This concern presents an important shift in the train of thought of MIP evaluation. Traditionally, the examination and reporting of MIPs can be typically classified into one of two categories. Researchers who approach them from a chromatography standpoint tend to view MIPs as solid sorbents; hence results are frequently reported in terms of adsorption isotherms, kinetics and morphology. On the other hand, those who look at MIP substrates as binding applications (for example as biomolecular analogues) view the results from a background of immunoanalysis, examining them as selective media or mixture of reagents possessing a binding affinity (constant) towards a particular analyte of interest.[96] The difference in the way these results are interpreted and expressed can depend on the principle the assay is coming from. Whether researchers choose to regard the developed MIP as a molecular sorbent or immunoaffinity extraction structure will affect the corresponding discussion and analysis.[404]

As we perform further investigations of MIPs, it becomes important to discern a relevant means of characterising the imprint recognition. While historically the literature has been focused on classifying the studies based from a choice in either of these two factions, a more appropriate perspective may be to re-examine past studies from a different viewpoint, considering the actual MIP performance to be a mixture of the two. It is therefore very useful that the developed method reported herein of investigating the binding and release behaviour in MIPs through impedance spectroscopy described in this work presents the ability to explore both aspects in a combined fashion. Because it allows for the observation of changes directly as they

occur, the adsorption/desorption behaviour of imprinted polymers can be evaluated in similar fashion to bulk chromatographic systems, while the sensitive measurements and flow properties allow one also to detect binding and exchange events of analyte. This allows better correlation with other models and provides an improved overall picture of the MIP activity.

Through the combination of studies with molecular imprinting using chitosan in bulk, thin layer, and a microsphere system, we were able to ascertain various key parameters in creating an aqueous-based chitosan MIP. Initial examination of polymerisation parameters suggested a strong relationship between the molecular weight of the polymer and template recognition. It also pointed to a limited contribution of the crosslinker in aiding the specific binding. We suggest that both of these factors related to creating a sufficiently tight physical network surrounding the template molecule. This was further confirmed by similar patterns in the recognition of larger molecular weight templates. Each of the studies suggested a strong dependence on the target size and molecular spacing of the polymer network to realising strong recognition. Whereas using imprinting offered an improvement in the amount of template captured by the MIP, through knowledge of the target molecule and depending on application, this allows the polymer to be adjusted to suit the desired degree. The base polymerisation model of chitosan presented by this work is versatile enough to encompass a wide range of potential targets and polymerisation methods. By simple controlled adjustment of the degree of substitution of the polymer and the molecular weight, one can adjust the molecular weight between crosslinks, to effectively expand or contract the molecular porosity. The matrix can be further refined by the additional use of crosslinking agents to achieve recognition selective to the templates chosen. The polymer itself possesses several key points of functionality for attempting additional chemical modifications to impart specific groups for targeted recognition of a desired template. These factors make the chitosan hydrogel a very useful model for aqueous molecular imprinting. The ability to apply a similar formula and methodology across a field of target applications is a central principle in molecular imprinting research and offers a focal benefit in the development of marketable products.

10 Contribution to the Knowledge

The work contained in this thesis contains several original contributions to the field. These are focused on the achievement of successful molecular imprinting of macromolecules in an aqueous-based chitosan system. The notable accomplishments of this research include:

1. Achieving molecular recognition of targets in aqueous media

While research into molecular imprinting spans a great share of the literature, it remains limited in terms of investigations involving molecular imprinting in aqueous media. There are few reports of forming effective MIPs in polar solvents, and having the recognition of them evaluated in the same. The majority of investigations done with water-based chemistries thus far principally involve using acrylamide as the recognition matrix; and these studies have been largely confined to applications in chromatographic separation methods of small molecules. This study demonstrated molecular imprinting in a crosslinked hydrogel using a long chain polymer. The modification of a water-soluble chitosan to form a polymerisable matrix was accomplished, and this was then used to form MIPs that showed higher capture of target molecules over control polymers, using aqueous solution environments, in both 2D planar visualisation, and 3D release studies. The molecularly imprinted chitosan hydrogels showed higher binding of target molecules over non-imprinted controls, for each of the evaluated models, and presented preferential interactions to the template molecule versus an analogue. No additional treatment or special solvent conditions were required to achieve and demonstrate the recognition. This is an especially relevant point for the development of this technology towards biological/biomedical applications. Being able to reach such recognition of a complex template in water, particularly with a swellable matrix like that presented here, is a notable accomplishment. While each molecular imprinting system developed is unique, the additional gain in knowledge of any new system greatly benefits the research area as a whole, and assists in the future development of other MIP systems.

2. Using chitosan as a molecularly imprinted polymer

Having a modified chitosan be the main recognition element in a MIP represents a strong realisation of the research goals. Only one other report involving chitosan as the principle polymer for imprint recognition has been made at this time, and in this case only for the capture of a small template molecule. It was also limited in its scope and targeted application. The results herein demonstrated an imprinting effect towards other, larger molecular weight targets, using constructs of the modified chitosan polymer in several forms. This outcome was particularly affirmative given the high degree of difficulty in using a weaker polymer network like a hydrogel to achieve MIP recognition, and attaining this same with an highly flexible and water-swellable polymer, as is glycol chitosan. The imprinting in bulk gels showed an improved capability in specific recognition for certain formulations of the polymer, and at the same time emphasised the importance of selecting and controlling the polymerisation conditions for optimum imprint effect. The roles of crosslinkers and polymer molecular weight in particular were shown to be critical factors in MIP design. These results were then supported by discerning the enhanced capture of labelled template molecules to imprinted polymers of chitosan, as visualised through 2D fluorescence of labelled targets. Finally, the study of template release from chitosan hydrogels in 3D confirmed higher amounts of target were released from an imprinted chitosan gel over non-imprinted formulations. Chitosan MIPs were shown to be possess selective and specific recognition of the template molecule versus analogous competitors. Altogether, each of these results confirms the ability of chitosan to form the basis of a MIP, which strongly adds to the argument to include consideration for it as a potential avenue when constructing MIPs, in addition to, or as a replacement for, other common imprinting polymers which have less functionality and versatility, and do not hold the same promise for biological applications.

3. The demonstration of a method to improve the functionality of chitosan as a biomaterial

The successful application of chitosan as a molecular imprinting matrix offers a new method to increase its use in the biomedical field. There is no question that chitosan, as

a molecule, has been extensively studied at length in scientific fields; and yet, despite this, there remains a gap of its reported use involving molecular imprinting. While already widely accepted as a useful and versatile biomaterial, the improvement in the recognition of a chitosan MIP over the controls seen in the results of this research signifies an important potential as a specialised matrix material. In each of the studies examined with chitosan MIPs, in bulk slabs, thin patterned surfaces, or microgels, the effect of imprinting showed equal or higher level of template captured than the NIPs. This verified the successful incorporation of an additional recognition capacity to chitosan. Creating this improved binding ability and selective recognition – through the process of molecular imprinting during polymerisation – lends itself to application in a variety of specialised areas. While chitosan itself is studied and applied at great length in biomedical applications, and it is for this reason and its beneficial properties it was selected as a starting point of this research, it is not conventionally utilised in intelligent or selective methodologies. Improving the biomaterial to have this characteristic greatly increases its function. In addition to the standard uses in resolving chemicals and separation needs, being able to improve chitosan with a recognition ability can enhance it as a tissue engineering support material, complement its use in specialised delivery vehicles for drugs and biomolecules, augment it as a binding matrix, and impart specific target capabilities for sensory applications. Whereas the polymer itself has an already proven strength in biomaterial application, due to is physical nature and biocompatibility, adding an additional feature of imparting specific and selective binding capability – as established by the results in each of the assay methods reported in this thesis – further bolsters its attractive properties.

4. Molecular imprinting of biomacromolecule templates

Whereas the use of larger molecular templates, having increased size and functionalities that can create impediments to binding in traditional molecular imprinting, is often a difficulty to achieve the same specific recognition aimed for by MIPs, these targets are not often successfully reported in the literature. Applying a variety of relevant model molecules to the chitosan imprinting system demonstrated that this matrix was capable of increased specific capture of targets as a result of the molecular imprinting

procedure, for even large macromolecules. The produced chitosan gels showing stronger recognition of heparin and cytochrome *C* (MWs of approximately 12 kDa), when imprinted microspheres were evaluated by dielectric spectroscopy, as well as of the larger protein albumin at a MW 66.5 kDa, verified by the imprinting effect calculated through the fluorescence changes in a 2D capture system. The successful imprinting of larger targets such as these, particularly those which represent models that might have biological significance, is a sound step forward in molecular imprinting research. This has repercussions for the recognition, capture, and delivery of highly complex molecules, imparted in a synthesised material, ultimately approaching the status of artificial antibodies.

5. Creation of chitosan microspheres and nanoparticles under neutral pH and using different gelation methods

Following successful modification of the chitosan polymer, two methods were used for the formation of varying hydrogel particles. The creation of chitosan microspheres via emulsification was achieved at a neutral pH, and gelation under these conditions formed a novel material. Control of the conditions during polymerisation (e.g. stirring speed, concentration, use of emulsifier) can produce particles of varying size depending on the need, as was verified through resultant particle imaging. Crosslinking of modified chitosan in a reverse emulsion was accomplished through photoinitiation by UV light as well as chemical initiation. Two different procedures were used which produced either micro-sized beads of chitosan gel, or nanoparticles. Examination by SEM confirmed the stability, shape, and size of the different spheres.

This gelation system offers a particular advantage in the molecular imprinting of large molecules (or even cells) as they can be localised at the surface of the microspheres. Which, in addition to allowances for significantly increased template size over other MIP forms, permits particular areas of interest on a template to be imprinted in a isolated or oriented fashion, removes the issue of impeded diffusion though the bulk polymer matrix, increases the degree of overall binding site accessibility, and finally, also allows for organic-soluble targets to be used. The high surface-to-volume

ratio of these microspheres and increased access to specific recognition sites was validated in the increased binding of macromolecular templates to chitosan MIPs over control non-imprinted polymers. Dielectric measurements showed a clear, more sustained and higher release profile of the corresponding templates, as well as confirming the selectivity of the imprint sites created.

An improvement in uniformity and size control was obtained through an encapsulation technique to create additional nano-sized chitosan spheres. Using a sacrificial liposomal shell to form photopolymerised nanoparticles of chitosan in this fashion has not yet been reported. This procedure had the ability to easily produce numerous and highly uniform chitosan beads, verified through image analysis and dynamic particle sizing, which could be then applied in any number of differing systems. As a result of their fine size, these nanoparticles have expanded functionality wherein they can be taken in by cells. When integrated with a molecular imprinting design, the chitosan nanogels offer a chance for intelligent drug delivery, molecular screening, or potential vector carriers.

6. The use of dielectric spectroscopy as a new technique for sensitive, real-time analysis of molecular imprinting

Incorporating a flow-through measurement cell and applying dielectric spectroscopy presented a novel method of using electrical impedance as an alternative method for detection of the typical binding and release studies used with MIPs. The augmentation in recognition imparted through the processes of molecular imprinting are at levels often very difficult to detect, and conventional means often prove insufficient to measure the changes between MIP and controls, particularly of small sample sizes and low sensitivity. DS offered a substantial improvement in the detection ability and versatility over the assays and equipment used conventionally for MIP assessment.

When examining the release of captured templates in MIP microspheres, the dielectric spectroscopy results were able to demonstrate, and observe in real time, the imprinting effect in chitosan hydrogels that was not easily assayed by other means (e.g.

RIfS, SPR, QCM). We were able to detect the patterns of varying release behaviour of heparin and cytochrome *C* from chitosan that showed a distinct heightened capture of the templates over the control polymers. It also showed selectivity towards the template molecule was present at the imprint sites, as the measured changes for a target did not show the same interaction with an imprinted sample of another biomolecular template. This was done using the small parallel plate chamber with only a small sample size of microspheres, and relatively low loading of test molecules, This improved sensitivity in detection allowed a clear distinction of the effect of molecularly imprinting biomolecules into the chitosan network, demonstrated through enhanced capture and release of the model protein templates. It also provides allowances towards time-dependent studies for binding assays and kinetics determination, as well as traditional sorbent characterisations.

Using the impedance changes in a system provided a viable means of data collection, for detecting even small changes in a polymer system. This is an important factor in supporting the ability to discretely distinguish the often small improvements in recognition capacity imparted by molecular imprinting. The developed approach further opens up more possibilities in terms of reactor design and optimisation. Additional measurement cells and electrode set-ups (e.g. multi-chamber measurement cell, interdigitated electrode surface) were designed that could be integrated with the dielectric spectroscopy detection system for versatile analyses of differing target substrate types (e.g. slabs, surfaces, particles, nanospheres, filaments, etc.), immediate in-line detection as a sensor component, and simultaneous multi-sample comparisons.

11 Conclusions and Perspectives

11.1 Summary of Achievements and Realisation of Objectives

This thesis represents a report of several key accomplishments in the field of molecular imprinting. Principally of these, we have demonstrated a base chemical system to achieve molecular imprinting with a chitosan polymer under aqueous conditions. Whereas there has been a dearth of reports concerning forming and applying MIP recognition in aqueous solvents, we set out to demonstrate this effect in a previously unpublished fashion. Beginning from the identification of chitosan as a strong candidate for molecular imprinting, and choosing a water-based chemistry to develop the recognition, we proceeded to build a polymerisable and customisable system that would allow fine tuning of the polymer matrix to particular applications, have demonstrated recognition ability towards biologically-relevant targets, and be versatile enough that a single starting material could be developed across a wide range of molecular imprinting platforms and detection systems.

Beginning with a water soluble chitosan derivative, we showed the ability to adjust the molecular weight of the polymer, as well as the degree of substitution using a functional radical-reactive moiety, such that the chain length and crosslink density of a resultant network made from the material could be controlled and used to develop desired properties of the polymerised matrix. This offers an important advantage in the development of a MIP formulation that can be used to recognise a range of targets. It also enables choice of physical characteristics through selection of modification parameters.

Studying the bulk gelation parameters and their influence on ultimate recognition of model templates demonstrated a distinct imprinting effect for particular formulations used. When the model was expanded upon with additional investigation parameters and an increase in the levels tested, we elucidated distinct trends in how the gelation conditions affected the release of a model template from the polymerised

chitosan. We also observed clear improvements in binding and capture of the target in MIPs versus controls made under the same conditions. Statistical evaluation of these results confirmed their relevance, and thus that we could derive several useful conclusions from the investigation. Firstly, as we had anticipated in setting up the adjustment of MW for the chitosan, we observed that stronger recognition of the template was achieved for polymers made using lower starting MWs. We also found a slight contribution to enhanced imprinting effect when using the DEGDMA crosslinker, and this was more pronounced for lower molecular weight chitosan samples. Incorporating EGDGE into the pre-polymerisation solution had little or negative effect on the bound doxorubicin, and was not seen to improve the molecular imprinting efficacy. Lastly, we observed no substantial effect in stronger target recognition of MIPs with increasing the amount of template used in the pre-polymerisation solution. In each of these results however, the key effect of note is that there was demonstration of recognition in the polymer that arose as a consequence of the molecular imprinting procedure. Ultimately, the aim of continuing to evaluate the experimental B-B design would be to develop a complete equation of the parameter set and full coefficients to be able to quantitatively define the system, however the importance of seeing the effect of the different model parameters is incredibly useful in optimising the systems and offering improvements in future studies.

We have presented a method for applying molecularly imprinted chitosan in a thin, two-dimensional surface, which could be shaped to nearly any desired pattern through the use of PDMS moulds. While this was used primarily as a simple and rapid assay for verifying MIP recognition, such techniques have abundant application in creating functionalised surfaces, microfluidic binding sites, localised arrays of different selectivity, and specialised sensing materials. The thin patterned layers of imprinted chitosan were shown to preferentially bind the target proteins. The fluorescence visualisation confirmed that the MIPs possessed both specificity, in higher binding of the target over a control surface, as well as selectivity, binding more template analogue than a competitor molecule when exposed in equal part simultaneous solution. We were able to use intensities gathered from the confocal imaging to calculate the imprint factor

for gel surfaces, which verified that there was a higher recaptured amount of protein on the MIP than control NIP. This assay also further confirmed the previous result that the incorporation of additional crosslinkers into the polymer formula does not necessarily improve the recognition achieved. The 2D binding system confirmed imprinting effectiveness of the chitosan gel and provided another means by which such a MIP could be applied in surface functionalisation or capture and detection systems.

In this research we introduced the use of dielectric spectroscopy as a new technique for sensitive, real-time analysis of molecular imprinting. We designed a small platinum electrode measurement cell capable of detecting minute concentration changes in bulk solution, which allowed us to study molecular changes in real-time using just a minor fraction of test sample. Incorporating dielectric spectroscopy presented a method of using the impedance analyser in a non-traditional fashion, while also yielding it as an alternative method having high detection sensitivity and versatility contrasted against the more conventional binding and release studies used with MIPs. We reported on the imprinting results with chitosan microspheres for their high surface-to-volume ratio and ability to be easily manipulated and suspended as required. From studying the release behaviour of two model proteins from chitosan microspheres using dielectric spectroscopy, our results were once more able to demonstrate and quantify a pattern of change in the recognition effect of the chitosan hydrogels that was relatable to the molecular imprinting procedure. Test samples of MIP microspheres displayed more gradual, sustained release of their templates, cytochrome *C* and heparin, respectively, than did the same polymers made without any molecular imprinting. Both formulations showed a strong degree of specificity over control polymers, as evidenced through the changes in relative capacitance of the test samples from having higher amounts of captured template. Further, when investigated against the opposing biomolecule, release performance from the imprinted microgels paralleled that of the NIP, signifying the presence of only a small degree of non-specific binding which had occurred to the oppositely-imprinted polymer, allowing us to conclude, accordingly, that a measure of selectivity also existed in the imprinted chitosan for these test templates of similar size. Whereas the DS technique allowed for sensitive measurements done in real-time, it can

be considered an advantageous design for conducting molecular imprinting comparisons. Producing time-dependent studies of template recognition is a constructive benefit for the investigation of binding and release studies and determination kinetic constants for the specific recognition behaviour. For future studies, the results of this report advocate that using observation of the impedance changes in a system provides a viable, and informative, means of data collection, for detecting even small changes in an imprinted polymer system.

Several main objectives were put forth in the proposal of this project. We hypothesised that the biopolymer chitosan would be an interesting choice for forming the base of a MIP, and offer a few key advantages over other imprinting materials. We therefore sought to demonstrate its efficacy in a molecularly imprinted network using model templates. We accomplished this by first verifying an improved capture effect of the imprinted polymer in bulk towards a selected target. We then proceeded to evaluate the molecular imprinting in a thin layer of chitosan hydrogel. Using two model proteins as macromolecular targets, we confirmed specificity and selectivity of the imprinted chitosan matrix. Following this, a third system using chitosan microspheres polymerised in a reverse emulsion was explored wherein the real time release of two biomolecules was tested via an electrical impedance flow cell. Once again, it was found that the gelled chitosan made in the presence of a template molecule, showed both a higher, and preferential, recognition of said template than did a control polymer or binding towards another molecule. These three methods established that there is strong indication for the molecular imprintability of chitosan.

As a demonstration of effective use of chitosan in a molecularly imprinted polymer, the report represents a substantial achievement in the field. To the author's knowledge, only one other report involving chitosan as the principle polymer for imprint recognition has been made at this time, and this only for a small glycoside (naringin). The results of this thesis research demonstrate an imprinting effect using constructs of the modified chitosan in several polymeric forms. Even attaining just a slight improvement in the recognition of an MIP over the controls, this signifies a new

and important step in the field, showing chitosan's potential as an MIP matrix. We demonstrated that from an identical starting material, a chitosan polymer could easily be modified and adapted into several different MIP systems, and therein established its potential as a molecular imprinting foundation polymer. The evidence gathered opens up numerous prospectives for its application and warrants further study of chitosan imprinting in many forms.

The three methods used in the course of this research for the formation of chitosan hydrogel particles were innovative developments not previously studied with this material. One approach, the creation of chitosan microspheres via emulsion was unique in that, although many chitosan microspheres have been made prior to this report, this system of using a chitosan soluble at neutral pH, and applying photogelation under these conditions forms a novel material. As mentioned in the discussion, this system offers a particular advantage in the molecular imprinting of large molecules (or even cells) as they can be localised at the surface of the microspheres, which, in addition to increased template size, permits particular areas of interest to be imprinted, removes the issue of impeded diffusion though the MIP matrix, increases overall binding site accessibility, allows for organic-soluble targets to be used, and can produce particles of varying size depending on the need, through adjustment of the emulsification conditions (e.g. speed, emulsifier). Similarly, using chemical crosslinking of the modified chitosan with high speed homogenisation produced uniform polymer nanoparticles. This form of reverse emulsion provided greater control over the reaction conditions, and yielded improvements in sphere generation, however was limited in the overall yield of MIP polymer able to be collected efficiently in a straightforward fashion.

As an additional means to create more homogeneous and smaller (nano-sized) chitosan spheres, a liposome technique was adapted for use in conjunction with the photocrosslinkable solution. This offered an improvement to the microspheres obtained by emulsion, in more uniform creation and control of size. Using a sacrificial liposomal shell to form polymerised nanoparticles is an uncommon procedure, and has not been

reported in this fashion with photopolymerisable chitosan. While previously applied in some few studies, when nanogels are formed in this fashion it is usually in a vehicle where the liposomal coating is intentionally used for a role in the application, whereas herein it is made use of solely as a templating mechanism/microreactor to create the chitosan particles. The generation of nanogels by this method is largely unexplored, and suggests a strong potential for further study, and latent ability to easily produce numerous and highly uniform chitosan beads. Their size offers advantage in surface imprinting density and access while also facilitating application in a number of specialty systems. As MIPs, such nanospheres carry potential for distinctive biomedical and biosensor devices.

We aimed to establish the promise of chitosan as a molecularly imprinted matrix, and demonstrate how this potential could be exploited in particular towards biomaterial applications. A major contribution this effect involved the study of molecular imprinting in an aqueous environment, which would closely resemble biological conditions, and achieving the selective and specific recognition that is the objective of MIPs with macromolecular targets. Both of these factors were accomplished in the demonstration of successful template recognition in the two dimensional surface and 3D microsphere studies. In both of these very different systems, we showed a higher level of a target model protein molecule was bound to the imprinted chitosan gels versus control polymers, showing a distinct marked improvement measured in aqueous solutions, and this particular preferential binding of the template did not apply likewise to a non-template protein. Because of the adaptable nature of the starting polymer, we were able to use multiple methods to demonstrate how such an imprinted chitosan could be incorporated as part of a biosensor. We have shown that the recognition feature of the MIP is retained in a thin layer as might be used on a planar sensor surface, and in microscale particles that could be easily loaded into detection devices. We have also established that the formulations can be equally effective for targeting rapid, visual observations of target presence, or highly sensitive and real-time detection of slight environmental changes from the MIP.

In each of investigations reported herein as part of this thesis research, it has been demonstrated that using a systematic modification of a water-soluble chitosan and carrying out polymerisation in a number of forms can be used to construct a specialised matrix with molecular imprinting characteristics. As suggested previously, the selective recognition imparted on such an hydrogel with this method can have numerous applications. While many other polymeric families might be available for uses in resolving chemicals and separation needs, the ability to use molecular imprinting with chitosan sets up a new theme for this proven biomaterial. By adding a degree of selective binding to it, we increase its already capable nature in the investigations in which it is regularly being studied as a biomaterial, as well as potentially adding others. Being able to improve chitosan with a recognition ability can enhance its use as a tissue engineering support, complement its use in delivery vehicles for drugs and biomolecules, augment it as a binding matrix, and impart specific capabilities for sensory applications, among many other usages. It is a compellingly novel improvement of an advantageous biomaterial.

As molecular imprinting research continues, scientists still wish to achieve the highly specific and selective binding capabilities of antibodies, using synthetically designed chemical structures that will recognise complex biomolecules in native environments. The results from this article offer a step forward in the pursuit of attaining this goal. As a starting material, chitosan, already known to be a worthy biomaterial, has now been shown to be a capable molecular imprinting matrix. Finally, this study involving imprinting in a crosslinked hydrogel illustrates that there lies the potential for extrapolating imprinting studies with another water soluble polymer, and indeed, many other matrices could be investigated for similar MIPs.

11.2 Future Outlook and Potential Improvement

11.2.1 Optimisation of Imprinting System

While some new concepts have been proposed and the different effects of polymerisation parameters on the MIP recognition were investigated, this work needs to

be further improved upon. The initial study of polymerisation parameters provided some important trends and information for establishing future experiments, but more repetitions and additional (and optimised) levels of effects should be use to draw conclusive arguments about the significance of specific conditions. By setting up a more developed model, with a greater number of factors, the importance of each could be better elucidated. In this way, the optimum amount of polymer, template, photoinitiator, crosslinking time, salt concentration, etc., could be determined. This would then form a crucial basis to using a set model to further evaluate subsequent imprinting experiments. An improvement on this experiment is to replace the previous capture-and-release study exploiting UV absorbance on a spectrophotometric plate reader, with a more accurate bulk release study and sensitive analysis by HPLC. This would provide much better quantitative results, and using greater numbers of samples should more clearly show the magnitude and effect of each contribution.

11.2.2 Revaluation of Imprint Matrix

While a primary objective of this research was to evaluate the developed chitosan polymer for molecular imprinting, additional steps could be taken to improve the recognition toward imprinted templates.

11.2.2.1 Chemical Modification

Using chemical modification of the chitosan backbone may enhance the possible interactions with the template during polymerisation. Whereas the modified chitosan polymer possesses a variety of chemical functionalities along its backbone, e.g. amine, alcohol, methacrylate, there is significant potential to further modify its structure. By using targeted chemistries, more functional groups could be added to the chitosan that would be chosen specifically to add stronger interactions with particular templates of interest. This would incorporate stronger and multipoint binding with the target, which would result in a more defined pre-polymerisation arrangement, and higher specificity and selectivity in subsequent capture experiments.

A major hurdle to specific template recognition in molecular imprinting is that the sites created through the imprint are very heterogeneous, and the distribution of highly-specific sites to non-specific binding sites is non-uniform.[215] It is this quality which leads to high non-specific binding of template analogues to MIPs. The imprinting process yields many sites of low- or partial-recognition, but the number of high-recognition sites is far less. One method of counteracting this trend would be to eliminate a percentage of the low-affinity sites in a MIP following polymerisation. This can be done by targeted chemistry against low binding sites in the polymer matrix.[405] Thus it may be possible to greatly increase the specificity of the MIP, without a loss to the binding of the template. This can shift the overall affinity distribution towards greater selectivity, although, depending on the ratio of low-to-high affinity sites, the magnitude of the resulting change may not be large. It is also possible to similarly identify proper functional monomer-template pairing ratios such that there is a strong enough complexation between the two, and functional monomer aggregation could be used to reduce the number of non-specific background binding sites.[59] This improves the overall selectivity of the matrix without significantly affecting the binding capacity.

11.2.2.2 Controlled Polymerisation

As was discussed in Section 2.3, current chemical knowledge allows very precise control of radical reactions, which can provide a great benefit towards building MIPs. Stringent control over the polymerisation mechanics not only allows regulation of the reaction rate, but supports methodical arrangement of the resultant polymer, and restriction of free radicals to a fixed local space. The report from Cutivet *et al*. provides an intriguing method of anchoring a template molecule with a target ligand, and building up an imprinted polymer microgel around it.[113] This produces a very specific orientation site that also remains open at the surface for access of large molecules. Using techniques such as atom transfer radical polymerisation (ATRP) and surface-initiated reversible addition-fragmentation chain transfer (RAFT) has allowed several groups to efficiently create imprinted polymers.[406, 407, 408, 409, 410, 411] As these generally result in thin layers of polymer, they are particularly effective for binding surfaces in

SPR[412] and QCM.[413] However, typical MIPs for protein separation can also be formed this way.[414]

Surface-initiated reactions are often used with silica supports, although gold is also a common base. The initiators are usually either drawn from the family of azo-compounds or a series of iniferters, each of which is not without certain drawbacks.[415] When combined with precipitation polymerisation, the regulated nature of ATRP[416] and RAFT[417] can produce imprinted polymer spheres of small diameters (< 10 μm) and of very uniform size distribution. A promising example of the so-called 'living' polymerisation (via ATRP) was reported by Fang *et al*. who generated photo- and thermo-regulated responsive MIP microspheres capable of controlled binding and release in aqueous media.[418] Similar light-, temperature-, and pH-responsive microspheres were imprinted with template during RAFT polymerisation.[419] These type of controlled and 'smart' polymer imprints have great potential for biomaterials. *S*-propranolol MIP-coated nanotubes, obtained through a surface-initiated ATRP strategy on anodic aluminium oxide membranes, have even been used in drug uploading and *in vivo* release studies.[420] Chang *et al*. have even combined the RAFT technique with 'click' chemistry, the rapid joining of small units, to achieve even greater reaction control.[421] As it has been shown that MIPs made via controlled 'living' polymerisations can show improved template recognition over similar MIPs formed in a traditional fashion, using these methods may be one avenue of improving the imprinting effect of a polymer system.[408, 422]

11.2.2.3 Template Immobilisation

Particularly for large macromolecules such as proteins, the size and specific orientation of the template can provide an impediment to easy recognition in imprinting designs. As a number of the most successful MIP systems and detection methods have been accomplished with thin layers of polymer, an idealised model of this would be one in which we have a near monolayer thickness of template which would be just effectually surrounded by polymer. This would provide an optimum availability of binding sites

per volume, and yield the highest access to imprint sites. This also minimises the amount of template needed for the MIP, and eliminates the consummation of any 'wasted' template too far recessed into the polymer from the surface to be removed or allow recapture of the target. A thin layer of MIP by this view could be regarded as a 'porous' film where template binding would effectively occur in an almost two-dimensional fashion.

Using a single layer of template molecules offers up an advantage towards MIP recognition beyond the physical improvement. When only a single layer of template is desired to be incorporated into the polymer matrix, this means that the molecule can be fixed to a surface prior to polymerisation. Whether using covalent, ionic, or hydrogen bonding, attaching the template first to a support base imparts some interesting benefits to the construction. For one, a surface can be specifically covered with template molecule in a controlled fashion (based on the local binding). This can then lead to the addition of multiple targets in a theoretical binding array, or specifically patterned areas for detection. Immobilisation at fixed points also eliminates template aggregation, and augments the complete surrounding of the imprint molecule by polymer, which improves the MIP-template interaction. Securing the template to the region ensures that it will remain present during the polymerisation process, and allows it to be controlled (e.g. stability, concentration) independent of the polymer ingredients and preparation steps. By having foreknowledge of the arrangement and density of binding sites, it also allows for more precise quantification of binding degree to the imprinted areas and against overall non-specific binding. But perhaps the most important advantage of fixing the template in this fashion is that it allows orientation of the molecule.

By carefully selecting the immobilisation chemistry, the template molecule can be attached to the surface in a controlled manner. A target site on the molecule would be chosen that would be able to form a bond with the service. This implies necessarily that the opposite side of the molecule would be that exposed to the greatest extent outwards (and into the polymer surrounding). In this way, careful design of the attachment procedure can attain an orientation wherein a specific part of the template

molecule is open for binding. This is not trivial, especially when working with large molecules. For example, by selecting a highly active or specific 'target' binding site of a protein, the opposite side of the protein would be fixed to the substrate, and, following MIP formation, the template would orient itself in a way where the active site is exposed. This is particularly useful for applications wherein the separation/detection of very similar molecules is desired. By controlling which part of a molecule will be exposed to the molecular imprinting, one can select particular regions of the molecule or amino acid sequence in a protein, to target that lone area for recognition, whereas competing molecules, identical in other parts but differing in a degree at that site, would only bind non-specifically. This is related to the epitope approach of molecular imprinting as well. Whereas it can be very difficult to achieve the overall recognition around the relatively large circumference of a protein, and the size of the target limits its release from the polymer, affixing the template first to a surface makes the partial imprinting of the molecule a much more facile operation.

11.2.2.4 Additional Monomer/Polymer Selection

While the degree of imprinting seen in this method remains relatively low, much of the low specific recognition may be attributable to the difficulty in using chitosan as the main polymer for an imprint matrix. The number of reported uses of chitosan in MIP is very minimal, and when it is present as part of an imprinted polymer, it usually forms a structural element only, and does not itself contribute to the binding recognition. Nearly all common imprinting schemes use polymers of comparably much lower molecular weight. The long chains of the chitosan, even after fractionation, may not provide a suitable degree of spatial closeness to provide the interaction needed for molecular imprinting. For strong specific recognition to take place, it may be necessary to form a tighter network around template molecules, else those few areas of interaction may not be sufficient to overcome the non-specific degree of binding elsewhere on various analytes.

There is the additional factor that the properties of the chitosan hydrogel, while making it an ideal material for tissue engineering, also make it less suitable towards achieving the chemical recognition derived from molecular imprinting. The hydrogel is a relatively loose network, with high equilibrium water content, and it swells and contracts significantly with changes in ionic strength, drying, and pH. Because of this, during intermediate stages of polymerisation and rebinding, it is extremely difficult to maintain the constant conditions in its environment. As the polymer matrix shifts, the imprint sites can easily be disrupted, and because the network is not completely elastic, the reorientation might not be sufficiently reversible to regenerate the specific arrangement necessary for recognition at these sites. Because of these factors, it is worth considering other polymer systems, or the addition of other monomers to supplement the chitosan that can help structure a more rigid/non-deformable matrix.

As previously discussed, the incorporation of supplementary monomers or crosslinkers can aid in template recognition, by maintaining a more specific arrangement of the matrix around the target. However, since the most commonly used crosslinkers in imprinting, such as ethyleneglycol dimethacrylate and divinylbenzene, have very low solubilities in water, if a crosslinker is used, it may call for one to be drawn from another chemical family. Unfortunately, most crosslinkers used in photoinitiated reactions still posses a degree of hydrophobic moiety which is responsible for the free-radical pre-cursor. Alternately, some chemical crosslinking agents could be examined, such as bis-succinimides, that would react with the other functional groups of glycol chitosan. They could be selected for higher water solubilities, allowing more incorporation into the MIP network. This does have to be balanced against increasing the hydrophilicity of the polymer matrix too much, as this will heighten water swelling and physical disruption of imprint sites.

Other monomers could be used to crosslink together with chitosan, forming a co-polymer, or simply to increase matrix density, e.g. in an interpenetrating network. Pyrrole is one possibility. Although it is a heterocyclic aromatic, it is soluble in water and is polymermisable; it has a pair of protons that are available for deprotonation and

nucleophilic substitution. Polypyrrole (PPy) is a conductive polymer, and has been employed in many applications due to its tuneable oxidation/reduction equilibrium and electrostatic properties. It has been extensively investigated in particular with biosensors, as it is electropolymerisable, and can easily be laid down in dense surface layers. Past studies including its use in MIPs have involved electrical impedance,[423] SPR,[424] and QCM[425] detection systems. Yu and Lai demonstrated its capacity in solid phase extraction with imprinted PPy coatings.[426] Their study slowed excellent long-term stability of the imprinted polymer, reaching up to 80% after 80 runs. Using cyclic and square wave voltammetry, Maouche *et al*. were able to achieve ultralow (pM) detection of dopamine in a PPy MIP.[427] The group of Yang *et al*. fashioned surface-imprinted pyrrole nanowires using alumina membrane as a sacrificial support.[428] While pyrrole itself has limited functionality, it can be modified by an overoxidation treatment to increase its recognition ability as an MIP matrix.[429] Singh *et al*. have specifically proposed pyrrole as a monomer for interfacial polymerisation for an organic-in-water emulsion, where the monomer and polymer are contained in separate phases.[430, 431] This scheme may provide an excellent method to overcome the imprinting difficulties with aqueous solutions, if the bonding can take place at an organic-aqueous interface.

It has been suggested by authors such as Rampey *et al*. that high affinity sites in MIPs are related to the heterogeneity of the polymer matrix.[215] This stands to reason, as a matrix with a wider distribution of shapes and functionalities would proffer a better chance at having those specific arrangements for highly attractive interactions, given that complete 'idealised' optimisation of the entire pre-polymer complex is highly unlikely from a practical perspective. Thus, making more modifications to the chitosan backbone, or adding in additional monomers and crosslinking agents should help to support increased selectivity with the imprinted chitosan hydrogel.

11.2.3 Template Selection

A further aspect to consider for future work with this imprinted chitosan gel is to select better model templates for evaluation of the imprinting effect. Because the proteins

used thus far have been general model molecules, with limited specific interaction between the template and imprint matrix, it may be beneficial to examine other sample templates. The proteins chosen for this thesis research were selected from common models used in molecular imprinting, and based on relative sizes and expected charge interactions (based on pIs). However, because the polymer matrix used in this case is more specific and unique compared to the conventional MIPs, selecting another template for achieving molecular recognition may offer considerably more specific interactions, while also more adequately reflecting the ultimate goal of this work.

11.2.3.1 Concanavalin *A*

One molecular identified as a potentially strong model target for this research is the lectin concanavalin *A*.[432] ConA is a homotetramer with a MW in the range of 53-92 kDa,[433, 434] and an isoelectric point (pI) between 4.5-5.5.[435] It is notable in that it interacts with many receptors and is a carbohydrate-binding protein. Specifically, ConA is known to bind α-*D*-mannosyl and α-*D*-glucosyl residues.[436] In fact, the strong binding ability of ConA has been exploited for the solid-phase immobilisation of glycoenzymes, including those which have proved difficult to immobilise by traditional covalent couplings.[437] It is hoped that this ability will allow it to interact in strong fashion with the *D*-glucosamine residues in chitosan. While not exactly the same molecular arrangement, the similarity should still provide a bond that can aid in template recognition for molecular imprinting. It is also well known that ConA undergoes significant conformational changes with pH, which may provide one simple method to achieve repeatable binding and removal between the template and the MIP.[438] The use of ConA as a template for molecularly imprinted polymers remains an unexplored area of study. While Zheng and Du have used its interaction to form binary monolayers of glycolipids, it relies on the selective arrangement of the chains according the binding sites of ConA, and does not involve polymerisation in the traditional sense.[439] Thus, as a potentially novel avenue of research, it would be prudent to look at using the strong binding ability of ConA in formation of MIPs.

11.2.3.2 Chondroitin-6-sulphate

Another strong target molecule for this research is the glycosaminoglycan chondroitin-6-sulphate (also known as chondroitin sulphate *C*). Chondroitin sulphates (CS) are sulphated chain sugars formed from alternating *N*-acetylgalactosamine and glucuronic acid residues. This particular form is sulphated on carbon 6 of the GalNAc sugar, as shown in Figure 11-1.

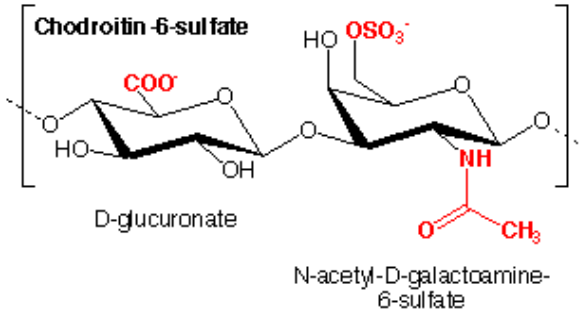


Figure 11-1. Chemical structure of chondroitin sulphate C/6.

Chondroitin-6-sulphate (C6S) possesses a net negative charge at physiologic pH,[440] making it a strong candidate for using electrostatic interaction with the positive charges on chitosan chains. The affinity between chitosan and chondroitin sulphates has been used for forming hydrogel complexes[441] and microencapsulated protein delivery.[442] In some applications requiring more robust structural support, such as nanoparticles[443] and membranes[444] the electrostatic binding has been enhanced with covalent linkages (e.g. EDC/NHS, tripolyphosphate). The combination of chitosan and C6S in an artificial ECM has been shown to stabilise growth factors for enhanced cell proliferation,[445] and similarly to support sustained insulin delivery in bead form.[446] C6S has also been proven to bind very strongly to antibodies, compared to other forms.[447] This particular form of chondroitin sulphate was also chosen for its well-established role in the support of cartilage tissue production and chondrogenic differentiation.[448, 449, 450] It has recently been shown that disaccharides, such as a heparan sulphate analogue, can be a target for MIP recognition.[451]

As a template molecule, the successful imprinting of C6S would provide a great advantage in many applications. Chondroitin sulphate is a very important structural component in the body, and particularly in articular cartilage, provides a significant contribution to the compressive strengths of that tissue.[452] As such, it would be very useful to have the ability to incorporate it within a tissue engineering matrix. Like chitosan, chondroitin sulphate has been investigated for different types of 'second generation' cartilage tissue repair. The use of chondroitin sulphate post-surgery has been shown to increase the synthesis of hyaluronan, glucosamine and collagen II, while inhibiting ECM degrading enzymes.[453] It has already been shown that the developed chitosan hydrogel construct can provide a means to grow *de novo* articular cartilage tissue in a 3D environment.[1] Combining this methodology with chondroitin sulphate (bound via molecular imprinting) would improve the tissue engineering ability, by more closely mimicking the extracellular environment and providing essential structural support to the overall implant. Similar saccharides molecules such as glucose have been previously used as imprint templates, although with limited success.[216, 454] While not directly exploring molecular imprinting, chondroitin sulphate has been use together with chitosan in a polyelectrolyte complex to bind and deliver theophylline, a bronchus-dilator drug and very common MIP template.[455] By virtue of their chemical functionalities, glycosaminoglycans also have significant potential for incorporation into biosensors.[456] Chondroitin sulphate has been used in conjunction with both QCM-D and SPR for interaction and detection of growth factor, e.g. BMP-2.[457] Accordingly, the investigation of C6S as a component in the chitosan MIP would provide several avenues of noteworthy research.

11.2.4 Further Investigation of Nanogels

As the creation of chitosan nanoparticles by chemical crosslinking in a reverse emulsion proved to be an effective method to generate small, relatively uniform gel spheres of high surface area, these still represent a great potential for further study as molecularly imprinted models. The principal drawback of using this method was that the yield of particles was very low, due to the need for excessive washing volumes and

losses in filtration and repeated transfers of the nanospheres. Because the chitosan gels are so small and have a density close to that of water, they became increasingly difficult to separate from solution as the purification progressed, while still maintaining them in a non-aggregated state. Further work in this area, such as slower removal of the supernatant by gradual evaporation or lyophilisation might help to concentrate the collected nanoparticles into a more efficient and usable form. They could also be packed into a contained unit, such as a mini-'filtration' cartridge, fixed with nanoporous membranes at the end, which would allow them to be kept without loss of material, and then used for MIP studies. In this fashion, solutions of target molecule (or a competitor analogue) could be passed through the unit of packed particles, and the binding analysed through changes in the solution concentration, or ultimately, as in chromatographic columns, through retention time by detection at the exit. This could be very useful for enhanced separation of a mixed feed stream of several similar components, the imprinted of which would be isolated and collected. Their very small size would allow such nanoparticles to be incorporated in an array of technologies, especially in the growing field of micro-scale devices, as well as biological applications involving ease of injection, targeted delivery, or cellular take up.

11.2.5 Detection Methods

Another area for potential improvement of this research model is to investigate different methodologies to measure the molecular imprinting recognition. There are a few ways in which the detection of molecular binding described in the thesis could be augmented to provide a more precise or enriched measurement system.

11.2.5.1 Microfluidics

The growing field of microfluidics offers many advantages in biosensors and lab-on-chip designs. With just a small amount of material, complex chemical reactions, manipulations, and detection cascades can be accomplished. In some cases, chitosan hydrogels can be fabricated directly inside the inner chamber of a microfluidic device.[458] In such a way, an imprinted polymer could be made directly on the surface of

a substrate or polymerised from a controlled mix of components inside the device. The latter would allow with ease different formulations to be tested and created alongside one another, and by manipulating the gradients possible from the laminar flow in microfluidic channels,[459, 460, 461, 462] several polymers could be compared together directly. It has been shown that manipulation of gel parameters via this method in combination with photocrosslinking can create effective gradients.[463] Many strategies are moving towards miniaturisation, and microfluidic platforms are introducing a new field of technical expertise for sensors and instrumentation.

As part of a larger analytical or detection system, MIPs could be incorporated in a variety of methods to offer an improved functionality. As a component in a lab-on-chip device, an imprinted polymer could be used for highly sensitive detection of an analyte in a diagnostic or chemosensory fashion. As on a macro scale, MIPs in a microfluidic system can be utilised for screening and sorting of cells or feed components, either as part of the sensor capacity, or to improve the purity of samples downstream. One of the foremost advantages of MIPs is their ease of production and simple customisability. This offers a prime benefit in microfluidics to use single formulations with template changes for high-throughput applications and multiplex detection arrays. With only slight adjustments, a large number of different targets can be created and used for manipulation in a microfluidic device.

11.2.5.2 Flow Cell

Along with advanced designs involving microfluidic flow, one might wish to consider the advantages offered by using flow through cells for measurement. Having continuous flow through the chamber being used for measurements enables several principles that would improve on the accuracy and effectiveness of MIP evaluation. Firstly, when incorporated into a real-time monitoring system, it enables observation of the removal of the template during the washing stages, and can directly monitor when the ‘plateau’ point is reached where no more template can be leached out of the polymer. Secondly, one can then measure the changes as the target molecule is captured

by the MIP, followed by its subsequent release. This is all controllable by flow of the template in solution, or a simple wash buffer, to the polymer. The key advantage here is the ability to change the concentration of solutes in the liquid phase during the experiment. By altering the amount of template and/or competitor molecules dissolved, one can directly visualise the changes in imprinting efficacy with concentration and time. It also importantly allows one to remove passive diffusion as a variable, such that either an infinite 'supply' or 'sink' can be applied, depending on the desired run. These dynamic abilities to control the system offer much more information and realistic applicability than can be gained from simple static conditions.

It was the intention to investigate the dynamic behaviour of the MIPs as an aspect of this research. As part of this, a continuous flow reactor was designed which could be integrated into the electrical impedance system. The single reaction chamber described previously to monitor the behaviour of MIP/NIP microspheres has also incorporated an inlet and outlet so that continuous flow could be used as a next step. Unfortunately, filtration of the gel particles to maintain them inside the measurement cell was an issue, which we did not have time to resolve. As the next development in this stage, a multi-chamber continuous flow cell was designed for dielectric spectroscopy measurements. This was a reflection of the ultimate goal of the work, and would have offered the ability to simultaneously observe the effects on several different gels concurrently. This model offers a considerable advantage, as it allows coincident measurement of both the imprinted polymer to its control, and several replicates to each other at once, all in reaction to the same feed stream. This not only simplifies the gathering of data, but improves it, since effects such as variation between samples and changes with buffer solution are minimised. A schematic of the multi-chamber flow-through reaction cell is shown in Figure 11-2.

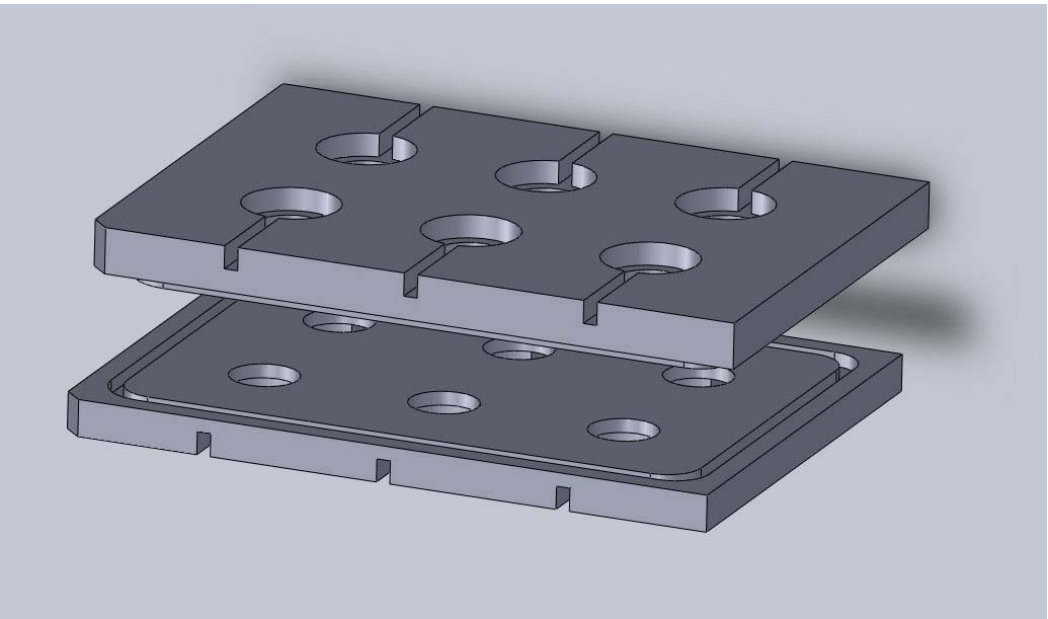


Figure 11-2. Six-chambered measurement cell designed for continuous flow and real-time monitoring by dielectric spectroscopy of molecularly imprinted hydrogels. This design has the ability to detect changes between samples in simultaneously and using parallel analyte streams.

11.2.5.3 Interdigitated Electrode

As one considers the miniaturised design applications of MIPs discussed, a principal component is the integration of a measurement/signal transduction element. Recently, the continuing improvements in integrated circuit design and ease of manufacture has given rise to the use of interdigitated electrodes for conductometric sensing applications.[464] While potentiometric immunosensors have been discussed since 1975,[465, 466] it was not until Taylor *et al*., who later reported on the development of a chip-based electrode sensor with interdigitated design,[467] that exploration of this method began. An example of a planar interdigitated electrode (IDE) designed and developed in our lab, and which has been used for preliminary testing of imprinted hydrogel layers (via dielectric spectroscopy) is shown in Figure 11-3.

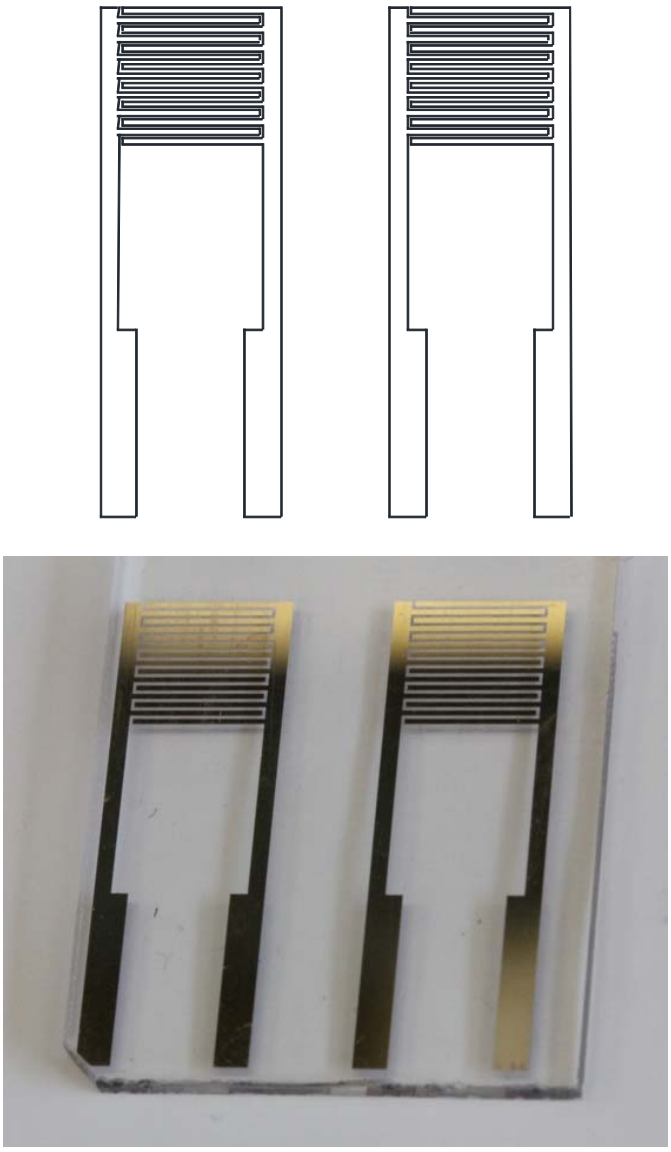


Figure 11-3. Sample design of interdigitated platinum electrodes printed on glass substrate.

Interdigitated electrodes have now been used to observe antibody binding,[468] DNA hybridisation,[469] pathogen detection,[470] bacterial sensing,[471] blood separation,[472] and cell sorting,[473] and have been used in conjunction with such methods as microfluidics,[474] surface patterning,[475] high-throughput screening,[476] and surface acoustic wave resonance.[477] These integrated arrays have been employed in the development of many impedimetric biosensors.[478] Currently, IDEs form a part of a great number microelectromechanical systems (MEMS).

Interdigitated electrodes (IDE) are generally laid down by deposition of thin metal layers of indium-tin oxide, gold, or platinum. Pyrrole-coated ceramic fingers have also been examined, thought this involves a more complex electrode synthesis stage.[479] Interestingly, Norman and Badia demonstrated an interdigitated electrode can be formed from self-assembled monolayers which they investigated via SPR. [480] While the substrate for IDEs is traditionally glass, chosen for its non-conductive nature, a polymer base can also be used,[481] which is convenient for rapid production and MEMS. The geometries of the electrodes (e.g. pitch, width, spacing) play a critical role in measurement, and must be finely tuned. An analytical comparison of planar and 3-D IDEs is available from Rana *et al*.[482] While many reports in the literature describe IDEs combined with polymer surfaces and membranes, few include the use of hydrogels.[483, 484] In one notable study, controlled photolithography was used during fabrication to localise different enzymes on adjacent electrodes, for multi-analyte detection.[485] Yang *et al*. have suggested when a layer of hydrogel is added to the electrodes, provided it is sufficiently thin, it can be modeled by a slight modification to the equivalent circuit, incorporating a membrane capacitance and resistance.[486] In combination with dielectric impedance, IDEs have been used to monitor and quantify cells (bacteria) in suspension.[487] In conjunction with a parallel plate capacitor an IDE has been used to characterise polymer mechanical properties, through dielectric permittivity.[488] The results from these combined studies suggest that employing a similar IDE design might be a very useful avenue of investigation for detecting the sensitive changes in MIPs during selectivity/specificity assays.

11.2.6 Potential of Future Work

This thesis project has great potential for achieving breakthroughs in a number of areas. If fully successful, it would mark a key point in achieving imprinting with a chitosan polymer matrix that has not yet been demonstrated, and offer support for the recognition of complex biomacromolecules through molecular imprinting in aqueous solution, and provides a novel medium for potential biosensors. The research offers many areas of improvement as discussed herein, and with more time and

experimentation put in, could vastly be improved upon. The developed chitosan has a demonstrated versatility as a biomaterial, and with the incorporation of target recognition though molecular imprinting, the potential applications for it continue to grow.

References

[1] Shapka, S.N. "A Bilayered Implant of Chitosan-Hydroxyapatite for the Tissue Engineering of Articular Cartilage." *MSc Thesis*. Department of Chemical Engineering, Queen's University. Kingston, ON. 2006.

[2] Mosbach, K., and Mosbach, R. "Entrapment of Enzymes and Microorganisms in Synthetic Cross-linked Polymers and their Application in Column Techniques." Acta Chemica Scandinavica. 20:10. pp 2807-2810. 1966.

[3] Mosbach, K. "Enzymes Bound to Artificial Matrices." Scientific American." 224:3. pp 26-33. 1971.

[4] Arshady, R., Mosbach, K. "Synthesis of Substrate-selective Polymers by Host-Guest Polymerization." Die Makromolekulare Chemie. 182:2. pp 687-692. 1981.

[5] Mosbach, K. "Novel Affinity Techniques." *in* Affinity Chromatography and Biological Recognition. Proceedings of the Fifth International Symposium on Affinity Chromatography and Biological Recognition. Eds. Chaiken, I.M., Wilchek, M., Parikh, I. pp 209-222. Academic Press, Inc. Montreal, QC. 1983.

[6] Wulff, G. "Fourty years of molecular imprinting in synthetic polymers: origin, features and perspectives." Microchimica Acta. pp 1-12. 2013.

[7] Polyakov, M.V. "Adsorption properties and structure of silica gel." Zhurnal Fizieskoj Khimii. 2. pp 799–805. 1931.

[8] Norrlow, O., Glad, M., Mosbach, K. "Acrylic Polymer Preparation Containing Recognition Sites Obtained by Imprinting with Substrates." Journal of Chromatography. 299:1. pp 29-41. 1984.

[9] Wang, Y., Zhang, Z., Jain, V., Yi, J., Mueller, S., Sokolov, J., Liu, Z., Levon, K., Rigas, B., Rafailovich, M.H. "Potentiometric sensors based on surface molecular imprinting: Detection of cancer biomarkers and viruses." Sensors and Actuators B: Chemical. 146:1. pp 381-387. 2010.

[10] Zimmerman, S.C., Wendland, M.S., Rakow, N.A., Zharov, I., Suslick, K.S. "Synthetic hosts by monomolecular imprinting inside dendrimers." Nature. 418:6896. 399-403. 2002.

[11] Pietrzyk-Le, A., Bikram, C.K.C., D'Souza, F., Kutner, W. "Supramolecular self-assembly governed molecularly imprinted polymers for selective chemical sensing." *in* Applications of Supramolecular Chemistry for 21st Century Technology. Ed. Schneider, H.-J. pp 105-128. CRC Press. Boca Raton, FL. 2012.

[12] Mosbach, K., Ramstrom, O. "The Emerging Technique of Molecular Imprinting and Its Future Impact on Biotechnology." Nature Biotechnology. 14:2. pp 163-170. 1996.

[13] Andersson, L., Sellergren, B., Mosbach, K. "Imprinting of Amino Acid Derivatives in Macroporous Polymers." Tetrahedron Letters. 25:45. pp 5211-5214. 1984.

[14] Striegler, S. "Designing selective sites in templated polymers utilizing coordinative bonds." Journal of Chromatography B. 804:1. pp 183-195. 2004.

[15] Wulff, G. "The Use of Polymers with Enzyme-Analogous Structures for the Resolution of Racemates." Angewandte Chemie International Edition. 11:4. pp 341. 1972.

[16] Wulff, G., Sarhan, A., Zabrocki, K. "Enzyme-Analogue Built Polymers and Their Use for the Resolution of Racemates." Tetrahedron Letters. 14:44. pp 4329-4332. 1973.

[17] Wulff, G. "Molecular Imprinting in Cross-Linked Materials with the Aid of Molecular Templates - A Way towards Artificial Antibodies." Angewandte Chemie International Edition. 34:17. pp 1812-1832. 1995.

[18] Ikegami, T., Mukawa, T., Nariai, H., Takeuchi, T. "Bisphenol A-recognition polymers prepared by covalent molecular imprinting." Analytica Chimica Acta. 504:1. pp 131-135. 2002.

[19] Shen, F., Ren, X. "Covalent molecular imprinting made easy: a case study of mannose imprinted polymer." RSC Advances. 4:25. pp 13123-13125. 2014.

[20] Prathish, K.P., Vishnuvardhan, V., Prasada Rao, T. "Rational Design of In Situ Monolithic Imprinted Polymer Membranes for the Potentiometric Sensing of Diethyl Chlorophosphate – a Chemical Warfare Agent Simulant." Electroanalysis. 21:9. pp 1048-1056. 2009.

[21] Sellergren, B., Andersson, L. "Molecular recognition in macroporous polymers prepared by a substrate analog imprinting strategy." The Journal of Organic Chemistry. 55:10. pp 3381-3383. 1990.

[22] Whitcombe, M.J., Rodriguez, M.E., Vulfson, E.N. "Polymeric adsorbents for cholesterol prepared by molecular imprinting." Special Publication - Royal Society of Chemistry. 158. *Separations for Biotechnology 3*. pp 565-571. 1994.

[23] Whitcombe, M.J., Rodriguez, M.E., Villar, P., Vulfson, E.N. "A New Method for the Introduction of Recognition Site Functionality into Polymers Prepared by Molecular Imprinting: Synthesis and Characterization of Polymeric Receptors for Cholesterol." Journal of the American Chemical Society. 117:27. pp 7105-7111. 1995.

[24] Alexander, C., Kirsch, N., Whitcombe, M. "New methodologies in the preparation of imprinted polymers." *in* Polymer Chemistry: A Practical Approach. Ed. Davis, F.J. pp 201-214. Oxford University Press, Inc. Toronto, ON. 2004.

[25] Knutsson, M., Andersson, H.S., Nicholls, I.A. "Novel chiral recognition elements for molecularly imprinted polymer preparation." Journal of Molecular Recognition. 11:1-6. pp 87-90. 1998.

[26] Ramstrom, O., Nicholls, I.A., Mosbach, K. "Synthetic Peptide Receptor Mimics: Highly Stereoselective Recognition in Non-Covalent Molecularly Imprinted Polymers." Tetrahedron: Asymmetry. 5:4. pp 649-656. 1994.

[27] Suedee, R., Srichana, T., Rattananont, T. "Enantioselective Release of Controlled Delivery Granules Based on Molecularly Imprinted Polymers." Drug Delivery. 9:1. pp 19-30. 2002.

[28] Allender, C.J., Richardson, C., Woodhouse, B., Heard, C.M., Brain, K.R. "Pharmaceutical applications for molecularly imprinted polymers." International Journal of Pharmaceutics. 195:1/2. pp 39-43. 2000.

[29] Owens, P.K., Karlsson, L., Lutz, E.S.M., Andersson, L.I. "Molecular imprinting for bio- and pharmaceutical analysis." Trends in Analytical Chemistry. 18:3. pp 146-154. 1999.

[30] Martín-Esteban, A., Sellergren, B. "2.17 - Molecularly Imprinted Polymers." *in* Comprehensive Sampling and Sample Preparation: Analytical Techniques for Scientists. *Volume 2: Theory of Extraction Techniques*. Ed. Janusz, P. pp 331-344. Academic Press. Oxford, UK. 2012.

[31] Sellergren, B. “Direct Drug Determination by Selective Sample Enrichment on an Imprinted Polymer.” Analytical Chemistry. 66:9. pp 1578-1582. 1994.

[32] Ulbricht, M. “Membrane separations using molecularly imprinted polymers.” Journal of Chromatography B. 804:1. pp 113-125. 2004.

[33] Pegoraro, C., Silvestri, D., Ciardelli, G., Cristallini, C., Barbani, N. “Molecularly imprinted poly(ethylene-co-vinyl alcohol) membranes for the specific recognition of phospholipids.” Biosensors and Bioelectronics. 24:4. pp 748-755. 2008.

[34] Wulff, G., Liu, J. “Design of Biomimetic Catalysts by Molecular Imprinting in Synthetic Polymers: The Role of Transition State Stabilization.” Accounts of Chemical Research. 45:2. pp 239-247. 2011.

[35] Bonini, F., Piletsky, S., Turner, A.P.F., Speghini, A., Bossi, A. “Surface imprinted beads for the recognition of human serum albumin.” Biosensors and Bioelectronics. 22:9. pp 2322-2328. 2007.

[36] Andersson, H.S., Koch-Schmidt, A.-C., Ohlson, S. “Study of the nature of Recognition in Molecularly Imprinted Polymers.” Journal of Molecular Recognition. 9:5/6. pp 675-682. 1996.

[37] Ye, L., Yu, Y., Mosbach, K. “Towards the development of molecularly imprinted artificial receptors for he screening of estrogenic chemicals.” The Analyst. 126:6. pp 760-765. 2001.

[38] El-Toufaili, F.-A., Visnjevski, A., Brüggemann, O. “Screening combinatorial libraries of molecularly imprinted polymer films casted on membranes in single-use membrane modules.” Journal of Chromatography B. 804:1. pp 135-139. 2004.

[39] Chen, L., Xu, S., Li, J. “Recent advances in molecular imprinting technology: current status, challenges and highlighted applications.” Chemical Society Reviews. 40:5. pp 2922-2942. 2011.

[40] Subrahmanyam, S., Piletsky, S.A., Piletska, E.V., Chen, B., Karim, K., Turner, A.P.F. “‘Bite-and-Switch’ approach using computationally designed molecularly imprinted polymers for sensing of creatinine.” Biosensors and Bioelectronics. 16:9. pp 631-637. 2001.

[41] Piletsky, S.A., Turner, A.P.F. “Electrochemical Sensors Based on Molecularly Imprinted Polymers.” Electroanalysis. 14:5. pp 317-323.2002.

[42] Slinchenko, O., Rachkov, A., Miyachi, H., Ogiso, M., Minoura, N. “Imprinted polymer layer for recognising double-stranded DNA.” Biosensors and Bioelectronics. 20:6. pp 1091-1097. 2004.

[43] Rachkov, A., McNiven, S., El’skaya, A., Yano, K., Karube, I. “Fluorescence detection of β-estradiol using a molecularly imprinted polymer.” Analytica Chimica Acta. 405:1. pp 23-29. 2000.

[44] Vlatakis, G., Andersson, L.I., Muller, R., Mosbach, K. “Drug assay using antibody mimics made by molecular imprinting.” Nature. 361:6413. pp 645-647. 1993.

[45] Mosbach, K. “The Promise of Molecular Imprinting.” Scientific American. 195:4. pp 86-91. 2006.

[46] Suedee, R., Jantarat, C., Lindner, W., Viernstein, H., Songkro, S., Srichana, T. “Development of a pH-responsive drug delivery system for enantioselective-controlled delivery of racemic drugs.” Journal of Controlled Release. 142:1. pp 122-131. 2010.

[47] Yamashita, K., Nishimura, T., Ohashi, K., Ohkouchi, H., Nango, M. “Two-step imprinting procedure of inter-penetrating polymer network-type stimuli-responsive hydrogel-adsorbents.” Polymer journal. 35:7. pp 545-550. 2003.

[48] Gong, C., Wong, K.-L., Lam, M.H.W. "Photoresponsive Molecularly Imprinted Hydrogels for the Photoregulated Release and Uptake of Pharmaceuticals in the Aqueous Media." Chemistry of Materials. 20:4. pp 1353-1358. 2008.

[49] Miyata, T., Jige, M., Nakaminami, T., Uragami, T. "Tumor marker-responsive behavior of gels prepared by biomolecular imprinting." Proceedings of the National Academy of Sciences of the United States of America. 103:5. pp 1190-1193. 2006.

[50] Qin, L., He, X.-W., Yuan, X., Li, W.-Y., Zhang, Y.-K. "Molecularly imprinted beads with double thermosensitive gates for selective recognition of proteins." Analytical & Bioanalytical Chemistry. 399:10. pp 3375-3385. 2011.

[51] Oya, T., Enoki, T., Grosberg, A.Y., Masamune, S., Sakiyama, T., Takeoka, Y., Tanaka, K., Wang, G., Yilmaz, Y., Feld, M.S., Dasari, R., Tanaka, T. "Reversible Molecular Adsorption Based on Multiple-Point Interaction by Shrinkable Gels." Science. 286:5444. pp 1543-1545. 1999.

[52] Ramström, O., Ye, L., Mosbach, K. "Artificial antibodies to corticosteroids prepared by molecular imprinting." Chemistry & Biology. 3:6. pp 471-477. 1996.

[53] Wu, X. Shimizu, K.D. "Molecular Imprinting for Sensor Applications." *in* Molecular Recognition and Polymers: Control of Polymer Structure and Self-Assembly. Eds. Rotello, V.M., Thayumanavan, S. pp 395-429. John Wiley & Sons, Inc. Hoboken, NJ. 2008.

[54] Umpleby II, R.J., Bode, M., Shimizu, K.D. "Measurement of the continuous distribution of binding sites in molecularly imprinted polymers." Analyst. 125:7. pp 1261-1265. 2000.

[55] Umpleby, R.J., Baxter, S.C., Chen, Y., Shah, R.N., Shimizu, K.D. "Characterization of Molecularly Imprinted Polymers with the Langmuir−Freundlich Isotherm." Analytical Chemistry. 73:19. pp 4584-4591. 2001.

[56] Andersson, H.S., Karlsson, J.G., Piletsky, S.A., Koch-Schmidt, A.-C., Mosbach, K., Nicholls, I.A. "Study of the nature of recognition in molecularly imprinted polymers, II : Influence of monomer–template ratio and sample load on retention and selectivity." Journal of Chromatography A. 848:1-2. pp 39-49. 1999.

[57] Ansell, R.J., Kuah, K.L. "Imprinted polymers for chiral resolution of (+/-)-ephedrine: understanding the pre-polymerisation equilibrium and the action of different mobile phase modifiers." Analyst. 130:2. pp 179-187. 2005.

[58] Umpleby II, R.J., Baxter, S.C., Rampey, A.M., Rushton, G.T., Chen, Y., Shimizu, K.D. "Characterization of the heterogeneous binding site affinity distributions in molecularly imprinted polymers." Journal of Chromatography B. 804:1. pp 141-149. 2004.

[59] Zhang, Y., Song, D., Brown, J.C., Shimizu, K.D. "Suppression of background sites in molecularly imprinted polymers via urea-urea monomer aggregation." Organic & Biomolecular Chemistry. 9:1. pp 120-126. 2011.

[60] Lee, K., Itharaju, R.R., Puleo, D.A. "Protein-imprinted polysiloxane scaffolds." Acta Biomaterialia. 3:4. pp 515-522. 2007.

[61] Yoshikawa, M., Ooi, T., Izumi, J.-I. "Alternative Molecularly Imprinted Membranes from a Derivative of Natural Polymer, Cellulose Acetate." Journal of Applied Polymer Science. 72:4. pp 493-499. 1999.

[62] Venkatesh, S., Sizemore, S.P., Byrne, M.E. "Biomimetic hydrogels for enhanced loading and extended release of ocular therapeutics." Biomaterials. 28:4. pp 717-724. 2007.

[63] Kryscio, D.R., Shi, Y., Ren, P., Peppas, N.A. "Molecular Docking Simulations for Macromolecularly Imprinted Polymers." Industrial & Engineering Chemistry Research. 50:24. pp 13877-13884. 2011.

[64] Barkaline, V.V., Douhaya, Y.V., Tsakalof, A. "Computer simulation based selection of optimal monomer for imprinting of tri-O-acetiladenosine in polymer matrix: vacuum calculations." Journal of Molecular Modeling. 19:1. pp 359-369. 2013.

[65] Yañez, F., Chianella, I., Piletsky, S.A., Concheiro, A., Alvarez-Lorenzo, C. "Computational modeling and molecular imprinting for the development of acrylic polymers with high affinity for bile salts." Analytica Chimica Acta. 659:1-2. pp 178-185. 2010.

[66] Henschel, H., Kirsch, N., Hedin-Dahlström, J., Whitcombe, M.J., Wikman, S., Nicholls, I.A. "Effect of the cross-linker on the general performance and temperature dependent behaviour of a molecularly imprinted polymer catalyst of a Diels–Alder reaction." Journal of Molecular Catalysis B: Enzymatic. 72:3-4. pp 199-205. 2011.

[67] De Luca, G., Donato, L., García Del Blanco, S., Tasselli, F., Drioli, E. "On the Cause of Controlling Affinity to Small Molecules of Imprinted Polymeric Membranes Prepared by Noncovalent Approach: A Computational and Experimental Investigation." The Journal of Physical Chemistry B. 115:30. pp 9345-9351. 2011.

[68] Żołek, T., Luliński, P., Maciejewska, D. "A computational model for selectivity evaluation of 2-(3,4-dimethoxyphenyl)ethylamine (homoveratrylamine) imprinted polymers towards biogenic compounds." Analytica Chimica Acta. 693:1-2. pp 121-129. 2011.

[69] Lousa, D., Baptista, A.M., Soares, C.M. "Structural determinants of ligand imprinting: A molecular dynamics simulation study of subtilisin in aqueous and apolar solvents." Protein Science. 20:2. pp 379-386. 2011.

[70] Levi, L., Raim, V., Srebnik, S. "A brief review of coarse-grained and other computational studies of molecularly imprinted polymers." Journal of Molecular Recognition. 24:6. pp 883-891. 2011.

[71] Olsson, G.D., Karlsson, B.C.G., Shoravi, S., Wiklander, J.G., Nicholls, I.A. "Mechanisms underlying molecularly imprinted polymer molecular memory and the role of crosslinker: resolving debate on the nature of template recognition in phenylalanine anilide imprinted polymers." Journal of Molecular Recognition. 25:2. pp 69-73. 2012.

[72] Mojica, E.-R. E., Autschbach, J., Bright, F.V., Aga, D.S. "Synthesis and evaluation of tetracycline imprinted xerogels: Comparison of experiment and computational modeling." Analytica Chimica Acta. 684:1-2. pp 72-80. 2011.

[73] Nicholls, I.A., Karlsson, B.C.G., Olsson, G.D., Rosengren, A.M. "Computational Strategies for the Design and Study of Molecularly Imprinted Materials." Industrial & Engineering Chemistry Research (in press)

[74] Golker, K., Karlsson, B.C.G., Olsson, G.D., Rosengren, A.M., Nicholls, I.A. "Influence of Composition and Morphology on Template Recognition in Molecularly Imprinted Polymers." Macromolecules. 46:4. pp 1408-1414. 2013.

[75] Nicholls, I.A., Andersson, H.S., Golker, K., Henschel, H., Karlsson, B.C.G., Olsson, G.D., Rosengren, A.M., Shoravi, S., Suriyanarayanan, S., Wiklander, J.G., Wikman, S. "Rational design of biomimetic molecularly imprinted materials: theoretical and computational strategies for guiding nanoscale structured polymer development." Analytical & Bioanalytical Chemistry. 400:6. pp 1771-1786. 2011.

[76] Azenha, M., Szefczyk, B., Loureiro, D., Kathirvel, P., Cordeiro, M.N.D.S., Fernando-Silva, A. "Molecular Dynamics Simulations of Pregelification Mixtures for the Production of Imprinted Xerogels." Langmuir. 27:8. pp 5062-5070. 2011.

[77] Zhang, Y., Song, D., Lanni, L.M., Shimizu, K.D. "Importance of Functional Monomer Dimerization in the Molecular Imprinting Process." Macromolecules. 43:15. pp 6284-6294. 2010.

[78] Meier, F., Schott, B., Riedel, D., Mizaikoff, B. "Computational and experimental study on the influence of the porogen on the selectivity of 4-nitrophenol molecularly imprinted polymers." Analytica Chimica Acta. 744:0. pp 68-74. 2012.

[79] Saloni, J., Walker, K., Hill, G. "Theoretical Investigation on Monomer and Solvent Selection for Molecular Imprinting of Nitrocompounds." The Journal of Physical Chemistry A. 117:7. pp 1531-1534. 2013.

[80] Xu, Z., Kuang, D., Feng, Y., Zhang, F. "Combination of hydrophobic effect and electrostatic interaction in imprinting for achieving efficient recognition in aqueous media." Carbohydrate Polymers. 79:3. pp 642-647. 2010.

[81] Deng, Q.-L., Lun, Z.-H., Gao, R.-Y., Zhang, L.-H., Zhang, W.-B., Zhang, Y.-K. "(S)-Ibuprofen-imprinted polymers incorporating γ-methacryloxypropyl-trimethoxysilane for CEC separation of ibuprofen enantiomers." Electrophoresis. 27:21. pp 4351-4358. 2006.

[82] Takátsy, A., Végvári, Á., Hjertén, S., Kilár, F. "Universal method for synthesis of artificial gel antibodies by the imprinting approach combined with a unique electrophoresis technique for detection of minute structural differences of proteins, viruses and cells (bacteria). Ib. Gel antibodies against proteins (hemoglobins)." Electrophoresis. 28:14. pp 2345-2350. 2007.

[83] Chassaing, C., Stokes, J., Venn, R. F., Lanza, F., Sellergren, B., Holmberg, A., Berggren, C. "Molecularly imprinted polymers for the determination of a pharmaceutical development compound in plasma using 96-well MISPE technology." Journal of Chromatography B. 804:1. pp 71-81. 2004.

[84] Dubey, L., Chianella, I., Dubey, I., Piletska, E., Whitcombe, M.J., Piletsky, S. "Selective Recognition of Bifunctional Molecules by Synthetic Polymers Prepared by Covalent Molecular Imprinting." Open Analytical Chemistry Journal. 6. pp 15-21. 2012.

[85] Lavignac, N., Allender, C.J., Brain, K.R. "Current status of molecularly imprinted polymers as alternatives to antibodies in sorbent assays." Analytica Chimica Acta. 510:2. pp 139-145. 2004.

[86] Bergmann, N.M., Peppas, N.A. "Configurational Biomimetic Imprinting for Protein Recognition: Structural Characteristics of Recognitive Hydrogels." Industrial & Engineering Chemistry Research. 47:23. pp 9099-9107. 2008.

[87] Baggiani, C., Giovannoli, C., Anfossi, L., Passini, C., Baravalle, P., Giraudi, G. "A Connection between the Binding Properties of Imprinted and Nonimprinted Polymers: A Change of Perspective in Molecular Imprinting." Journal of the American Chemical Society. 134:3. pp 1513-1518. 2011.

[88] Castell, O.K., Barrow, D.A., Kamarudin, A.R., Allender, C.J. "Current practices for describing the performance of molecularly imprinted polymers can be misleading and may be hampering the development of the field." Journal of Molecular Recognition. 24:6. pp 1115-1122. 2011.

[89] Vasapollo, G., Del Sole, R., Mergola, L., Lazzoi, M.R., Scardino, A., Scorrano, S., Mele, G. "Molecularly Imprinted Polymers: Present and Future Prospective." International Journal of Molecular Sciences. 12:9. pp 5908-5945. 2011.

[90] Sanbe, H., Haginaka, J. "Uniformly sized molecularly imprinted polymers for bisphenol A and β-estradiol: retention and molecular recognition properties in hydro-organic mobile phases." Journal of Pharmaceutical and Biomedical Analysis. 30:6. pp 1835-1844. 2003.

[91] Muhammad, T., Nur, Z., Piletska, E.V., Yimit, O., Piletsky, S.A. "Rational design of molecularly imprinted polymer: the choice of cross-linker." Analyst. 137:11. pp 2623-2628. 2012.

[92] Haupt, K., Mayes, A.G., Mosbach, K. "Herbicide Assay Using an Imprinted Polymer-Based System Analogous to Competitive Fluoroimmunoassays." Analytical Chemistry. 70:18. pp 3936-3939. 1998.

[93] Yang, L., Chen, Z. "Direct Evaluation of Ligand-Receptor Interaction by a Novel Imaging Model Based on Fluorescent Silica Nanoparticle as Ligand Probe and Molecular Imprinting Polymer as Artificial Receptor." Analytical Letters. 44:4. 687-697. 2011.

[94] Haupt, K. "Molecularly imprinted sorbent assays and the use of non-related probes." Reactive and Functional Polymers. 41:1-3. pp 125-131. 1999.

[95] Molinelli, A. "Molecularly Imprinted Polymers: Towards a Rational Understanding of Biomimetic Materials." *PhD Thesis*. School of Chemistry and Biochemistry. Georgia Institute of Technology. Atlanta, GA. October 2004.

[96] Pap, T., Horvai, G. "Binding assays with molecularly imprinted polymers—why do they work?" Journal of Chromatography B. 804:1. pp 167-172. 2004.

[97] Allender, C.J., Castell, O.K., Davies, P.R., Fiddy, S., Hedin-Dahlstrom, J., Stockenhuber, M. "A glimpse of the inner workings of the templated site." Chemical Communications. 0:2. pp 165-167. 2009.

[98] Wei, S., Jakusch, M., Mizaikoff, B. "Capturing molecules with templated materials – Analysis and rational design of molecularly imprinted polymers." Analytica Chimica Acta. 578:1. pp 50-58. 2006.

[99] Hilt, J.Z., Byrne, M.E. "Configurational biomimesis in drug delivery: molecular imprinting of biologically significant molecules." Advanced Drug Delivery Reviews. 56:11. pp 1599-1620. 2004.

[100] Hansen, D.E. "Recent developments in the molecular imprinting of proteins." Biomaterials. 28:29. pp 4178-4191. 2007.

[101] Kryscio, D.R., Peppas, N.A. "Critical review and perspective of macromolecularly imprinted polymers." Acta Biomaterialia. 8:2. pp 461-473. 2012.

[102] Ge, Y., Turner, A.P.F. "Too large to fit? Recent developments in macromolecular imprinting." Trends in Biotechnology. 26:4. pp 218-224. 2008.

[103] Tan, C.J., Tong, Y.W. "The Effect of Protein Structural Conformation on Nanoparticle Molecular Imprinting of Ribonuclease A Using Miniemulsion Polymerization." Langmuir. 23:5. pp 2722-2730. 2007.

[104] Kryscio, D.R., Fleming, M.Q., Peppas, N.A. "Conformational studies of common protein templates in macromolecularly imprinted polymers." Biomedical Microdevices. 14:4. pp 679-687. 2012.

[105] Maier, N.M., Lindner, W. "Chiral recognition applications of molecularly imprinted polymers: a critical review." Analytical & Bioanalytical Chemistry. 389:2. pp 377-397. 2007.

[106] Carter, S.C., Rimmer, S. "Aqueous compatible polymers in bionanotechnology." IEE Proceedings on Nanobiotechnology. 152:5. 2005.

[107] Yilmaz, E., Haupt, K., Mosbach, K. "The Use of Immobilized Templates—A New Approach in Molecular Imprinting." Angewandte Chemie International Edition. 39:12. pp 2115-2118. 2000.

[108] Nishino, H., Huang, C.-S., Shea, K.J. "Selective Protein Capture by Epitope Imprinting." Angewandte Chemie International Edition. 45:15. pp 2392-2396. 2006.

[109] Nematollahzadeh, A., Sun, W., Aureliano, C.S.A., Lütkemeyer, D., Stute, J., Abdekhodaie, M.J., Shojaei, A., Sellergren, B. "High-Capacity Hierarchically Imprinted Polymer Beads for Protein Recognition and Capture." Angewandte Chemie International Edition. 50:2. pp 495-498. 2011.

[110] Liu, L., Zheng, J., Fang, G., Xie, W. "Improvement of the homogeneity of protein-imprinted polymer films by orientated immobilization of the template." Analytica Chimica Acta. 726:0. pp 85-92. 2012.

[111] Lu, Y., Yan, C.-L., Gao, S.-Y. "Preparation and recognition of surface molecularly imprinted core–shell microbeads for protein in aqueous solutions." Applied Surface Science. 255:12. pp 6061-6066. 2009.

[112] Dechtrirat, D., Jetzschmann, K.J., Stöcklein, W.F.M., Scheller, F.W., Gajovic-Eichelmann, N. "Protein Rebinding to a Surface-Confined Imprint. Advanced Functional Materials. 22:24. pp 5231-5237. 2012.

[113] Cutivet, A., Schembri, C., Kovensky, J., Haupt, K. "Molecularly Imprinted Microgels as Enzyme Inhibitors." Journal of the American Chemical Society. 131:41. pp 14699-14702. 2009.

[114] Rosellini, E., Barbani, N., Giusti, P., Ciardelli, G., Cristallini, C. "Molecularly imprinted nanoparticles with recognition properties towards a laminin H–Tyr–Ile–Gly–Ser–Arg–OH sequence for tissue engineering applications." Biomedical Materials. 5:6. pp 1-11. 2010.

[115] Kempe, M. "Antibody-Mimicking Polymers as Chiral Stationary Phases in HPLC." Analytical Chemistry. 68:11. pp 1948-1953. 1996.

[116] Gao, J., Tian, H., Wang, Y., Yang, Q., Liu, D., Wang, Y., Mi, H. "The design of protein-imprinted polymers as antibody substitutes for investigating protein–protein interactions." Biomaterials. 33:11. pp 3344-3352. 2012.

[117] Surugiu, I., Ye, L., Yilmaz, E., Dzgoev, A., Danielsson, B., Mosbach, K., Haupt, K. "An enzyme-linked molecularly imprinted sorbent assay." Analyst. 125:1. pp 13-16. 2000.

[118] Díaz-Díaz, G., Antuña-Jiménez, D., Carmen Blanco-López, M., Jesús Lobo-Castañón, M., Miranda-Ordieres, A.J., Tuñón-Blanco, P. "New materials for analytical biomimetic assays based on affinity and catalytic receptors prepared by molecular imprinting." TrAC Trends in Analytical Chemistry. 33:0. pp 68-80. 2012.

[119] Lv, Y., Tan, T., Svec, F. "Molecular imprinting of proteins in polymers attached to the surface of nanomaterials for selective recognition of biomacromolecules." Biotechnology Advances. 31:8. pp 1172-1186. 2013.

[120] Linares, A.V., Vandevelde, F., Pantigny, J., Falcimaigne-Cordin, A., Haupt, K. "Polymer Films Composed of Surface-Bound Nanofilaments with a High Aspect Ratio, Molecularly Imprinted with Small Molecules and Proteins." Advanced Functional Materials. 19:8. pp 1299-1303. 2009.

[121] El Kirat, K., Bartkowski, M., Haupt, K. "Probing the recognition specificity of a protein molecularly imprinted polymer using force spectroscopy." Biosensors and Bioelectronics. 24:8. pp 2618-2624. 2009.

[122] Takeuchi, T., Goto, D., Shinmori, H. "Protein profiling by protein imprinted polymer array." Analyst. 132:2. pp 101-103. 2007.

[123] Piletsky, S.A., Piletska, E.V., Chen, B., Karim, K., Weston, D., Barrett, G., Lowe, P., Turner, A.P.F. "Chemical Grafting of Molecularly Imprinted Homopolymers to the Surface of Microplates. Application of Artificial Adrenergic Receptor in Enzyme-Linked Assay for β-Agonists Determination." Analytical Chemistry. 72:18. pp 4381-4385. 2000.

[124] Bossi, A., Piletsky, S.A., Piletska, E.V., Righetti, P.G., Turner, A.P.F. "Surface-Grafted Molecularly Imprinted Polymers for Protein Recognition." Analytical Chemistry. 73:21. pp 5281-5286. 2001.

[125] Wang, Y., Zhou, Y., Sokolov, J., Rigas, B., Levon, K., Rafailovich, M. "A potentiometric protein sensor built with surface molecular imprinting method." Biosensors and Bioelectronics. 24:1. pp 162-166. 2008.

[126] Verheyen, E., Schillemans, J.P., van Wijk, M., Demeniex, M.-A., Hennink, W.E., van Nostrum, C.F. "Challenges for the effective molecular imprinting of proteins." Biomaterials. 32:11. pp 3008-3020. 2011.

[127] Hillberg, A.L., Tabrizian, M. "Biomolecule imprinting: Developments in mimicking dynamic natural recognition." Ingenierie et Recherche Biomedicale. 29:2-3. pp 89-104. 2008.

[128] Takeuchi, T., Haginaka, J. "Separation and sensing based on molecular recognition using molecularly imprinted polymers." Journal of Chromatography B: Biomedical Sciences and Applications. 728:1. pp 1-20. 1999.

[129] Zhang, H., Ye, L., Mosbach, K. "Non-covalent molecular imprinting with emphasis on it application in separation and drug development." Journal of Molecular Recognition. 19:4. pp 248-259. 2006.

[130] Schrader, T., and Koch, S. "Artificial protein sensors." Molecular Biosystems. 3:4. pp 241-248. 2007.

[131] Wehner, M., Janssen, D., Schafer, G., Schrader, T. "Sequence-Selective Peptide Recognition with Designed Modules." European Journal of Organic Chemistry. 1. pp 138-153. 2006.

[132] Renner, C., Piehler, J., Schrader, T. "Arginine- and Lysine-Specific Polymers for Protein Recognition and Immobilization." Journal of the American Chemical Society. 128:2. pp 620-628. 2006.

[133] Schmuck, C., Rupprecht, D., Junkers, M., Schrader, T. "Artificial Ditopic Arg-Gly-Asp (RGD) Receptors." Chemistry - A European Journal. 13:24. pp 6864-6873. 2007.

[134] Koch, S.J., Renner, C., Xie, X., Schrader, T. "Tuning Linear Copolymers into Protein-Specific Hosts." Angewandte Chemie International Edition. 45:38. pp 6352-6355. 2006.

[135] Wenck, K., Koch, S., Renner, C., Sun, W., Schrader, T. "A Noncovalent Switch for Lysosyme." Journal of the American Chemical Society. 129:51. pp 16015-16019. 2007.

[136] Ying, X., Cheng, G., Li, X. "The imprinting induce-fit model of specific rebinding of macromolecularly imprinted polymer microspheres." Journal of Applied Polymer Science. 122:3. pp 1847-1856. 2011.

[137] Kobayashi, T., Fukaya, T., Abe, M., Fujii, N. "Phase Inversion Molecular Imprinting by Using Template Copolymers for High Substrate Recognition." Langmuir. 18:7. pp 2866-2872. 2002.

[138] Tang, Q., Gong, C., Lam, M.H.-W., Fu, X. "Photoregulated uptake and release of drug by an organic–inorganic hybrid sol–gel material." Journal of Sol-Gel Science and Technology. 59:3. pp 495-504. 2011.

[139] Gore, M.A., Karmalkar, R.N., Kulkarni, M.G. "Enhanced capacities and selectivities for cholesterol in aqueous media by molecular imprinting: role of novel cross-linkers." Journal of Chromatography B. 804:1. pp 211-221. 2004.

[140] Bompart, M., Haupt, K. "Molecularly Imprinted Polymers and Controlled/Living Radical Polymerization." Australian Journal of Chemistry. 62:8. pp 751-761. 2009.

[141] Sasaki, S., Ooya, T., Takeuchi, T. "Highly selective bisphenol A-imprinted polymers prepared by atom transfer radical polymerization." Polymer Chemistry. 1:10. pp 1684-1688. 2010.

[142] Gonzato, C., Courty, M., Pasetto, P., Haupt, K. "Magnetic Molecularly Imprinted Polymer Nanocomposites via Surface-Initiated RAFT Polymerization." Advanced Functional Materials. 21:20. pp 3947-3953. 2011.

[143] Salian, V.D., Byrne, M.E. "Living Radical Polymerization and Molecular Imprinting: Improving Polymer Morphology in Imprinted Polymers." Macromolecular Materials and Engineering. 298:4. pp 379-390. 2013.

[144] Takeuchi, T., Hishiya, T. "Molecular imprinting of proteins emerging as a tool for protein recognition." Organic & Biomolecular Chemistry. 6:14. pp 2459-2467. 2008.

[145] Yu, C., Mosbach, K. "Molecular Imprinting Utilizing an Amide Functional Group for Hydrogen Bonding Leading to Highly Efficient Polymers." Journal of Organic Chemistry. 62:12. pp 4057-4064. 1997.

[146] Enoki, T., Tanaka, K., Watanabe, T., Oya, T., Sakiyama, T., Takeoka, Y., Ito, K., Wang, G., Annaka, M., Hara, K., Du, R., Chuang, J., Wasserman, K., Grosberg, A.Y., Masamune, S., Tanaka, T. "Frustrations in Polymer Conformation in Gels and their Minimization through Molecular Imprinting." Physical Review Letters. 85:23. pp 5000-5003. 2000.

[147] Ito, K., Chuang, J., Alvarez-Lorenzo, C., Watanabe, T., Ando, N., Grosberg, A.Y. "Multiple point adsorption in a heteropolymer gel and the Tanaka approach to imprinting: experiment and theory." Progress in Polymer Science. 28:10. pp 1489-1515. 2003.

[148] Tong, D., Hetenyi, Cs., Bikadim Zs., Gao, J.-P., Hjerten, S. "Some Studies of the Chromatographic Properties of Gels ('Artificial Antibodies/Receptors') for Selective Adsorption of Proteins." Chromatographia. 54:1/2. pp 7-14. 2001.

[149] Hiratani, H., Alvarez-Lorenzo, C. "Timolol uptake and release by imprinted soft contact lenses made of *N,N*-diethylacrylamide and methacrylic acid." Journal of Controlled Release. 83:2. pp 223-230. 2002.

[150] Hjerten, S. Liao, J.-L., Nakazato, K., Wang, Y., Zamaratskaia, G., Zhang, H.-X. "Gels Mimicking Antibodies in Their Selective Recognition of Proteins." Chromatographia. 44:5/6. pp 227-234. 1997.

[151] Byrne, M.E., Park, K., Peppas, N.A. "Molecular imprinting within hydrogels." Advanced Drug Delivery Reviews. 54:1. pp 149-161. 2002.

[152] Byrne, M.E., Salian, V. "Molecular Imprinting Within Hydrogels II: Progress and Analysis of the Field." International Journal of Pharmaceutics. 364:2. pp 188-212. 2008.

[153] Ali, M., Horikawa, S., Venkatesh, S., Saha, J., Hong, J.W., Byrne, M.E. "Zero-order therapeutic release from imprinted hydrogel contact lenses within *in vitro* physiological ocular tear flow." Journal of Controlled Release. 124:3. pp 154-162. 2007.

[154] Alvarez-Lorenzo, C., Hiratani, H., Gomez-Amoza, J.L., Martinez-Pacheco, R., Souto, C., Conchiero, A. "Soft Contact Lenses Capable of Sustained Delivery of Timolol." Journal of Pharmaceutical Sciences. 91:10. pp 2182-2192. 2002.

[155] Hiratani, H., Alvarez-Lorenzo, C. "The nature of backbone monomers determines the performance of imprinted soft contact lenses as timolol drug delivery systems." Biomaterials. 25:6. pp 1105-1113. 2004.

[156] Hiratani, H., Fujiwara, A., Tamiya, Y., Mizutani, Y., Alvarez-Lorenzo, C. "Ocular release of timolol from molecularly imprinted soft contact lenses." Biomaterials. 26:11. pp 1293-1298. 2005.

[157] Alvarez-Lorenzo, C., Yañez, F., Barreiro-Iglesias, R., Concheiro, A. "Imprinted soft contact lenses as norfloxacin delivery systems." Journal of Controlled Release. 113:3. pp 236-244. 2006.

[158] Li, S., Tiwari, A., Ge, Y., Fei, D. "A pH-responsive, low crosslinked, molecularly imprinted insulin delivery system." Advanced Materials Letters. 1:2. pp 4-10. 2010.

[159] Kryscio, D.R., Peppas, N.A. "Mimicking biological delivery through feedback-controlled drug release systems based on molecular imprinting." American Institute of Chemical Engineers Journal. Bioengineeing, Food, and Natural Products. 55:6. pp 1311-1324. 2009.

[160] Kanekiyo, Y., Naganawa, R., Tao, H. "pH-Responsive Molecularly Imprinted Polymers." Angewandte Chemie International Edition. 42:26. pp 3014-3016. 2003.

[161] Fu, G., Zhao, J., Yu, H., Liu, L., He, B. "Bovine serum albumin-imprinted polymer gels prepared by graft polymerization of acrylamide on chitosan." Reactive and Functional Polymers. 67:5. pp 442-450. 2007.

[162] Espinosa-Garcia, B., Arguelles-Monal, W.M., Hernandez, J., Felix-Valenzuela, L., Acosta, N., Goyocoolea, F.M. "Molecularly Imprinted Chitosan-Genipin Hydrogels with Recognition Capacity toward o-Xylene." Biomacromolecules. 8:11. pp 3355-3364. 2007.

[163] Aburto, J., Mendez-Orozco, A., Le Borgne, S. "Hydrogels as adsorbents of organosulphur compounds currently found in diesel." Chemical Engineering and Processing. 43:12. pp 1587-1595. 2004.

[164] Shi, H., Tsai, W.-B., Garrison, M.D., Ferrari, S., Ratner, B.D. "Template-imprinted nanostructured surfaces for protein recognition." Nature. 398:6728. pp 593-597. 1999.

[165] Madihally, S.V., Matthew, H.W.T. "Porous chitosan scaffolds for tissue engineering." Biomaterials. 20:12. pp 1133-1142. 1999.

[166] Fradet, G., Brister, S., Mulder, D.S., Lough, J., Averbach, B.L. "Evaluation of chitosan as a new hemostatic agent: in vitro and in vivo experiments." *in* Chitin in Nature and Technology. Eds. Muzzarelli, R., Jeuniaux, C., Gooday, G.W. pp 443-451. Plenum Press. New York, NY. 1986.

[167] Olsen, R., Schwartzmiller, D., Weppner, W., Winandy, R. "Biomedical applications of chitin and its derivatives." *in* Chitin and Chitosan: sources, biochemistry, physical properties, and applications. Eds. Skjaak-Braek, G., Anthonsen, T., Sandford, P. pp 813-828. New York, NY. 1989.

[168] Rao, S.B., Sharma, C.P. "Use of chitosan as a biomaterial: Studies on its safety and hemostatic potential." Journal of Biomedical Materials Research. 34:1. pp 21-28. 1997.

[169] Jarry, C., Chaput, C., Chenite, A., Renaud, M.-A., Buschmann, M., Leroux, J.-C. *Effects of Steam Sterilization on Thermogelling Chitosan-Based Gels*. Journal of Biomedical Materials Research – Part B: Applied Biomaterials. 58:1. pp 127-135. 2001.

[170] Chatelet, C., Damour, O., Domard, A. "Influence of the degree of acetylation on some biological properties of chitosan films." Biomaterials. 22:3. pp 261-268. 2001.

[171] Haque, S., Shadab, M., Fazil, M., Sahni, J.K., Baboota, S., Dang, S., Ali, J. "Role of chitosan biomaterials in drug delivery systems: a patent perspective." Recent Patents on Materials Science. 4:3. pp 209-223. 2011.

[172] Liu, X., Ma, L., Mao, Z., Gao, C. "Chitosan-Based Biomaterials for Tissue Repair and Regeneration." *in* Chitosan for Biomaterials II. Eds. Jayakumar, R., Prabaharan, M., Muzzarelli, R.A.A. *Advances in Polymer Science*. Vol. 244. pp 81-127. Springer Berlin Heidelberg. Berlin, DE. 2011.

[173] Croisier, F., Jérôme, C. "Chitosan-based biomaterials for tissue engineering." European Polymer Journal. 49:4. pp 780-792. 2013.

[174] Aiba, S.-I. "Studies on chitosan: 6. Relationship between N-acetyl group distribution pattern and chitinase digestibility of partially N-acetylated chitosans." International Journal of Biological Macromolecules. 15:4. pp 241-245. 1993.

[175] Nordtveit, R.J., Varum, K.M., Smidsrod, O. "Degradation of fully water-soluble, partially N-acetylated chitosans with lysozyme." Carbohydrate Polymers. 23:4. pp 253-260. 1994.

[176] Chen, X.-G., Park, H.-J. "Chemical characteristics of O-carboxymethyl chitosans related to the preparation conditions." Carbohydrate Polymers. 53:4. pp 355-359. 2003.

[177] Auzely, R., Rinaudo, M. "Controlled Chemical Modifications of Chitosan. Characterization and Investigation of Original Properties." Macromolecular Bioscience. 3:10. pp 562-565. 2003.

[178] Kurita, K., Hirakawa, M., Aida, K., Yang, J., Nishiyama, Y. "Trimethylsilylated Chitosan: A Convenient Precursor for Chemical Modifications." Chemistry letters. 32:11. pp 1074-1075. 2003.

[179] Kato, Y., Onishi, H., Machida, Y. "Review. N-succinyl-chitosan as a drug carrier: water-insoluble and water-soluble conjugates." Biomaterials. 25:5. pp 907-915. 2004.

[180] Makuska, R., Gorochovceva, N. "Regioselective grafting of poly(ethylene glycol) onto chitosan through C-6 position of glucosamine units." Carbohydrate Polymers. 64:2. pp 319-327. 2006.

[181] Sashiwa, H., Shigemasa, Y., Roy, R. "Chemical modification of chitosan 11: chitosan-dendrimer hybrid as a tree like molecule." Carbohydrate Polymers. 49:2. pp 195-205. 2002.

[182] Kweon, D.K., Kang, D.W. "Drug-Release Behaviour of Chitosan-g-Poly(vinyl alcohol) Copolymer Matrix." Journal of Applied Polymer Science. 74:2. pp 458-464. 1999.

[183] Morimoto, M., Saimoto, H., Sigemasa, Y. "Control of Chitin and Chitosan by Chemical Modification." Trends in Glycoscience and Glycotechnology. 14:78. pp 205-222. 2002.

[184] Anon. "Limited Summary of Data for Chemical Selection: Chitosan (9012-76-4)." *http://ntp-server.niehs.nih.gov/ntp/htdocs/Chem_Background/ExSumPdf/Chitosan.pdf*. National Toxicology Program. Department of Health and Human Services. National Institute of Environmental Health Sciences. National Institutes of Health. Research Triangle Park, NC.

[185] Anon. "Press Release: HemCon Receives Canadian medical Device License for Unique Hemorrhage Control Bandage." *http://www.hemcon.com/PDF/HemCon%20Press%20Release%20-%20Canada.pdf*. Hemcon Control Technologies. Portland, OR. 15 March 2006.

[186] Anon. "Premarket Approval (PMA) Database – Surestat Arterial Closure System." *http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfPMA/PMASimpleSearch.cfm?db=PMA&id=12283#AOStatement*. Premarket Approval P010049, Supplement S012. Center for Devices and Radiological Health. U.S. Food and Drug Administration. Rockville, MD. 31 August 2004.

[187] http://patft.uspto.gov/netacgi/nph-Parser?Sect1=PTO2&Sect2=HITOFF&p=1&u=%2Fnetahtml%2FPTO%2Fsearch-bool.html&r=0&f=S&l=50&TERM1=chitosan&FIELD1=TI&co1=AND&TERM2=&FIELD2=&d=PTXT . Results of search in 1790-2013 db for: chitosan in [TITLE]. USPTO Patent Full-Text and Image Database. US Patents and Trademark Office. Alexandria, VA. 16 April 2013.

[188] Di Martino, A., Sittinger, M., Risbud, M.V. "Chitosan: A versatile biopolymer for orthopaedic tissue-engineering." Biomaterials 26:30. pp 5983-5990. 2005.

[189] Kubota, N., Eguchi, Y. "Facile Preparation of Water-Soluble N-Acetyalted Chitosan and Molecular Weight Dependence on Its Water-Solubility." Polymer Journal. 29:2. pp 123-127. 1997.

[190] Kubota, N., Tatsumoto, N., Sano, T., Toya, K. "A simple preparation of half N-acetylated chitosan highly soluble in water and aqueous organic solvents." Carbohydrate Research. 324:4. pp 268-274. 2000.

[191] Carreno-Gomez, B., Duncan, R. "Evaluation of the biological properties of soluble chitosan and chitosan microspheres." International Journal of Pharmaceutics. 148:2. pp 231-240. 1997.

[192] Park, H., Park, K., Kim, D. "Preparation and swelling behaviour of chitosan-based superporous hydrogels for gastric retention application." Journal of Biomedical Materials Research. 76(A):1. pp 144-150. 2006.

[193] Noble, L., Gray, A.I., Sadiq, L., Uchegbu, I.F. "A non-covalently cross-linked chitosan based hydrogel. International Journal fop Pharmaceutics." 192:2. pp 173-182. 1999.

[194] Kubota, N., Ohga, K., Moriguchi, M. "Permeability Properties of Glycol Chitosan Membrane Modified with Thiol Groups." Journal of Applied Polymer Science. 42:2. pp 495-501. 1991.

[195] Martin, L., Wilson, C.G., Koosha, F., Uchegbu, I.F. "Sustained buccal delivery of the hydrophobic drug denbufylline using physically cross-linked palmitoyl glycol chitosan hydrogels." European Journal of Pharmaceutics and Biopharmaceutics. 55:1. pp 35-45. 2003.

[196] Huang, J., Xing, X., Zhang, X., He, X., Lin, Q., Lian, W., Zhu, H. "A molecularly imprinted electrochemical sensor based on multiwalled carbon nanotube-gold nanoparticle composites and chitosan for the detection of tyramine." Food Research International. 44:1. pp 276-281. 2011.

[197] Guo, T.-Y., Xia, Y.-Q., Hao, G.-J., Zhang, B.-H., Fu, G-Q., Yuan, Z., He, B.-L., Kennedy, J.F. "Chemically modified chitosan beads as matrices for adsorptive separation of proteins by molecularly imprinted polymer." Carbohydrate Polymers. 62:3. pp 214-221. 2005.

[198] Okutucu, B., Zihnioglu, F., Telefoncu, A. "Shell-core imprinted polyacrylamide crosslinked chitosan for albumin removal from plasma." Journal of Biomedical Materials Research. 84A:3. pp 842-845. 2008.

[199] Wang, H., He, Y., He, X., Li., W., Chen, L., Zhang, Y. "BSA-imprinted synthetic receptor for reversible template recognition." Journal of Separation Science. 32:11. pp 1981-1986. 2009.

[200] Chen, S., Du, D., Huang, J., Zhang, A., Tu, H., Zhang, A. "Rational design and application of molecularly imprinted sol–gel polymer for the electrochemically selective and sensitive determination of Sudan I." Talanta. 84:2. pp 451-456. 2011.

[201] Dai, C., Liu, C., Wei, J., Hong, H., Zhao, Q. "Molecular imprinted macroporous chitosan coated mesoporous silica xerogels for hemorrhage control." Biomaterials. 31:30. pp 7620-7630. 2010.

[202] Monier, M., El-Sokkary, A.M.A. "Preparation of molecularly imprinted cross-linked chitosan/glutaraldehyde resin for enantioselective separation of l-glutamic acid." International Journal of Biological Macromolecules. 47:2. pp 207-213. 2010.

[203] Xia, Y.Q., Guo, T.-Y., Song, M.-D., Zhang, B.-H., Zhang, B.-L. "Hemoglobin Recognition by Imprinting in Semi-Interpenetrating Polymer Network Hydrogel Based on Polyacrylamide and Chitosan. Biomacromolecules. 6:5. pp 2601-2606. 2005.

[204] Guo, T.-Y., Xia, Y.-Q., Wang, J., Song, M.-D., Zhang, B.-H. "Chitosan beads as molecularly imprinted polymer matrix for selective separation of proteins." Biomaterials. 26:28. pp 2537-5745. 2005.

[205] Aburto, J., Le Borgne, S. "Selective Adsorption of Dibenzothiophene Sulfone by an Imprinted and Stimuli-Responsive Chitosan Hydrogel." Macromolecules. 37:8. pp 2938-2943. 2004.

[206] Chen, C.-Y., Chang, J.-C., Chen, A.-H. "Competitive biosorption of azo dyes from aqueous solution on the templated crosslinked-chitosan nanoparticles." Journal of Hazardous Materials. 185:1. pp 430-441. 2011.

[207] Ma, X., Chen, R., Zheng, X., Youn, H., Chen, Z. "Preparation of molecularly imprinted CS membrane for recognizing naringin in aqueous media." Polymer Bulletin. 66:6. pp 853-863. 2011.

[208] Ramstrom, O., Ansell, R.J. "Molecular Imprinting Technology: Challenges and Prospects for the Future." Chirality. 10:3. pp 195-209. 1998.

[209] Oral, E. and Peppas, N.A. "Hydrophilic molecularly imprinted poly(hydroxyethyl methacrylate) polymers. Journal of Biomedical Materials Research. 78A:1. pp 205-210. 2006.

[210] Rachkov, A., Minoura, N. "Recognition of oxytocin and oxytocin-related peptides in aqueous media using a molecularly imprinted polymer synthesized by the epitope approach." Journal of Chromatography A. 889:1/2. pp 111-118. 2000.

[211] Liao, J.-L., Wang, Y., Hjerten, S. "A Novel Support with Artificially Created Recognition for the Selective Removal of Proteins and for Affinity Chromatography." Chromatographia. 42:5/6. pp 259-262. 1996.

[212] Janiak, D.S., Kofinas, P. "Molecular imprinting of peptides and proteins in aqueous media." Analytical and Bioanalytical Chemistry. 389:2. pp 399-404. 2007.

[213] Piletska, E.V., Guerreiro, A.R., Romero-Guerra, M., Chianella, I., Turner, A.P.F., Piletsky, S.A. "Design of molecularly imprinted polymers compatible with aqueous environment." Analytica Chimica Acta. 607:1. pp 54-60. 2008.

[214] Xu, Z., Kuang, D., Liu, L., Deng, Q. "Selective adsorption of norfloxacin in aqueous media by an imprinted polymer based on hydrophobic and electrostatic interactions." Journal of Pharmaceutical and Biomedical Analysis. 45:1. pp 54-61. 2007.

[215] Rampey, A.M., Umpleby, R.J., Rushton, G.T., Iseman, J.C., Shah, R.N., Shimizu, K.D. "Characterization of the Imprint Effect and the Influence of Imprinting Conditions on Affinity, Capacity, and heterogeneity in Molecularly Imprinted Polymers Using the Freundlich Isotherm-Affinity Distribution Analysis." Analytical Chemistry. 76:4. pp 1123-1133. 2004.

[216] Byrne, M.E., Hilt, J.Z., Peppas, N.A. "Recognitive biomimetic networks with moiety imprinting for intelligent drug delivery." Journal of Biomedical Materials Research. 84A:1. pp 137-147. 2008.

[217] Fu, G.-Q., Yu, H., Zhu, J. "Imprinting effect of protein-imprinted polymers composed of chitosan and polyacrylamide: A re-examination." Biomaterials. 19:13. pp 2138-2142. 2008.

[218] Rechichi, A., Cristallini, C., Vitale, U., Ciardelli, G., Barbani, N., Vozzi, G., Giusti, P. "New biomedical devices with selective peptide recognition properties. Part 1: Characterization and cytotoxicity of molecularly imprinted polymers." Journal of Cellular and Molecular Medicine." 11:6. pp 1367-1376. 2007.

[219] Dickert, F.L., Lieberzeit, P., Hayden, O. "Sensor strategies for microorganism detection—from physical principles to imprinting procedures." Analytical and Bioanalytical Chemistry. 377:3. pp 540-549. 2003.

[220] Hayden, O., Dickert, F.L. "Selective Microorganism Detection with Cell Surface Imprinted Polymers." Advanced Materials. 13:19. pp 1480-1483. 2001.

[221] Dickert, F.L., Hayden, O., Halikias, K.P. "Synthetic receptors as sensor coatings for molecules and living cells." The Analyst. 126:6. pp 766-771. 2001.

[222] Aherne, A., Alexander, C., Payne, M.J., Perez, N., Vulfson, E.N. "Bacteria-Mediated Lithography of Polymer Surfaces." Journal of the American Chemical Society. 118:36. pp 8771-8772. 1996.

[223] Harvey, S.D., Mong, G.M., Ozanich, R.M., Mclean, J.S., Goodwin, S.M., Valentine, N.B., Fredrickson, J.K. "Preparation and evaluation of spore-specific affinity-augmented bio-imprinted beads." Analytical and Bioanalytical Chemistry. 386:2. pp 211-219. 2006.

[224] Backsay, I., Takatsy, A., Vegvari, A., Elfwing, A., Ballagi-Pordany, A., Kilar, F., Hjerten, S. "Universal method for synthesis of artificial gel antibodies by the imprinting approach combined with a unique electrophoresis technique for detection of minute structural difference of proteins, viruses, and cells (bacteria). III: Gel antibodies against cells (bacteria). Electrophoresis. 27:23. pp 4682-4687. 2006.

[225] Wandelt, B., Sadowska, M., Cywinski, P., Hachulka, K. "Polymeric Sensory Systems Based on Molecular Imprinting for Identification and Separation of Molecules and Bigger Biological Objects." Molecular Crystals and Liquid Crystals. 486:1. pp 257/[1299]-270/[1312]. 2008.

[226] Ren, K., Zare, R.N. "Chemical Recognition in Cell-Imprinted Polymers." ACS Nano. 6:5. pp 4314-4318. 2012.

[227] Bolisay, L.D., Culver, J.N., Kofinas, P. "Optimization of Virus Imprinting Methods to Improve Selectivity and Reduce Nonspecific Binding." Biomacromolecules. 8:12. pp 3893-3899. 2007.

[228] Takatsy, A., Sedzik, J., Kilar, F., Hjerten, S. "Universal method for synthesis of artificial gel antibodies by the imprinting approach combined with a unique electrophoresis technique for detection of minute structural difference of proteins, viruses, and cells (bacteria). II: Gel antibodies against virus (Semliki Forest Virus)." Journal of Separation Science. 29:18. pp 2810-2815. 2006.

[229] Bengtsson, H., Roos, U., Andersson, L.I. "Molecular Imprint Based Radioassay for Direct Determination of S-Propranolol in Human Plasma." Analytical Communications. 34:9. pp 233-235. 1997.

[230] Shinkai, S., Takeuchi, M. "Molecular design of synthetic receptors with dynamic, imprinting, and allosteric functions." Biosensors and Bioelectronics. 20:6. pp 1250-1259. 2004.

[231] Tan, J., Wang, H.-F., Yan, X.-P. "Discrimination of Saccharides with a Fluorescent Molecular Imprinting Sensor Array Based on Phenylboronic Acid Functionalized Mesoporous Silica." Analytical Chemistry. 81:13. pp 5273-5280. 2009.

[232] Sunayama, H., Ooya, T., Takeuchi, T. "Fluorescent protein recognition polymer thin films capable of selective signal transduction of target binding events prepared by molecular imprinting with a post-imprinting treatment." Biosensors and Bioelectronics. 26:2. pp 458-462. 2010.

[233] Cywinski, P. "Fluorescent Molecularly Imprinted Polymers in Sensing of cAMP and cGMP." Journal of Physical Chemistry & Biophysics. 3:111. pp 1000111-2. 2013.

[234] Guan, G., Liu, B., Wang, Z., Zhang, Z. "Imprinting of Molecular Recognition Sites on Nanostructures and Its Applications in Chemosensors." Sensors. 8:12. pp 8291-8320. 2008.

[235] Hillberg, A.L., Brain, K.R., Allender, C.J. "Molecular imprinted polymer sensors: Implications for therapeutics." Advanced Drug Delivery Reviews. 57:12. pp 1875-1889. 2005.

[236] Hu, X., Li, G., Li, M., Huang, J., Li, Y., Gao, Y., Zhang, Y. "Ultrasensitive Specific Stimulant Assay Based on Molecularly Imprinted Photonic Hydrogels." Advanced Functional Materials. 18:4. pp 575-583. 2008.

[237] Anon. "4294A Precision Impedance Analyzer, 40 Hz to 100 MHz." *http://www.home.agilent.com/en/pd-1000000858%3Aepsg%3Apro-pn-4294A/precision-impedance-analyzer-40-hz-to-110-mhz?&cc=CA&lc=eng*. Agilent Technologies Canada Inc. Mississauga, ON. (2 May 2013)

[238] Anon. "16092A Spring Clip Fixture." *http://www.home.agilent.com/agilent/product.jspx?cc=CA&lc=eng&nid=-34051.536879621&pageMode=OV&cc=CA&lc=eng*. Agilent Technologies Canada Inc. Mississauga, ON. (2 May 2013)

[239] Debye, P. "Part I. Dielectric constant. Energy absorption in dielectrics with polar molecules." Transactions of the Faraday Society. 30. pp 679-684. 1934.

[240] Razaz, M., Davies, J. B. "Capacitance of the Abrupt Transition from Coaxial-to-Circular Waveguide." IEEE Transactions on Microwave Theory and Techniques. 27:6. pp 564-569. 1979.

[241] Dibeneditto, J., Uhlir, A. "Frequency dependence of 50-Ohm coaxial open-circuit reflection standard." IEEE Transactions on Instrumentation and Measurement. IM-30:3. pp 228-229. 1981.

[242] Bussey, H.E. "Dielectric Measurements in a Shielded Open Circuit Coaxial Line." IEEE Transactions on Instrumentation and Measurement. 29:2. 120-124. 1980.

[243] Asami, K. "Characterization of heterogeneous systems by dielectric spectroscopy." Progress in Polymer Science. 27:8. pp 1617-1659. 2002.

[244] Davey, C.L., Kell, D.B. "The influence of electrode polarisation on dielectric spectra, with special reference to capacitive biomass measurements: I. Quantifying the effects on electrode polarisation of factors likely to occur during fermentations." Bioelectrochemistry and Bioenergetics. 46:1. pp 91-103. 1998.

[245] Feldman, Y., Polygalov, E., Ermolina, I., Polevaya, Y., Tsentsiper, B. "Electrode polarization correction in time domain dielectric spectroscopy." Measurement Science and Technology. 12:8. pp 1355-1364.

[246] Bordi, F., Cametti, C., Gili, T. "Dielectric spectroscopy of erythrocyte cell suspensions. A comparison between Looyenga and Maxwell–Wagner–Hanai effective medium theory formulations." Journal of Non-Crystalline Solids. 305:1-3. pp 278-284. 2002.

[247] Asami, K., Yamaguchi, T. "Electrical and Morphological Changes of Human Erythrocytes under High Hydrostatic Pressure Followed by Dielectric Spectroscopy." Annals of Biomedical Engineering. 27:4. pp 427-435. 1999.

[248] Zoltowski, P. "On the electrical capacitance of interfaces exhibiting constant phase element behaviour." Journal of Electroanalytical Chemistry. 443:1. pp 149-154. 1998.

[249] Bordi, F., Cametti, C., Paradossi, G. "Dielectric behavior of polyelectrolyte solutions: the role of proton fluctuation." The Journal of Physical Chemistry. 95:12. pp 4883-4889. 1991.

[250] Bordi, F., Cametti, C., Paradossi, G. "High-frequency dielectric relaxation measurements of side-chain dynamics of branched chitosan derivatives in aqueous solutions." Macromolecules. 26:13. pp 3363-3368. 1993.

[251] Osman, Z., Ibrahim, Z. A., Arof, A. K. "Conductivity enhancement due to ion dissociation in plasticized chitosan based polymer electrolytes." Carbohydrate Polymers. 44:2. pp 167-173. 2001.

[252] Khiar, A. S. A., Puteh, R., Arof, A. K. "Conductivity studies of a chitosan-based polymer electrolyte." Physica B: Condensed Matter. 373:1. pp 23-27. 2006.

[253] Winie, T., Arof, A. "Dielectric behaviour and AC conductivity of LiCF3SO3 doped H-chitosan polymer films." Ionics. 13:3. pp 193-199. 2004.

[254] Nogales, A., Ezquerra, T., Rueda, D., Martinez, F., Retuert, J. "Influence of water on the dielectric behaviour of chitosan films." Colloid & Polymer Science. 275:5. pp 419-425. 1997.

[255] Hanai, T. "Theory of the dielectric dispersion due to the interfacial polarization and its application to emulsions." Colloid & Polymer Science. 171:1. pp 23-31. 1960.

[256] Hanai, T. "A remark on the "Theory of the dielectric dispersion due to the interfacial polarization". " Colloid & Polymer Science. 175:1. pp 61-62. 1961.

[257] Ni, N., Zhao, K. "Dielectric analysis of chitosan gel beads suspensions: Influence of low crosslinking agent concentration on the dielectric behaviour." Journal of Colloid and Interface Science. 312:2. pp 256-264. 2007.

[258] Schwarz, G. "A theory of the low-frequency dielectric dispersion of colloidal particles in electrolyte solution." The Journal of Physical Chemistry. 66:12. pp 2636-2642. 1962.

[259] Schurr, J. M. "On the Theory of the Dielectric Dispersion of Spherical Colloidal Particles in Electrolyte Solution." The Journal of Physical Chemistry. 68:9. pp 2407-2413. 1964.

[260] Zhao, K., Asami, K., Lei, J. "Dielectric analysis of chitosan microsphere suspensions: study on its ion adsorption." Colloid & Polymer Science. 280:11. pp 1038-1044. 2002.

[261] Hanai, T. "Electrical properties of emulsions." *in* Emulsion Science. Ed. Sherman, P. pp 353-478. Academic Press. London. 1968.

[262] Bagnaninchi, P.-O., Dikeakos, M., Veres, T., Tabrizian, M. "Towards on-line monitoring of cell growth in microporous scaffolds: Utilization and interpretation of complex permittivity measurements." Biotechnology and Bioengineering. 84:3. pp 343-350. 2003.

[263] Bagnaninchi, P. O., Dikeakos, M., Veres, T., Tabrizian, M. "Complex permittivity measurement as a new noninvasive tool for monitoring in vitro tissue engineering and cell signature through the detection of cell proliferation, differentiation, and pretissue formation." IEEE Transactions on NanoBioscience. 3:4. pp 243-250. 2004.

[264] Piletsky, S.A., Panasyuk, T.L., Piletskaya, E.V., Nicholls, I.A., Ulbricht, M. "Receptor and transport properties of imprinted polymer membranes – a review." Journal of Membrane Science. 157:2. pp 263-278. 1999.

[265] Horemans, F., Diliën, H., Wagner, P., Cleij, T.J. "Chapter 5 - MIP-based Sensor Platforms for Detection of Analytes in Nano- and Micromolar Range." *in* Molecularly Imprinted Sensors. Ed. Li, S. pp 91-124. Elsevier. Amsterdam. 2012.

[266] Panasyuk-Delaney, T., Mirsky, V.M., Wolfbeis, O.S. "Capacitive Creatinine Sensor Based on a Photografted Molecularly Imprinted Polymer." Electroanalysis. 14:3. pp 221-224. 2002.

[267] Apodaca, D.C., Pernites, R.B., Del Mundo, F.R., Advincula, R.C. "Detection of 2,4-Dinitrotoluene (DNT) as a Model System for Nitroaromatic Compounds via Molecularly Imprinted Short-Alkyl-Chain SAMs." Langmuir. 27:11. pp 6768-6779. 2011.

[268] Apodaca, D.C., Pernites, R.B., Ponnapati, R., Del Mundo, F.R., Advincula, R.C. "Electropolymerized Molecularly Imprinted Polymer Film: EIS Sensing of Bisphenol A." Macromolecules. 44:17. 6669-6682. 2011.

[269] Li, J., Zhang, Z., Xu, S., Chen, L., Zhou, N., Xiong, H., Peng, H. "Label-free colorimetric detection of trace cholesterol based on molecularly imprinted photonic hydrogels." Journal of Materials Chemistry. 21:48. pp 19267-19274. 2011.

[270] Gao, N., Dong, J., Liu, M., Ning, B., Cheng, C., Guo, C., Zhou, C., Peng, Y., Bai, J., Gao, Z. "Development of molecularly imprinted polymer films used for detection of profenofos based on a quartz crystal microbalance sensor." Analyst. 137:5. pp 1252-1258. 2012.

[271] Kan, X., Xing, Z., Zhu, A., Zhao, Z., Xu, G., Li, C., Zhou, H. "Molecularly imprinted polymers based electrochemical sensor for bovine hemoglobin recognition." Sensors and Actuators B: Chemical. 168:0. pp 395-401. 2012.

[272] Peeters, M., Troost, F. J., van Grinsven, B., Horemans, F., Alenus, J., Murib, M. S., Keszthelyi, D., Ethirajan, A., Thoelen, R., Cleij, T. J., Wagner, P. "MIP-based biomimetic sensor for the electronic detection of serotonin in human blood plasma." Sensors and Actuators B: Chemical. 171-172:0. pp 602-610. 2012.

[273] Liu, B., Lian, H.T., Yin, J.F., Sun, X.Y. "Dopamine molecularly imprinted electrochemical sensor based on graphene–chitosan composite." Electrochimica Acta. 75:0. pp 108-114. 2012.

[274] Peeters, M., Troost, F.J., Mingels, R.H.G., Welsch, T., van Grinsven, B., Vranken, T., Ingebrandt, S., Thoelen, R., Cleij, T.J., Wagner, P. "Impedimetric Detection of Histamine in Bowel Fluids Using Synthetic Receptors with pH-Optimized Binding Characteristics." Analytical Chemistry. 85:3. pp 1475-1483. 2012.

[275] Viswanathan, S., Rani, C., Ribeiro, S., Delerue-Matos, C. "Molecular imprinted nanoelectrodes for ultra sensitive detection of ovarian cancer marker." Biosensors and Bioelectronics. 33:1. pp 179-183. 2012.

[276] Cai, D., Ren, L., Zhao, H., Xu, C., Zhang, L., Yu, Y., Wang, H., Lan, Y., Roberts, M.F., Chuang, J.H., Naughton, M.J., Ren, Z., Chiles, T.C. "A molecular-imprint nanosensor for ultrasensitive detection of proteins." Nature Nanotechnology. 5:8. pp 597-601. 2010.

[277] Kochkodan, V., Hilal, N., Melnik, V., Kochkodan, O., Vasilenko, O. "Selective recognition of organic pollutants in aqueous solutions with composite imprinted membranes." Advances in Colloid and Interface Science. 159:2. pp 180-188. 2010.

[278] Morelli, I., Chiono, V., Vozzi, G., Ciardelli, G., Silvestri, D., Giusti, P. "Molecularly imprinted submicronspheres for applications in a novel model biosensor-film." Sensors and Actuators B: Chemical. 150:1. pp 394-401. 2010.

[279] Bayer, C.L., Peppas, N.A. "Recognitive hydrogel comprising molecularly imprinted polymer." US Patent WO2008131123A1. 30 October 2008.

[280] Haupt, K., Linares, A.V., Bompart, M., Tse Sum Bui, B. "Molecularly Imprinted Polymers." *in* Molecular Imprinting. Ed. Haupt. K. *Topics in Current Chemistry*. Vol. 325. pp 1-28. Berlin, DE. 2012.

[281] Fukazawa, K., Ishihara, K. "Fabrication of a cell-adhesive protein imprinting surface with an artificial cell membrane structure for cell capturing." Biosensors and Bioelectronics. 25:3. pp 609-614. 2009.

[282] Wu, X. "Molecular imprinting for anion recognition in aqueous media." Microchimica Acta. 176:1/2. pp 23-47. 2012.

[283] Haginaka, J. "Molecularly imprinted polymers as affinity-based separation media for sample preparation." Journal of Separation Science. 32:10. pp 1548-1565. 2009.

[284] Buszewski, B., Ričanyová, J., Gadzała-Kopciuch, R., Szumski, M. "Supramolecular recognition of estrogens via molecularly imprinted polymers." Analytical and Bioanalytical Chemistry. 397:7. pp 2977-2986. 2010.

[285] Nilsson, J., Spégel, P., Nilsson, S. "Molecularly imprinted polymer formats for capillary electrochromatography." Journal of Chromatography B. 804:1. pp 3-12. 2004.

[286] Trotta, F., Biasizzo, M., Caldera, F. "Molecularly Imprinted Membranes." Membranes. 2:3. pp 440-477. 2012.

[287] Wu, S., Tan, W., Xu, H. "Protein molecularly imprinted polyacrylamide membrane: for hemoglobin sensing." Analyst. 135:10. pp 2523-2527. 2010.

[288] Nicholls, I.A., Rosengren, J.P. "Molecular imprinting of surfaces." Bioseparation. 10:6. pp 301-305. 2001.

[289] Lin, Z., Xia, Z., Zheng, J., Zheng, D., Zhang, L., Yang, H., Chen, G. "Synthesis of uniformly sized molecularly imprinted polymer-coated silica nanoparticles for selective recognition and enrichment of lysozyme." Journal of Materials Chemistry. 22:34. pp 17914-17922. 2012.

[290] Jakusch, M., Janotta, M., Mizaikoff, B., Mosbach, K., Haupt, K. "Molecularly Imprinted Polymers and Infrared Evanescent Wave Spectroscopy. A Chemical Sensors Approach." Analytical Chemistry. 71:20. pp 4786-4791. 1999.

[291] Panasyuk, T.L., Mirsky, V.M., Piletsky, S.A., Wolfbeis, O.S. "Electropolymerized Molecularly Imprinted Polymers as Receptor Layers in Capacitive Chemical Sensors." Analytical Chemistry. 71:20. pp 4609-4613. 1999.

[292] Schweitz, L. "Molecularly Imprinted Polymer Coatings for Open-Tubular Capillary Electrochromatography Prepared by Surface Initiation." Analytical Chemistry. 74:5. pp 1192-1196. 2002.

[293] Rückert, B., Hall, A.J., Sellergren, B. "Molecularly imprinted composite materials via iniferter-modified supports." Journal of Materials Chemistry. 12:8. pp 2275-2280. 2002.

[294] Liang, C., Peng, H., Bao, X., Nie, L., Yao, S. "Study of a molecular imprinting polymer coated BAW bio-mimic sensor and its application to the determination of caffeine in human serum and urine." Analyst. 124:12. pp 1781-1785. 1999.

[295] Schmidt, R.H., Haupt, K. "Molecularly Imprinted Polymer Films with Binding Properties Enhanced by the Reaction-Induced Phase Separation of a Sacrificial Polymeric Porogen." Chemistry of Materials. 17:5. pp 1007-1016. 2005.

[296] Kan, X., Zhao, Y., Geng, Z., Wang, Z., Zhu, J.-J. "Composites of Multiwalled Carbon Nanotubes and Molecularly Imprinted Polymers for Dopamine Recognition." Journal of Physical Chemistry C. 112:13. pp 4849-4854. 2008.

[297] Ouyang, R.Z., Lei, J.P., Ju, H.X. "Surface molecularly imprinted nanowire for protein specific recognition." Chemical Communications. 44. pp 5761-5763. 2008.

[298] Li, Y., Yang, H.-H., You, Q.-H., Zhuang, Z.-X., Wang, X.-R. "Protein Recognition via Surface Molecularly Imprinted Polymer Nanowires." Analytical Chemistry. 78:1. pp 317-320. 2006.

[299] Chou, T.-C., Rick, J., Weng, Y.-C. "Nanocavity protein biosensor - fabricated by molecular imprinting." Nanotechnology, 2007. IEEE-NANO 2007. *Proceedings of the 7th IEEE International Conference on Nanotechnology*. pp 16-20. 2007.

[300] Tovar, G.E.M., Krauter, I., Gruber, C. "Molecularly Imprinted Polymer Nanospheres as Fully Synthetic Affinity Receptors." *in* Topics in Current Chemistry: (Volume 227) Colloid Chemistry II. Ed. Antonietti, M. pp 125-144. Springer-Verlag. Berlin. 2003.

[301] Amsden, B.G., Sukarto, A., Knight, D.K., Shapka, S.N. "Methacrylated glycol chitosan as a photopolymerizable biomaterial." Biomacromolecules. 8:12. pp 3758-3766. 2007.

[302] Sellergren, B., Shea, K.J. "Influence of polymer morphology on the ability of imprinted network polymers to resolve enantiomers." Journal of Chromatography. 635:1. pp 31-49. 1993.

[303] Puoci, F., Iemma, F., Cirillo, G., Curcio, M., Parisi, O.I., Spizzirri, U.G., Picci, N. "New restricted access materials combined to molecularly imprinted polymers for selective recognition/release in water media." European Polymer Journal. 45:6. pp 1634-1640. 2009.

[304] Wang, Y., Zhang, J., Zhu, X.X., Yu, A. "Specific binding of cholic acid by cross-linked polymers prepared by the hybrid imprinting method." Polymer. 48:19. pp 5565-5571. 2007.

[305] Knight, D.K., Shapka, S.N., Amsden, B.G. "Structure, depolymerization, and cytocompatibility evaluation of glycol chitosan." Journal of Biomedical Materials Research. 83(A):3. pp 787-798. 2007.

[306] Allan, G.G., Peyron, M. "Molecular weight manipulation of chitosan I: kinetics of depolymerization by nitrous acid." Carbohydrate Research. 277:2. pp 257-272. 1995.

[307] Hsu, S.-C., Don, T.-M., Chiu, W.-Y. "Synthesis of chitosan-modified poly(methyl methacrylate) by emulsion polymerization." Journal of Applied Polymer Science. 86:12. pp 3047-3056. 2002.

[308] Knight, D.K., Shapka, S.N., Amsden, B.G. "Characterization of glycol chitosan: a potential material for use in biomedical and pharmaceutical applications: a comparison of fractionation techniques." *in* ACS Symposium Series 934 – Polysaccharides for Drug Delivery and Pharmaceutical Applications. Eds. Marchessault, R., Ravenelle, F., Zhu, X.X. pp 227-242. American Chemical Society. Washington, DC. 2006.

[309] Rinaudo, M. "Chitin and chitosan: Properties and applications." Progress in Polymer Science. 31:7. pp 603-632. 2006.

[310] Navarro-Villoslada, F., Vicente, B.S., Moreno-Bondi, M.C. "Application of multivariate analysis to the screening of molecularly imprinted polymers for bisphenol A." Analytica Chimica Acta. 504:1. pp 149-162. 2004.

[311] Jia, X., Li, H., Luo, J., Lu, Q., Peng, Y., Shi, L., Liu, L., Du, S., Zhang, G., Chen, L. "Rational design of core–shell molecularly imprinted polymer based on computational simulation and Doehlert experimental optimization: application to the separation of tanshinone IIA from Salvia miltiorrhiza Bunge." Analytical and Bioanalytical Chemistry. 403:9. pp 2691-2703. 2012.

[312] Rahiminejad, M., Shahtaheri, S.J., Ganjali, M.R., Rahimi Forushani, A., Golbabaei, F. "Molecularly Imprinted Solid Phase Extraction for Trace Analysis of Diazinon in Drinking Water." Iranian Journal of Environmental Health Science & Engineering. 6:2. pp 97-106. 2009.

[313] Schillinger, E., Möder, M., Olsson, G.D., Nicholls, I.A., Sellergren, B. "An Artificial Estrogen Receptor through Combinatorial Imprinting." Chemistry – A European Journal. 18:46. pp 14773-14783. 2012.

[314] Batra, D., Shea, K.J. "Combinatorial methods in molecular imprinting." Current Opinion in Chemical Biology. 7:3. pp 434-442. 2003.

[315] Si, H.Z., Zhang, K.J., Hu, Z.D., Fan, B.T. "QSAR Model for Prediction Capacity Factor of Molecular Imprinting Polymer Based on Gene Expression Programming." QSAR & Combinatorial Science. 26:1. pp 41-50. 2007.

[316] Rossi, C., Haupt, K. "Application of the Doehlert experimental design to molecularly imprinted polymers: surface response optimization of specific template recognition as a function of the type and degree of cross-linking." Analytical & Bioanalytical Chemistry. 382:2. pp 455-460. 2007.

[317] Eppler, S., Schröder, T., Friedle, J., Michl, S., Dangel, W., Mizaikoff, B. "A strategy for high-throughput screening of ligands suitable for molecular imprinting of proteins." Biosensors and Bioelectronics. 35:1. pp 27-32. 2012.

[318] Navarro-Villoslada, F., Takeuchi, T. "Multivariate Analysis and Experimental Design in the Screening of Combinatorial Libraries of Molecular Imprinted Polymers." Bulletin of the Chemical Society of Japan. 78:7. pp 1354-1361. 2005.

[319] Dirion, B., Cobb, Z., Schillinger, E., Andersson, L.I., Sellergren, B. "Water-Compatible Molecularly Imprinted Polymers Obtained via High-Throughput Synthesis and Experimental Design." Journal of the American Chemical Society. 125:49. pp 15101-15109. 2003.

[320] Hillberg, A.L., Brain, K.R., Allender, C.J. "Design and evaluation of thin and flexible theophylline imprinted polymer membrane materials." Journal of Molecular Recognition. 22:3. pp 223-231. 2009.

[321] Spivak, D.A. "Optimization, evaluation, and characterization of molecularly imprinted polymers." Advanced Drug Delivery Reviews. 57:12. pp 1779-1794. 2005.

[322] Levi, L., Srebnik, S. "Simulation of Protein-Imprinted Polymers. 1. Imprinted Pore Properties." Journal of Physical Chemistry. 114:1. pp 107-114. 2010.

[323] Mrksich, M., Dike, L.E., Tien, J., Ingber, D.E., Whitesides, G.M. "Using Microcontact Printing to Pattern the Attachment of Mammalian Cells to Self-Assembled Monolayers of Alkanethiolates on Transparent Films of Gold and Silver." Experimental Cell Research. 235:2. pp 305-313. 1997.

[324] Rozkiewicz, D.I., Kraan, Y., Werten, M.W.T., de Wolf, F.A., Subramaniam, V., Ravoo, B.J., Reinhoudt, D.N. "Covalent Microcontact Printing of Proteins for Cell Patterning." Chemistry – A European Journal. 12:24. pp 6290-6297. 2006.

[325] Gomez, E.W., Nelson, C.M. "Lithographically Defined Two- and Three-Dimensional Tissue Microarrays." Biological Microarrays. 671:1. pp 107-116. 2011.

[326] Raghavan, S., Desai, R.A., Kwon, Y., Mrksich, M., Chen, C.S. "Micropatterned Dynamically Adhesive Substrates for Cell Migration." Langmuir. 26:22. pp 17733-17738. 2010.

[327] Saliba, A.-E., Saias, L., Psychari, E., Minc, N., Simon, D., Bidard, F.-C., Mathiot, C., Pierga, J.-Y., Fraisier, V., Salamero, J., Saada, V., Farace, F., Vielh, P., Malaquin, L., Viovy, J.-L. "Microfluidic sorting and multimodal typing of cancer cells in self-assembled magnetic arrays." Proceedings of the National Academy of Sciences. 107:33. pp 14524-14529. 2010.

[328] Ricoult, S.G., Goldman, J.S., Stellwagen, D., Juncker, D., Kennedy, T.E. "Generation of microisland cultures using microcontact printing to pattern protein substrates." Journal of Neuroscience Methods. 208:1. pp 10-17. 2012.

[329] Kane R.S., Takayama S., Ostuni E., Ingber D.E., Whitesides G.M. "Patterning proteins and cells using soft lithography." Biomaterials. 20:23-24. pp 2363-2376. 1999.

[330] Jacobs, H.O., Whitesides, G.M. "Submicrometer Patterning of Charge in Thin-Film Electrets." Science. 291:5509. pp 1763-1766. 2001.

[331] Renault, J.P., Bernard, A., Bietsch, A., Michel, B., Bosshard, H.R., Delamarche, E., Kreiter, M., Hecht, B., Wild, U.P. "Fabricating Arrays of Single Protein Molecules on Glass Using Microcontact Printing." The Journal of Physical Chemistry B. 107:3. pp 703-711. 2002.

[332] Kaufmann, T., Ravoo, B.J. "Stamps, inks and substrates: polymers in microcontact printing." Polymer Chemistry. 1:4. pp 371-387. 2010.

[333] Moon, J.J., Hahn, M.S., Kim, I., Nsiah, B.A., West, J.L. "Micropatterning of Poly(Ethylene Glycol) Diacrylate Hydrogels with Biomolecules to Regulate and Guide Endothelial Morphogenesis." Tissue Engineering: Part A. 15:3. pp 579-585. 2009.

[334] Park, K.H., Park, H.G., Kim, J.-H., Seong, K.H. "Poly(dimethyl siloxane)-based protein chip for simultaneous detection of multiple samples: Use of glycidyl methacrylate photopolymer for site-specific protein immobilization." Biosensors and Bioelectronics. 22:5. pp 613-620. 2006.

[335] Paik, I., Scurr, D.J., Morris, B., Hall, G., Denning, C., Alexander, M.R., Shakesheff, K.M., Dixon, J.E. "Rapid micropatterning of cell lines and human pluripotent stem cells on elastomeric membranes." Biotechnology and Bioengineering. 109:10. pp 2630-2641. 2012.

[336] Unger, M.A., Chou, H.-P., Thorsen, T., Scherer, A., Quake, S.R. "Monolithic Microfabricated Valves and Pumps by Multilayer Soft Lithography." Science. 288:5463. pp 113-116. 2000.

[337] Whitesides, G., Ostuni, E., Takayama, S., Jiang, X., Ingber, D.E. "Soft Lithography in Biology and Biochemistry." Annual Review of Biomedical Engineering. 3:1. pp 335-373. 2001.

[338] Rogers, J.A., Nuzzo, R.G. "Recent progress in soft lithography." Materials Today. 8:2. pp 50-56. 2005.

[339] Qin, D., Xia, Y., Whitesides, G.M. "Soft lithography for micro- and nanoscale patterning." Nature Protocols. 5:3. pp 491-502. 2010.

[340] Li, H.-W., Muir, B.V.O., Fichet, G., Huck, W.T.S. "Nanocontact Printing: A Route to Sub-50-nm-Scale Chemical and Biological Patterning." Langmuir. 19:6. pp 1963-1965. 2003.

[341] Co, C.C., Wang, Y.-C., Ho, C.-C. "Biocompatible Micropatterning of Two Different Cell Types." Journal of the American Chemical Society. 127:6. pp 1598-1599. 2005.

[342] Elloumi Hannachi, I., Itoga, K., Kumashiro, Y., Kobayashi, J., Yamato, M., Okano, T. "Fabrication of transferable micropatterned-co-cultured cell sheets with microcontact printing." Biomaterials. 30:29. pp 5427-5432. 2009.

[343] Luz, G.M., Boesel, L., Campo, A.d., Mano, J.F. "Micropatterning of Bioactive Glass Nanoparticles on Chitosan Membranes for Spatial Controlled Biomineralization." Langmuir. 28:17. pp 6970-6977. 2012.

[344] Eichinger, C.D., Hsiao, T.W., Hlady, V. "Multiprotein Microcontact Printing with Micrometer Resolution." Langmuir. 28:4. 2238-2243. 2011.

[345] Hui, C. Y., Jagota, A., Lin, Y.Y., Kramer, E.J. "Constraints on Microcontact Printing Imposed by Stamp Deformation." Langmuir. 18:4. pp 1394-1407. 2002.

[346] Sharp, K.G., Blackman, G.S., Glassmaker, N.J., Jagota, A., Hui, C.-Y. "Effect of Stamp Deformation on the Quality of Microcontact Printing: Theory and Experiment." Langmuir. 20:15. pp 6430-6438. 2004.

[347] Xu, H., Huskens, J. "Versatile Stamps in Microcontact Printing: Transferring Inks by Molecular Recognition and from Ink Reservoirs." Chemistry – A European Journal. 16:8. pp 2342-2348. 2010.

[348] Faid, K., Voicu, R., Bani-Yaghoub, M., Tremblay, R., Mealing, G., Py, C., Barjovanu, R. "Rapid Fabrication and Chemical Patterning of Polymer Microstructures and their Applications as a Platform for Cell Cultures." Biomedical Microdevices. 7:3. pp 179-184. 2005.

[349] Moeller, H.-C., Mian, M.K., Shrivastava, S., Chung, B.G., Khademhosseini, A. "A microwell array system for stem cell culture." Biomaterials. 29:6. pp 752-763. 2008.

[350] Dickinson, L.E., Rand, D.R., Tsao, J., Eberle, W., Gerecht, S. "Endothelial cell responses to micropillar substrates of varying dimensions and stiffness." Journal of Biomedical Materials Research Part A. 100A:6. pp 1457-1466. 2012.

[351] Feng, C.L., Vancso, G.J., Schönherr, H. "Reactive μCP on Ultrathin Block Copolymer Films: Investigation of the μCP Mechanism and Application to Sub-μm (Bio)molecular Patterning." Langmuir. 23:3. pp 1131-1140. 2006.

[352] Sharma, A., Gonuguntla, M., Mukherjee, R., Subramanian, S.A., Pangule. R.C. "Self-organized meso-patterning of soft solids by controlled adhesion: elastic contact lithography." Journal of Nanoscience and Nanotechnology. 7:6. pp 1744-1752. 2007.

[353] Yu, H., Xiong, S., Tay, C.Y., Leong, W.S., Tan, L.P. "A novel and simple microcontact printing technique for tacky, soft substrates and/or complex surfaces in soft tissue engineering." Acta Biomaterialia. 8:3. pp 1267-1272. 2012.

[354] Yeh, J., Ling, Y., Karp, J.M., Gantz, J., Chandawarkar, A., Eng, G., Blumling Iii, J., Langer, R., Khademhosseini, A. "Micromolding of shape-controlled, harvestable cell-laden hydrogels." Biomaterials. 27:31. pp 5391-5398. 2006.

[355] Kobel, S., Limacher, M., Gobaa, S., Laroche, T., Lutolf, M.P. "Micropatterning of Hydrogels by Soft Embossing." Langmuir. 25:15. pp 8774-8779. 2009.

[356] Tang, M.D., Golden, A.P., Tien, J. "Molding of Three-Dimensional Microstructures of Gels." Journal of the American Chemical Society. 125:43. pp 12988-12989. 2003.

[357] Karp, J.M., Yeh, J., Eng, G., Fukuda, J., Blumling, J., Kahp-Yang, S., Cheng, J., Mahdavi, A., Borenstein, J., Langer, R., Khademhosseini, A. "Controlling size, shape and homogeneity of embryoid bodies using poly(ethylene glycol) microwells." Lab on a Chip. 7:6. pp 786-794. 2007.

[358] Ruiz, S.A., Chen, C.S. "Microcontact printing: A tool to pattern." Soft Matter. 3:2. pp 167-177. 2007.

[359] Huang, H.C., Lin, C.I., Joseph, A.K., Lee, Y.D. "Photo-lithographically impregnated and molecularly imprinted polymer thin film for biosensor applications." Journal of Chromatography A. 1027:1-2. 263-268. 2004.

[360] Voicu, R., Faid, K., Farah, A.A., Bensebaa, F., Barjovanu, R., Py, C., Tao, Y. "Nanotemplating for Two-Dimensional Molecular Imprinting." Langmuir. 23:10. pp 5452-5458. 2007.

[361] Forchheimer, D., Luo, G., Ye, L., Montelius, L. "Molecularly selective nanopatterns using nanoimprint lithography: A label-free sensor architecture." *Presented at the 54th International Conference on Electron, Ion, and Photon Beam Technology and Nanofabrication Conference, Anchorage, AK, June 1–4, 2010*. Journal of Vacuum Science & Technology B. 29:1. pp 011021-5. 2011.

[362] Liu, Z., Yi, Y., Xu, H., Zhang, X., Ngo, T.H., Smet, M. "Cation-Selective Microcontact Printing Based on Surface-Molecular-Imprinted Layer-by-Layer Films." Advanced Materials. 22:24. pp 2689-2693. 2010.

[363] Alexander, C., Vulfson, E.N. "Spatially functionalized polymer surfaces produced via cell-mediated lithography." Advanced Materials. 9:9. pp 751-755. 1997.

[364] Seidler, K., Lieberzeit, P.A., Dickert, F.L. "Application of yeast imprinting in biotechnology and process control." Analyst. 134:2. pp 361-366. 2009.

[365] Lin, H.-Y., Hsu, C.-Y., Thomas, J.L., Wang, S.-E., Chen, H.-C., Chou, T.-C. "The microcontact imprinting of proteins: The effect of cross-linking monomers for lysozyme, ribonuclease A and myoglobin." Biosensors and Bioelectronics. 22:4. pp 534-543. 2006.

[366] Lin, H.-Y., Rick, J., Chou, T.-C. "Optimizing the formulation of a myoglobin molecularly imprinted thin-film polymer—formed using a micro-contact imprinting method." Biosensors and Bioelectronics. 22:12. pp 3293-3301. 2007.

[367] Lalo, H., Ayela, C., Dague, E., Vieu, C., Haupt, K. "Nanopatterning molecularly imprinted polymers by soft lithography: a hierarchical approach." Lab on a Chip. 10:10. pp 1316-1318. 2010.

[368] Fu, G.-Q., Yu, H., Zhu, J. "Imprinting effect of protein-imprinted polymers composed of chitosan and polyacrylamide: A re-examination." Biomaterials. 19:13. pp 2138-2142. 2008.

[369] Guillon, S., Lemaire, R., Linares, A.V., Haupt, K., Ayela, C. "Single step patterning of molecularly imprinted polymers for large scale fabrication of microbiochips." Lab on a Chip. 9:20. pp 2987-2991. 2009.

[370] Turner, N.W., Jeans, C.W., Brain, K.R., Allender, C.J., Hlady, V., Britt, D.W. "From 2D to 3D: A Review of the Molecular Imprinting of Proteins." Biotechnology Progress. 22:6. pp 1474-1489. 2006.

[371] Liao, H., Zhang, Z., Li, H., Nie, L., Yao, S. "Preparation of the molecularly imprinted polymers-based capacitive sensor specific for tegafur and its characterization by electrochemical impedance and piezoelectric quartz crystal microbalance." Electrochimica Acta. 49:30. pp 4101-4107. 2004.

[372] Tao, Z., Tehan, E.C., Bukowski, R.M., Tang, Y., Shughart, E.L., Holthoff, W.G., Cartwright, A.N., Titus, A.H., Bright, F.V. "Templated xerogels as platforms for biomolecule-less biomolecue sensors." Analytica Chimica Acta. 564:1. pp 59-65. 2006.

[373] Mayes, A.G., Whitcombe, M.J. "Synthetic strategies for the generation of molecularly imprinted organic polymers." Advanced Drug Delivery Reviews. 57:12. pp 1742-1778. 2005.

[374] Chen, Z., Ni, N., Zhao, K. "Real-time monitor on the release of salicylic acid from chitosan gel beads by means of dielectric spectroscopy." Colloid & Polymer Science. 288:12-13. pp1245-1253. 2010.

[375] Cheng, Z., Wang, E., Yang, X. "Capacitive detection of glucose using molecularly imprinted polymers." Biosensors and Bioelectronics. 16:3. pp 179-185. 2001.

[376] Aghaei, A., Milani Hosseini, M.R., Najafi, M. "A novel capacitive biosensor for cholesterol assay that uses an electropolymerized molecularly imprinted polymer." Electrochimica Acta. 55:5. pp 1503-1508. 2010.

[377] Thoelen, R., Vansweevelt, R., Duchateau, J., Horemans, F., D'Haen, J., Lutsen, L., Vanderzande, D., Ameloot, M., vandeVen, M., Cleij, T.J., Wagner, P. "A MIP-based impedimetric sensor for the detection of low-MWQ molecules." Biosensors and Bioelectronics. 23:6. pp 913-918. 2008.

[378] Zhang, Z., Long Y., Nie, L., Yao, S. "Molecularly imprinted thin film self-assembled on piezoelectric quartz crystal surface by the sol–gel process for protein recognition." Biosensors and Bioelectronics. 21:7. pp 1244-1251. 2006.

[379] Panasyuk-Delaney, T., Mirsky, V.M., Ulbricht, M., Wolfbeis, O.S. "Impedometric herbicide chemosensors based on molecularly imprinted polymers." Analytica Chimica Acta. 435:1. pp 157-162. 2001.

[380] BelBruno, J.J., Zhang, G., Gibson, U.J. "Capacitive sensing of amino acids in molecularly imprinted nylon films." Sensors and Actuators. 155:2. pp 915-918. 2011.

[381] Horemans, F., Alenus, J., Bongaers, E., Weustenraed, A., Thoelen, R., Duchateau, J., Lutsen, L., Vanderzande, D., Wagner, P., Cleij, T.J. "MIP-based sensor platforms for the detection of histamine in the nano- and micromolar range in aqueous media." Sensors and Actuators B: Chemical. 148:2. pp 392-398. 2010.

[382] Bongaers, E., Alenus, J., Horemans, F., Weustenraed, A., Lutsen, L., Vanderzande, D., Cleij, T.J., Troost, F.J., Brummer, R.-J., Wagner, P. "A MIP-based biomimetic sensor for the impedimetric detection of histamine in different pH environments." physica status solidi (a). 207:4. pp 837-843. 2010.

[383] Suedee, R., Intakong, W., Dickert, F.L. "Molecularly imprinted polymer-modified electrode for on-line conductometric monitoring of haloacetic acids in chlorinated water." Analytica Chimica Acta. 569:1-2. pp 66-75. 2006.

[384] Birnbaumer, G.M., Lieberzeit, P.A., Richter, L., Schirhagl, R., Milnera, M., Dickert, F.L., Bailey, A., Ertl, P. "Detection of viruses with molecularly imprinted polymers integrated on a microfluidic biochip using contact-less dielectric microsensors." Lab on a Chip. 9:24. pp 3549-3556. 2009.

[385] Broeders, J., Duchateau, S., Van Grinsven, B., Vanaken, W., Peeters, M., Cleij, T., Thoelen, R., Wagner, P., De Cuennick, W. "Miniaturised eight-channel impedance spectroscopy unit as a sensor platform for biosensor applications." physica status solidi (a). 208:6. pp 1357-1363. 2011.

[386] Fang, M., Gao, J.L., Wang, S., Lian, Y.W., Zhao, K.S. "Dielectric monitoring method for the drug release mechanism of drug-loading chitosan microspheres." Chinese Science Bulletin. 55:13. pp 1246-1254. 2010.

[387] Skop, N.B., Calderon, F., Levison, S.W., Gandhi, C.D., Cho, C.H. "Heparin crosslinked chitosan microspheres for the delivery of neural stem cells and growth factors for central nervous system repair." Acta Biomaterialia. 9:6. pp 6834-6843. 2013.

[388] Hirayama, K., Sakai, Y., Kameoka, K. "Synthesis of polymer particles with specific lysozyme recognition sites by a molecular imprinting technique." Journal of Applied Polymer Science. 81:14. pp 3378-3387. 2001.

[389] Kimhi, O., Bianco-Peled, H. "Study of the Interactions between Protein-Imprinted Hydrogels and Their Templates." Langmuir. 23:11. pp 6329-6335. 2007.

[390] Poma, A., Turner, A.P.F., Piletsky, S.A. "Advances in the manufacture of MIP nanoparticles." Trends in Biotechnology. 28:12. pp 629-637. 2010.

[391] Flores, A., Cunliffe, D., Whitcombe, M.J., Vulfson, E.N. "Imprinted polymers prepared by aqueous suspension polymerization." Journal of Applied Polymer Science. 77:8. pp 1841-1850. 2000.

[392] Silvestri, S., Borelli, C., Giusti, P., Cristallini, C., Ciardelli, G. "Polymeric devices containing imprinted nanospheres: a novel approach to improve recognition in water for clinical uses." Analytica Chimica Acta. 542:1. pp 3-13. 2005.

[393] Perez, N., Whitcombe, M.J., Vulfson, E.N. "Molecularly imprinted nanoparticles prepared by core-shell emulsion polymerization." Journal of Applied Polymer Science. 77:8. pp 1851-1859. 2000.

[394] Perez, N., Whitcombe, M.J., Vulfson, E.N. "Surface Imprinting of Cholesterol on Submicrometer Core-Shell Emulsion Particles." Macromolecules. 34:4. pp 830-836. 2001.

[395] Curcio, P., Zandanel, C., Wagner, A., Miskowski, C., Baati, R. "Semi-Covalent Surface Molecular Imprinting of Polymers by One-Sage Mini-emulsion Polymerization: Glucopyranoside as a Model Analyte." Macromolecular Bioscience. 9:6. pp 596-604. 2009.

[396] Heris, H.K., Rahmat, M., Mongeau, L. "Characterization of a Hierarchical Network of Hyaluronic Acid/Gelatin Composite for use as a Smart Injectable Biomaterial." Macromolecular Bioscience. 12:2. pp 202-212. 2012.

[397] Kotrotsiou, O., Chaitidou, S., Kiparissides, C. "On the synthesis of peptide imprinted polymers by a combined suspension-Epitope polymerization method." Materials Science and Engineering: B. 165:3. pp 256-260. 2009.

[398] Koohpei, A.R., Shahtaheri, S.J., Ganjali, M.R., Rahimi Forushani, A., Golbabaei, F. "Application of multivariate analysis to the screening of molecularly imprinted polymers (MIPs) for ametryn." Talanta. 75:4. pp 978-986. 2008.

[399] Kowalska, A., Stobiecka, A., Wysocki, S. "A computational investigation of the interactions between harmane and the functional monomers commonly used in molecular imprinting." Journal of Molecular Structure: THEOCHEM. 901:1-3. pp 88-95. 2009.

[400] Oral, E., Peppas, N.A. "Dynamic studies of molecular imprinting polymerisations." Polymer. 45:18. pp 6163-6173. 2004.

[401] Korytkowska-Wałach, A. "Molecularly imprinted hydrogels for application in aqueous environment." Polymer Bulletin. 70:5. pp 1647-1657. 2013.

[402] Gómez-Pineda, L.E., Pina-Luis, G.E., Cuán, Á., García-Calzón, J.A., Díaz-García, M.E. "Physico-chemical characterization of flavonol molecularly imprinted polymers." Reactive and Functional Polymers. 71:4. pp 402-408. 2011.

[403] Sajonz, P., Kele, M., Zhong, G., Sellergren, B., Guiochon, G. "Study of the thermodynamics and mass transfer kinetics of two enantiomers on a polymeric imprinted stationary phase." Journal of Chromatography A. 810:1-2. pp 1-17. 1998.

[404] Andersson, L.I. "Molecular imprinting for drug bioanalysis: A review on the application of imprinted polymers to solid-phase extraction and binding assay." Journal of Chromatography B: Biomedical Sciences and Applications. 739:1. pp 163-173. 2000.

[405] Umpleby, R.J., Rushton, G.T., Shah, R.N., Rampey, A.M., Bradshaw, J.C., Berch, J.K., Shimizu, K.D. "Recognition Directed Site-Selective Chemical Modification of Molecularly Imprinted Polymers." Macromolecules. 34:24. pp 8446-8452. 2001.

[406] Wei, X., Li, X., Husson, S.M. "Surface Molecular Imprinting by Atom Transfer Radical Polymerization." Biomacromolecules. 6:2. pp 1113-1121. 2005.

[407] Wang, H.-J., Zhou, W.-H., Yin, X.-F., Zhuang, Z.-X., Yang, H.-H., Wang, X.-R. "Template Synthesized Molecularly Imprinted Polymer Nanotube Membranes for Chemical Separations." Journal of the American Chemical Society. 128:50. pp 15954-15955. 2006.

[408] Ban, L., Zhao, L., Deng, B.-L., Huang, Y.-P., Liu, Z.-S. "Preparation and characterization of an imprinted monolith by atom transfer radical polymerization assisted by crowding agents." Analytical and Bioanalytical Chemistry. 405:7. pp 2245-2253. 2013.

[409] Titirici, M.-M., Sellergren, B. "Thin Molecularly Imprinted Polymer Films via Reversible Addition−Fragmentation Chain Transfer Polymerization." Chemistry of Materials. 18:7. pp 1773-1779. 2006.

[410] Li, Y., Zhou, W.-H., Yang, H.-H., Wang, X.-R. "Grafting of molecularly imprinted polymers from the surface of silica gel particles via reversible addition-fragmentation chain transfer polymerization: A selective sorbent for theophylline." Talanta. 79:2. pp 141-145. 2009.

[411] Pan, G., Ma, Y., Zhang, Y., Guo, X., Li, C., Zhang, H. "Controlled synthesis of water-compatible molecularly imprinted polymer microspheres with ultrathin hydrophilic polymer shells via surface-initiated reversible addition-fragmentation chain transfer polymerization." Soft Matter. 7:18. pp 8428-8439. 2011.

[412] Zhao, N., Chen, C., Zhou, J. "Surface plasmon resonance detection of ametryn using a molecularly imprinted sensing film prepared by surface-initiated atom transfer radical polymerization." Sensors and Actuators B: Chemical. 166-167:0. pp 473-479. 2012.

[413] Madhuri, R., Tiwari, M.P., Kumar, D., Mukharji, A., Prasad, B.B. "Biomimetic piezoelectric quartz sensor for folic acid based on a molecular imprinting technology." Advanced Materials Letters. 2:4. pp 264-267. 2011.

[414] Gai, Q.-Q., Qu, F., Liu, Z.-J., Dai, R.-J., Zhang, Y.-K. "Superparamagnetic lysozyme surface-imprinted polymer prepared by atom transfer radical polymerization and its application for protein separation." Journal of Chromatography A. 1217:31. pp 5035-5042. 2010.

[415] Halhalli, M.R., Aureliano, C.S.A., Schillinger, E., Sulitzky, C., Titirici, M.M., Sellergren, B. "An improved grafting technique for producing imprinted thin film composite beads." Polymer Chemistry. 3:4. pp 1033-1042. 2012.

[416] Zu, B., Pan, G., Guo, X., Zhang, Y., Zhang, H. "Preparation of molecularly imprinted polymer microspheres via atom transfer radical precipitation polymerization." Journal of Polymer Science Part A: Polymer Chemistry. 47:13. pp 3257-3270. 2009.

[417] Pan, G., Zhang, Y., Ma, Y., Li, C., Zhang, H. "Efficient One-Pot Synthesis of Water-Compatible Molecularly Imprinted Polymer Microspheres by Facile RAFT Precipitation Polymerization." Angewandte Chemie International Edition. 50:49. pp 11731-11734. 2011.

[418] Fang, L., Chen, S., Guo, X., Zhang, Y., Zhang, H. "Azobenzene-Containing Molecularly Imprinted Polymer Microspheres with Photo- and Thermoresponsive Template Binding Properties in Pure Aqueous Media by Atom Transfer Radical Polymerization." Langmuir. 28:25. pp 9767-9777. 2012.

[419] Ma, Y., Zhang, Y., Zhao, M., Guo, X., Zhang, H. "Efficient synthesis of narrowly dispersed molecularly imprinted polymer microspheres with multiple stimuli-responsive template binding properties in aqueous media." Chemical Communications. 48:50. pp 6217-6219. 2012.

[420] Yin, J., Cui, Y., Yang, G., Wang, H. "Molecularly imprinted nanotubes for enantioselective drug delivery and controlled release." Chemical Communications. 46:41. pp 7688-7690. 2010.

[421] Chang, L., Li, Y., Chu, J., Qi, J., Li, X. "Preparation of core-shell molecularly imprinted polymer via the combination of reversible addition-fragmentation chain transfer polymerization and click reaction." Analytica Chimica Acta. 680:1-2. pp 65-71. 2010.

[422] Xu, S., Li, J., Chen, L. "Molecularly imprinted polymers by reversible addition–fragmentation chain transfer precipitation polymerization for preconcentration of atrazine in food matrices." Talanta. 85:1. pp 282-289. 2011.

[423] Kan, X., Zhou, H., Li, C., Zhu, A., Xing, Z., Zhao, Z. "Imprinted electrochemical sensor for dopamine recognition and determination based on a carbon nanotube/polypyrrole film." Electrochimica Acta. 63:0. pp 69-75. 2012.

[424] Choi, S.-W., Chang, H.-J., Lee, N., Chun, H.S. "A Surface Plasmon Resonance Sensor for the Detection of Deoxynivalenol Using a Molecularly Imprinted Polymer." Sensors. 11:9. pp 8654-8664. 2011.

[425] Syritski, V., Reut, J., Menaker, A., Gyurcsányi, R.E., Öpik, A. "Electrosynthesized molecularly imprinted polypyrrole films for enantioselective recognition of l-aspartic acid." Electrochimica Acta. 53:6. pp 2729-2736. 2008.

[426] Yu, J.C.C., Lai, E.P.C. "Molecularly imprinted polypyrrole modified carbon nanotubes on stainless steel frit for selective micro solid phase pre-concentration of ochratoxin A." Reactive and Functional Polymers. 66:7. pp 702-711. 2006.

[427] Maouche, N., Guergouri, M., Gam-Derouich, S., Jouini, M., Nessark, B., Chehimi, M.M. "Molecularly imprinted polypyrrole films: Some key parameters for electrochemical picomolar detection of dopamine." Journal of Electroanalytical Chemistry. 685:0. pp 21-27. 2012.

[428] Yang, H-H., Zhang, S.-Q., Tan, F., Zhuang, Z.-X., Wang, X.-R. "Surface Molecularly Imprinted Nanowires for Biorecognition." Surface Molecularly Imprinted Nanowires for Biorecognition. 127:5. pp 1378-1379. 2005.

[429] Tokonami, S., Shiigi, H., Nagaoka, T. "Chapter 4 - Molecularly Imprinted Overoxidized Polypyrrole Films for Sensor Applications from Enantiorecognition to Trace Analysis." *in* Molecularly Imprinted Sensors. Eds. Li, S., Ge, Y., Piletsky, S.A., Lunec, J. pp 73-89. Elsevier. Amsterdam, NL. 2012.

[430] Singh, W.P., Giletto, A., Gaskin, D., Fyffe, J. "Imprinting large molecular weight compounds in polymer composites." United States Patent # 6,582,971. United States Patent and Trademark Office. June 24, 2003.

[431] Singh, W.P., Giletto, A., Gaskin, D., Fyffe, J. "Imprinting large molecular weight compounds in polymer composites." United States Patent Application # 20030319828. United States Patent and Trademark Office. November 27, 2003.

[432] Hardman, K.D., Ainsworth, C.F. "Structure of concanavalin A at 2.4-Ang resolution." Biochemistry. 11:26. pp 4910-4919. 1972.

[433] McKenzie, G. H., Sawyer, W. H., Nichol, L. W. "The molecular weight and stability of concanavalin A." Biochimica et Biophysica Acta. 263:2. pp 283-293. 1972.

[434] Sumner, J.B., Gralën, N., Eriksson-Quensel, I.-B. "The Molecular Weights of Urease, Canavalin, Concanavalin A and Concanavalin B." Science. 87:2261. pp 395-396. 1938.

[435] Entlicher, G., Koštíř, J. V., Kocourek, J. "Studies on phytohemagglutinins. VIII. Isoelectric point and multiplicity of purified concanavalin A." Biochimica et Biophysica Acta. 236:3. pp 795-797. 1971.

[436] Goldstein I.J., Poretz R.D. "Isolation, physicochemical characterization, and carbohydrate-binding specificity of lectins." *in* The Lectins. Properties, Functions, and Applications in Biology and Medicine. Eds. Liener, I.E., Sharon, N., Goldstein, I.J. pp 233-247. Orlando, Fla. 1986.

[437] Saleemuddin, M., Husain, Q. "Concanavalin A: A useful ligand for glycoenzyme immobilization—A review." Enzyme and Microbial Technology. 13:4. pp 290-295. 1991.

[438] Zand, R., Agrawal, B.B.L., Goldstein, I.J. "pH-Dependent Conformational Changes of Concanavalin A." Proceedings of the National Academy of Sciences. 68:9. pp 2173-2176. 1971.

[439] Zheng, H.F., Du, X.Z. "Enhanced Binding and Biosensing of Carbohydrate-Functionalized Monolayers to Target Proteins by Surface Molecular Imprinting."Journal of Physical Chemistry B. 113:32. pp 11330-11337. 2009.

[440] Rueda, C., Arias, C., Galera, P., López-Cabarcos, E., Yagüe, A. "Mucopolysaccharides in aqueous solutions: effect of ionic strength on titration curves." Il Farmaco. 56:5-7. pp 527-532. 2001.

[441] Piai, J.F., Rubira, A.F., Muniz, E.C. "Self-assembly of a swollen chitosan/chondroitin sulfate hydrogel by outward diffusion of the chondroitin sulfate chains." Acta Biomaterialia. 5:7. pp 2601-2609. 2009.

[442] Maculotti, K., Tira, E.M., Sonaggere, M., Perugini, P., Conti, B., Modena, T., Pavanetto, F. "In vitro evaluation of chondroitin sulphate-chitosan microspheres as carrier for the delivery of proteins." Journal of Microencapsulation. 26:6. pp 535-543. 2009.

[443] Hou, Y., Hu, J., Park, H., Lee, M. "Chitosan-based nanoparticles as a sustained protein release carrier for tissue engineering applications." Journal of Biomedical Materials Research Part A. 100A:4. pp 939-947. 2012.

[444] Yuan, N.-Y., Tsai, R.-Y., Ho, M.-H., Wang, D.-M., Lai, J.-Y., Hsieh, H.-J. "Fabrication and characterization of chondroitin sulfate-modified chitosan membranes for biomedical applications." Desalination. 234:1-3. pp 166-174. 2008.

[445] Mi, F.-L., Shyu, S.-S., Peng, C.-K., Wu, Y.-B., Sung, H.-W., Wang, P.-S., Huang, C.-C. "Fabrication of chondroitin sulfate-chitosan composite artificial extracellular matrix for stabilization of fibroblast growth factor." Journal of Biomedical Materials Research Part A. 76A:1. pp 1-15. 2006.

[446] Kofuji, K., Akamine, H., Oshirabe, H., Maeda, Y., Murata, Y., Kawashima, S. "Retention and release behavior of insulin in chitosan gel beads." Journal of Biomaterials Science - Polymer Edition. 14:11. pp 1243-1253. 2003.

[447] Takada, W., Fukushima, M., Pothacharoen, P., Kongtawelert, P., Sugahara, K. "A sulfated glycosaminoglycan array for molecular interactions between glycosaminoglycans and growth factors or anti-glycosaminoglycan antibodies." Analytical Biochemistry. 435:2. pp 123-130. 2013.

[448] Nishimoto, S., Takagi, M., Wakitani, S., Nihira, T., Yoshida, T. "Effect of chondroitin sulfate and hyaluronic acid on gene expression in a three-dimensional culture of chondrocytes." Journal of Bioscience and Bioengineering. 100:1. pp 123-126. 2005.

[449] Wu, C.-H., Ko, C.-S., Huang, J.-W. "Effects of exogenous glycosaminoglycans on human chondrocytes cultivated on type II collagen scaffolds." Journal of Materials Science: Materials in Medicine. 21:2. pp 725-729. 2010.

[450] Chen, W.-C., Yao, C.-L., Chu, I.M., Wei, Y.-H. "Compare the effects of chondrogenesis by culture of human mesenchymal stem cells with various type of the chondroitin sulfate C." Journal of Bioscience and Bioengineering. 111:2. pp 226-231. 2011.

[451] Singabraya, D., Bultel, L., Siñeriz, F., Mothéré, M., Lesur, D., Kovensky, J., Papy-Garcia, D. "Molecular imprinting technology for specific recognition of heparan sulfate like disaccharides." Talanta. 99:0. pp 833-839. 2012.

[452] Baeurle, S.A., Kiselev, M.G., Makarova, E.S., Nogovitsin, E.A. "Effect of the counterion behavior on the frictional–compressive properties of chondroitin sulfate solutions." Polymer. 50:7. pp 1805-1813. 2009.

[453] Muzzarelli, R.A.A., Greco, F., Busilacchi, A., Sollazzo, V., Gigante, A. "Chitosan, hyaluronan and chondroitin sulfate in tissue engineering for cartilage regeneration: A review." Carbohydrate Polymers. 89:3. pp 723-739. 2012.

[454] Wu, W., Shen, J., Li, Y., Zhu, H., Banerjee, P., Zhou, S. "Specific glucose-to-SPR signal transduction at physiological pH by molecularly imprinted responsive hybrid microgels." Biomaterials. 33:29. pp 7115-7125. 2012.

[455] Muniz, E.C., Lopes, L.C., Piai, J.F., Fajardo, A.R., Rubira, A.F. "Incorporation of theophylline in a hydrogel based on polyelectrolyte complex of chitosan/chondroitin sulfate: mechanical properties and release profiles at different pH conditions." Transactions of the Annual Meeting of the Society for Biomaterials. 32. *Annual Meeting of the Society for Biomaterials: Giving Life to a World of Materials, vol 2*. pp 647. 2010.

[456] Altgärde, N. "Designing biosensor platforms to study glycosaminoglycan Interactions." *Dissertation*. Department of Applied Physics. Chalmers University of Technology. Göteborg, Sweden. 2012.

[457] Altgärde, N., Becher, J., Möller, S., Weber, F.E., Schnabelrauch, M., Svedhem, S. "Immobilization of chondroitin sulfate to lipid membranes and its interactions with ECM proteins." Journal of Colloid and Interface Science. 390:1. pp 258-266. 2013.

[458] Cheng, Yi., Luo, X., Payne, G.F., Rubloff, G.W. "Biofabrication: programmable assembly of polysaccharide hydrogels in microfluidics as biocompatible scaffolds." Journal of Materials Chemistry. 22:16. pp 7659-7666. 2012.

[459] Dertinger, S.K.W., Chiu, D.T., Jeon, N.L., Whitesides, G.M. "Generation of Gradients Having Complex Shapes Using Microfluidic Networks." Analytical Chemistry. 73:6. pp 1240-1246. 2001.

[460] Chung, B.G., Choo, J. "Microfluidic gradient platforms for controlling cellular behaviour." Electrophoresis. 31:18. pp 3014-3027. 2010.

[461] Kim, S., Kim, H.J., Jeon, N.L. "Biological applications of microfluidic gradient devices." Integrative Biology. 2:11-12. pp 584-603. 2010.

[462] Didar, T.F., Tabrizian, M. "Generating multiplex gradients of biomolecules for controlling cellular adhesion in parallel microfluidic channels." Lab on a Chip. 12:21. pp 4363-4371. 2012.

[463] Zaari, N., Rajagopalan, P., Kim, S.K., Engler, A.J., Wong, J. Y. "Photopolymerization in Microfluidic Gradient Generators: Microscale Control of Substrate Compliance to Manipulate Cell Response." Advanced Materials. 16:23-24. pp 2133-2137. 2004.

[464] Sheppard, N.F., Tucker, R.C., Wu, C. "Electrical conductivity measurements using microfabricated interdigitated electrodes." Analytical Chemistry. 65:9. pp 1199-1202. 1993.

[465] Janata, J. "Immunoelectrode." Journal of the American Chemical Society. 97:10. pp 2914-2916. 1975.

[466] Aizawa, M., Morioka, A., Matsuoka, H., Suzuki, S., Nagamura, Y., Shinohara, R., Ishiguro, I. "An enzyme immunosensor for IgG." Applied Biochemistry and Biotechnology. 1:4. pp 319-328. 1976.

[467] Taylor, R., F., Marenchic, I.G., Spencer, R.H. "Antibody- and receptor-based biosensors for detection and process control." Analytica Chimica Acta. 249:1. pp 67-70. 1991.

[468] Fang, X., Tan, Ooi K., Tse, M.S., Ooi, E.E. "A label-free immunosensor for diagnosis of dengue infection with simple electrical measurements." Biosensors and Bioelectronics. 25:5. pp 1137-1142. 2010.

[469] Berdat, D., Martin Rodriguez, A.C., Herrera, F., Gijs, M.A.M. "Label-free detection of DNA with interdigitated micro-electrodes in a fluidic cell." Lab on a Chip. 8:2. pp 302-308. 2008.

[470] de la Rica, R., Baldi, A., Fernández-Sánchez, C., Matsui, H. "Selective Detection of Live Pathogens via Surface-Confined Electric Field Perturbation on Interdigitated Silicon Transducers." Analytical Chemistry. 81:10. pp 3830-3835. 2009.

[471] Suehiro, J., Ohtsubo, A., Hatano, T., Hara, M. "Selective detection of bacteria by a dielectrophoretic impedance measurement method using an antibody-immobilized electrode chip." Sensors and Actuators B: Chemical. 119:1. pp 319-326. 2006.

[472] Nam, J., Lim, H., Kim, D., Shin, S. "Separation of platelets from whole blood using standing surface acoustic waves in a microchannel." Lab on a Chip. 11:19. 3361-3364. 2011.

[473] Wang, L., Lu, J., Marchenko, S.A., Monuki, E.S., Flanagan, L.A., Lee, A.P. "Dual frequency dielectrophoresis with interdigitated sidewall electrodes for microfluidic flow-through separation of beads and cells." Electrophoresis. 30:5. pp 782-791. 2009.

[474] Huang, C.-C., Bazant, M.Z., Thorsen, T. "Ultrafast high-pressure AC electro-osmotic pumps for portable biomedical microfluidics." Lab on a Chip. 10:1. pp 80-85. 2010.

[475] Yamamoto, M., Yasukawa, T., Suzuki, M., Kosuge, S., Shiku, H., Matsue, T., Mizutani, F. "Patterning with particles using three-dimensional interdigitated array electrodes with negative dielectrophoresis and its application to simple immunosensing." Electrochimica Acta. 82:0. pp 35-42. 2012.

[476] Ino, K., Saito, W., Koide, M., Umemura, T., Shiku, H., Matsue, T. "Addressable electrode array device with IDA electrodes for high-throughput detection." Lab on a Chip.11:3. pp 385-388. 2011.

[477] Lee, J., Choi, Y.-S., Lee, Y., Lee, H.J., Lee, J.N., Kim, S.K., Han, K.Y., Cho, E.C., Park, J.C., Lee, S.S. "Sensitive and Simultaneous Detection of Cardiac Markers in Human Serum Using Surface Acoustic Wave Immunosensor." Analytical Chemistry. 83:22. pp 8629-8635. 2011.

[478] Singh, K.V., Bhura, D.K., Nandamuri, G., Whited, A.M., Evans, D., King, J., Solanki, R. "Nanoparticle-Enhanced Sensitivity of a Nanogap-Interdigitated Electrode Array Impedimetric Biosensor." Langmuir. 27:22. pp 13931-13939. 2011.

[479] Pini, N., Busato, S., Elsener, H.-R., Ermanni, P. "In situ growth of interdigitated electrodes made of polypyrrole for active fiber composites." Polymers for Advanced Technologies. 18:3. pp 249-253. 2007.

[480] Norman, L.L., Badia, A. "Electrochemical Surface Plasmon Resonance Investigation of Dodecyl Sulfate Adsorption to Electroactive Self-Assembled Monolayers via Ion-Pairing Interactions." Langmuir. 23:20. pp 10198-10208. 2007.

[481] Zou, Z., Kai, J., Rust, M.J., Han, J., Ahn, C.H. "Functionalized nano interdigitated electrodes arrays on polymer with integrated microfluidics for direct bio-affinity sensing using impedimetric measurement." Sensors and Actuators A: Physical. 136:2. pp 518-526. 2007.

[482] Rana, S., Page, R.H., McNeil, C.J. "Comparison of Planar and 3-D Interdigitated Electrodes as Electrochemical Impedance Biosensors." Electroanalysis. 23:10. pp 2485-2490. 2011.

[483] Guiseppi-Elie, A., Lesho, M.J., Sheppard, N.F., Jr. "Electrical impedance properties of chemically responsive hydrogels." *in* Electrical and Optical Polymer Systems: Fundamentals: Methods, and Applications. Eds. Wise, D.L., Wnek, G.E., Trantolo, D.J., Cooper, T.M., Gresser, J.D. *Plastics Engineering*. Vol. 45. pp 1187-1211. Marcel Dekker, Inc. New York. 1998.

[484] Sridharamurthy, S.S., Agarwal, A.K., Beebe, D.J., Jiang, H. "Dissolvable membranes as sensing elements for microfluidics based biological/chemical sensors." Lab on a Chip. 6:7. pp 840-842. 2006.

[485] Yan, J., Pedrosa, V.A., Simonian, A.L., Revzin, A. "Immobilizing Enzymes onto Electrode Arrays by Hydrogel Photolithography to Fabricate Multi-Analyte Electrochemical Biosensors." ACS Applied Materials & Interfaces. 2:3. pp 748-755. 2010.

[486] Yang, L., Guiseppi-Wilson, A., Guiseppi-Elie, A. "Design considerations in the use of interdigitated microsensor electrode arrays (IMEs) for impedimetric characterization of biomimetic hydrogels." Biomedical Microdevices." 13:2. pp 279-289. 2011.

[487] Junya, S., Ryuichi, Y., Ryo, H., Masanori, H. "Quantitative estimation of biological cell concentration suspended in aqueous medium by using dielectrophoretic impedance measurement method." Journal of Physics D: Applied Physics. 32:21. pp 2814-2820. 1999.

[488] Patel, K.S., Kohl, P.A., Bidstrup Allen, S.A. "Dual capacitor technique for measurement of through-plane modulus of thin polymer films." Journal of Polymer Science Part B: Polymer Physics. 38:12. pp 1634-1644. 2000.

APPENDICES

Appendix A: Quantification of Binding to MIPs

For further development of the chitosan MIP as a potential biosensor, as well as means of quantifying the binding of target molecule to the hydrogel, several techniques were investigated to exploit advanced detection technologies. It was hoped that studies of these methods would provide a three-fold benefit to the research project as a whole. Firstly and foremost, they would allow measurement of any recognition effect seen in the chitosan MIPs, to verify success of molecular imprinting. Secondly, they would demonstrate the ability of a chitosan MIP to act as a biosensor for model bimolecular targets. Finally, successful confirmation of MIP specificity via these methods would allow the eventual development of a chitosan MIP component to be incorporated into a greater in-line biodetection and assay system.

A 1.1 Quartz Crystal Microbalance

Quartz crystal microbalance (QCM) is a sensitive analytical technique which uses the change in frequency resonance of a crystal surface to determine localised mass changes. Exploiting the piezoelectric effect for microgravimetric sensing, QCM has become a standard application for sensing applications, including many commercial processes.[i, ii] The principle is based on the application of an electrical current to a crystal (in this case a thin surface). When this happens, the electric potential induces mechanical stress and results in an oscillating wave within the crystal lattice. In QCM, this shear wave propagates in the direction perpendicular to the surface. Key to this principle is attaining resonance oscillation, which depends on the fundamental frequency of the crystal. This, in turn, is affected by the thickness of the wafer, its chemical structure, shape, and mass. In identical quartz crystals, the thickness, density, and shear modulus will remain constant, thus leaving unique changes in the oscillation frequency affected by the physical properties of the adjacent media (density and viscosity). By applying the

Sauerbrey equation, the changes in resonant frequency are related to mass accumulated on the crystal surface:[iii]

$$\Delta f = -\frac{2 f_o^{\,2}}{A\sqrt{\rho_q \mu_q}} \Delta m \qquad \textbf{[A-1]}$$

where Δf is the frequency change, f_o is the resonant frequency, A is the piezoelectrically active crystal surface area, ρ_q is the density of the quartz crystal, μ_q is the shear modulus of quartz, and Δm is the mass change. As this relationship was initially developed for air, such analysis can be simplified to:

$$\Delta f \propto K \Delta m \qquad \textbf{[A-2]}$$

However, in most biological cases, the analytes of concern are not solely in air or in solid masses on the surface. When liquid solvents are used, the coupling of the crystal surface to the liquid occurs as the quartz is in oscillating contact with it, generating plane-laminar flow in the liquid and dramatically changing the frequency by a decrease proportional to the liquid's density and viscosity:[ii]

$$\Delta f = f_o^{3/2}\left(\frac{\rho\eta}{\pi\eta_q\rho_q}\right)^{1/2} \qquad \textbf{[A-3]}$$

where ρ and η and the density and viscosity of the contacting liquid, respectively. Film thickness also plays a key role. For thinner films, the resonance frequency is generally inversely proportional to the total plate thickness. Deposition of monolayers can reach an ideal sensitivity of measurement. However, when thicker coatings are employed, the viscoelastic effects of the damping these cause against the oscillations must be accounted for.[iv]

For advanced QCM instruments, both the resonance frequency (f_r) and bandwidth (w) are available for analysis. The latter is used as a measurement of damping by the holder and ohmic loss inside the crystal. This is especially important in the analysis of non-rigid films, where the Sauerbrey equation does

not hold, as the adsorbed layer will not be truly coupled to the oscillation of the surface (rendering the relation invalid). Thus we can define a damping, or dissipation factor, that relates to the film's softness:[v]

$$D = \frac{E_{lost}}{2\pi E_{stored}} \qquad \textbf{[A-4]}$$

where D is the dissipation, E_{lost} is energy lost during one oscillation cycle, and E_{stored} is total energy stored in the oscillator. The dissipation is inversely related to the quality factor, Q, which is in turn expressible as a function of the bandwidth:

$$D = Q^{-1} = \frac{w}{f_r} \qquad \textbf{[A-5]}$$

Methods of measuring the exponential decay of the oscillations in the quartz crystal, also called 'ring-down,' were first proposed by Sittel *et al.*[vi] Later descriptions by Hirao *et al.*[vii] and Rodahl *et al.*[viii] applied this theory to QCM instrumentation. While certain QCM units determine the bandwidth from the conductance spectra, currently, QCM with dissipation monitoring, *QCM-D*, is only available via instruments from Q-sense, a division of Biolin Scientific AB.

As a sensitive mass sensor, QCM-D is highly effective at determining real-time deposition behaviour. This makes it a key tool for studying adsorption/desorption behaviour, and allowing real-time observation of binding kinetics on various surfaces. While these changes are most effectively measured under vacuum, researchers determined that the damped nature of liquids could be accounted for to overcome this phase barrier,[ix, x] and the principle has recently been exploited for determining biomolecule affinity and protein binding on surfaces.[xi, xii] Thus, in addition to being greatly beneficial in the fields of materials science and biophysics, QCM-D is useful in the studies of biomaterials, cell adhesion, and drug discovery. The principle for study of molecular interactions with surfaces (interfacial) as well as interactions between molecules, the

parameter sensitivity, and its label-free and real-time operation, make it an ideal method for evaluating binding of templates at imprint recognition sites.

As a very common technique for measuring slight changes in chemical bonding, the combination of QCM with molecular imprinting has been exploited for chemosensors.[xiii] The potential of this method, exploiting the solution-surface interface with imprinted polymers, was theorised early on by some of the pioneers in the field.[xiv] An early cursory report by Malitesta *et al*. described a glucose sensor based on an electropolymermised MIP.[xv] This was closely followed by Haupt *et al*. using more traditional MIP chemistry.[xvi] Friggeri *et al*. were able to demonstrate preferential binding of target to imprinted surfaces via QCM, and selectivity between the structurally similar *D*-glucose and *D*-fructose.[xvii] Further enantioselectivity has been reported via this method by Liu *et al*.[xviii]

Whereas the surface used for QCM analysis is the relatively inert quartz, and may often be coated by gold, the measurement chips remain resilient to alterable polymerisation methods. QCM sensors can incorporate the MIP surface through dip-casting,[xvii] polyelectrolyte layer-by-layer build-up,[xix] radical initiation,[xx] high temperature curing,[xxi] photocrosslinking,[xxii] or electropolymerisation,[xxiii] without serious concern to affecting the underlying surface. It has also been shown that nanoparticles, which represent a growing field of interest, including within the imprinting field, can be effectively fixed to the microbalance surfaces. Reimhult *et al*. prepared a chirally selective TRIM/MAA MIP through precipitation polymerisation, which demonstrated high fidelity when fixed in a practical fashion using an inert polymer layer.[xxiv] Recent growth in the research continues to expand the number of applications incorporating MIP detection with microgravimetry.[xxv]

QCM has been shown to be a useful tool in evaluating larger biomolecules as well.[xxvi, xxvii] In 1999, an important paper by Kugimiya and Takeuchi recognised the great promise of developing a synthetic polymer receptor on QCM

towards label-free biodetection.[xxviii] It has been especially exploited for monitoring imprinting studies by Hayden and Dickert,[xxix, xxx] most notably in imprinting studies for the recognition of entire yeast cells, and viruses.[xxxi, xxxii] Similar 'stamping' methods of molecular imprinting have been applied using dual channel instruments for detection of viruses in aqueous buffer, which allows simultaneous examination of NIP and MIP surfaces, and wherein non-specific effects can be accounted for (e.g. temperature, viscosity, or non-selective adhesion).[xxxiii, xxxiv] Lin *et al*. have shown that a QCM sensor towards albumin showed a capable binding selectively towards the target in human serum samples, but notably this study reflects the importance of the crosslinker being used to form the MIP, and the effect of surface functionalisations.[xxxv] Generally these studies explore rebinding of the template through conventional non-covalent interactions, although semi-covalent[xxxvi] and metal-complexation ligand exchange[xxxvii] have also been investigated.

A 1.2 Surface Plasmon Resonance

In recent decades, optical methods for detection have played a key role in numerous chemosensors and biosensors. While first developed in the 1970s for measurements of CO_2 and oxygen levels,[xxxviii] new methodologies soon emerged, including spectroscopy (luminescence, phosphorescence, fluorescence, Raman), ellipsometry, interferometry (white light and modal interferometry in optical waveguide structures), spectroscopy of guided modes in optical waveguide structures (grating coupler, resonant mirror), and surface plasmon resonance.[xxxix, xl] Of these, the latter is especially of interest to scientists for its sensitivity and versatility across a varied range of analyses. While the phenomenon of excited surface plasma waves leading to anomalous diffraction on surface gratings was first observed at the turn of the century,[xli] it was not until much later, in 1968, that the manipulation of this phenomenon for measurements of attenuated total reflection was reported.[xlii, xliii] It quickly became evident that manipulation of SPR

properties could be used to evaluate processes at the interface of metals,[xliv] and for characterising thin films.[xlv]

When focused light is bombarded on a surface (e.g. metal film), the photons can interact with delocalised electrons, creating a plasmon. The principle behind this relies on total internal reflection. When the light beam passes through a medium of high refractive index (e.g. glass) and meets a medium of lower refractive index (e.g. dielectric), if the incident angle of the light is above a certain critical angle, the wave will not pass out of the material, and the light will be totally reflected back into the highly refractive medium.

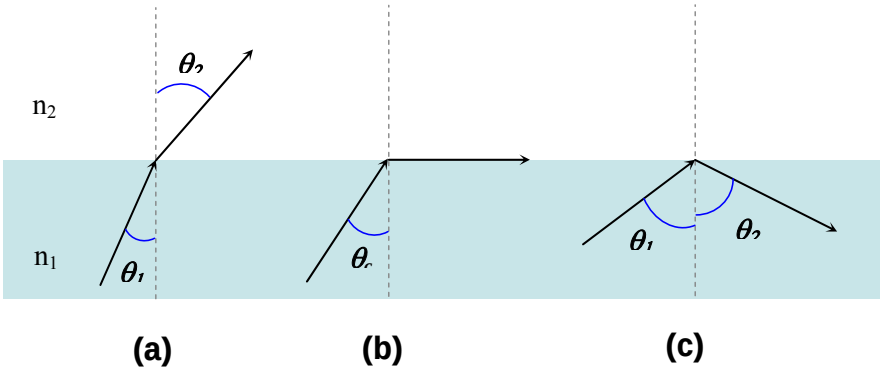


Figure A-1. Principles of the refraction of light at the interface between two different mediums. Illustration of (a) refraction of incident ray, (b) critical angle, and (c) total internal reflection.

The critical angle is given by Snell's Law:

$$n_1 \sin\theta_i = n_2 \sin\theta_t \qquad \textbf{[A-6]}$$

where n_1 is the refractive index for the incident material, n_2 is the refractive index for the secondary (transmitted) material, θ_i is the angle of incidence, and θ_t is the angle of transmission. For total internal reflection (TIR), the condition necessitates an angle of refraction at 90°, therefore the above relation simplifies to:

$$\theta_c = \theta_i = \arcsin\left(\frac{n_1}{n_2}\right) \qquad \textbf{[A-7]}$$

Upon TIR, there is no net loss of energy of the reflected light beam, however it does create an electrical field intensity (referred to as evanescent field wave) into the lower refractive dielectric. The amplitude of the evanescent wave will decrease exponentially away from its starting point, generally decaying in one light wavelength from the surface. If, however, a suitable conducting material is present, the polarised component may excite this layer, and the electromagnetic wave may continue to propagate along the low-refractive medium.[xlvi]

Surface plasmon resonance (SPR) refers to the oscillation of charge density (e.g. valence electrons) at the interface of two dissimilar materials caused by bombardment with incident light. This oscillation usually results from two materials with oppositely charge dielectric constants, such as a metal and a dielectric fluid. When the plasmon is excited, the resonance condition is met if the frequency of light photons matches the natural frequency of surface electrons oscillating against the restoring force of positive nuclei. Such matching of the incident beam to the momentum of the plasmon can be achieved using polarization; specifically, through *p-polarised* light (parallel to the plane of incidence). By passing the beam through a glass block, the wavenumber can be increased to attain resonance for a given wavelength and angle. Once excited, the surface plasmon proceeds as an electromagnetic wave propagating in the direction parallel to the interface of the dielectric and metal. This wave has a propagation constant defined by:[xl]

$$\beta = k\sqrt{\frac{\varepsilon_m {n_s}^2}{\varepsilon_m + {n_s}^2}} \qquad \textbf{[A-8]}$$

where k denotes the free space wavenumber, ε_m is the dielectric constant of the metal given by $(\varepsilon_m = \varepsilon_{m,r} + i\varepsilon_{m,i})$, and n_s is the refractive index of the dielectric. Owing to high loss in the metal, propagation of the surface plasmon wave occurs with high attenuation in the visible and near-infrared spectral regions.

Two common configurations exist for light-excited SPR (depicted graphically below in Figure A-2): the Otto configuration,[xliii] in which the glass block, usually a prism, is placed directly in contact with the metal, and the polarised light undergoes total internal reflectance at the interface of the glass, and surface plasmons are excited by an associated evanescent wave at the metal-dielectric layer sandwiched between the glass; or the Kretschmann configuration,[xlvii] wherein a thin metal layer is directly deposited on the prism (usually by evaporation), and the plasmons become excited on the opposing side of the metal, at the interface in contact with the dielectric. The Kretschmann is often chosen for surface functional measurements, as it allows SPR observation without the need to pass light through the absorbate, which is oftentimes opaque, and is simple to construct as the analyte surface can simply be placed on already constructed and interchangeable prism systems.

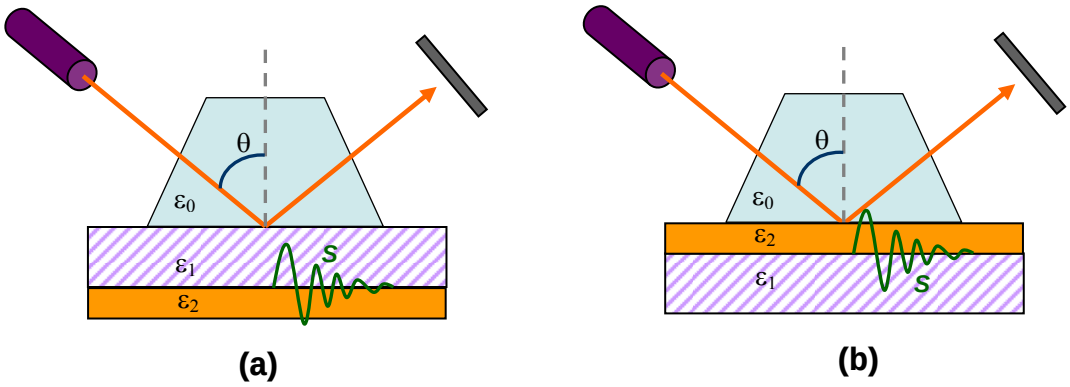


Figure A-2. Schematic representation of typical SPR design setups: (a) Otto, and (b) Kretschmann configurations.

When a plasmon in the dielectric is excited by photons, the path of the photon is changed, and a dip in the reflected light at that specific angle is observed. By optically interrogating the surface plasmon wave (SPW), an electronic signal is generated which can then be processed. During wave propagation, the electromagnetic field adjacent to the dielectric is highly concentrated, and subsequently the propagation constant is very sensitive to variations in the optical properties of the dielectric adjacent to the metal layer. Thus, any slight changes near the surface along the evanescent wave will result in

transformation of the interaction between the optical wave and the resonant SPW, which can then be correlated in terms of a signal. Most detection strategies in SPR employ monitoring of the intensity of the optical wave near resonance, or measurement of the resonant momentum of the optical wave (including angular and wavelength interrogation).[xl]

Owing to the high degree of sensitivity in detection, and its aspect of measuring slight changes on a surface in real-time, SPR has become a boon in the field of chemical and biological sensors. It is a highly efficacious tool for measuring binding events of surfaces, which allows it to be used for detection of molecules of interest, drug-screenings, bioassays, protein-cell interactions, kinetics, layer-by-layer assembly, among others. Because changes in SPR signal are directly proportional to the mass change on the detection surface, interactions can be assessed in terms of stoichiometry of immobilisation; the data can then be interpreted into mass and thickness of adsorbed layers. Typical surface plasmons reach depths of 200-300 nm of penetration into the dielectric media, meaning a strong detection limit for submicron changes. Sensitive instrumentation can even allow internal conformational changes to be detected, such as for the binding behaviour of cells and proteins in layers.[xlvi]

Recent increased interest in the field of surface plasmon resonance has brought about the application of SPR for biomolecule detection in MIPs. For one, using MIPs in conjunction with SPR replaces the need for other receptor molecules (usually antibodies) which are costly and unstable; a severe limitation of current biosensor coatings. MIPs, on the other hand, offer a low-cost and stable alternative. Like QCM, the nature of using glass and gold substrates as part of SPR detection makes many different chemistries, functionalisations (e.g. silanes, thiols, amination, vinylation), and polymerisation types possible. While having an initial high detection limit, an early report from Lai *et al*. verified the feasibility of combining 'anti-polymer' technology with SPR via photothermal deflection spectrometry (PDS) and photodiode array (PDA) for a novel assay.[xlviii] Recently,

thin layer MIPs have been imprinted for SPR detection of DNA, enzymes, and various hormones.[xlix, l] Wu *et al*. have reported on the development of an SPR/MIP for sensitive glucose detection under physiologic conditions using a responsive hydrogel.[li] Imprinted polymer nanoparticles can also be affixed to SPR sensors surfaces.[lii] These methods allow rapid diffusion of the analyte into the polymer surface, providing for the rapid real time responses that are key in biosensing. Such systems are capable of biosensing of targets in biological solutions down to 10^{-9} M.[liii] SPR detection has been integrated directly into microfluidic platforms, wherein multiple imprinted regions can be used to detect for a variety of different biomolecules from a single source.[liv] Zheng and Du have demonstrated the ability of SPR to recognise protein adherence and, importantly, to observe the lateral behaviour of surface ligands during binding event interactions involved in MIP films.[lv] It is also possible to use common photolithographic techniques to form detection arrays of imprinted polymer.[lvi] While only a cursory study, such patterned surfaces or oriented 'stamping' on SPR slides suggest potential towards multi-target detection surfaces in the future.

As the changes in reflective angles are very slight with binding events, it is often desired when using SPR to use some supplemental methods to increase the signal (and subsequently observed changes). In some cases, positional bands or nanostructures can be used on SPR sensor surfaces to enhance detection through intensifying localised signals.[lvii] Gold nanoparticles can also be used to improve the detection of binding events, through either enhancing the local electromagnetic field[lviii] or causing changes in signal due to local proximity to the sensor surface as the material swells/de-swells with the presence of the analyte.[lix] This amplification of signal allowed Riskin *et al*. to distinguish changes in SPR binding curves of a MIP that suggested stereo- and chiral-selectivity towards the amino acid cysteine and analogues.[lx] The use of quantum dots for enhancing transducer sensing of binding on MIPs has been reported,[lxi, lxii] and this strategy can be combined with SPR detection, as the increased quantum dot luminescence from excitation[lxiii] is one method of enhancing localised SPR signal.[lxiv]

One of the limitations of SPR, as it is based on an optical evanescent wave, lies in that for most setups the detectable region is generally limited to about 100 nm distant from the plasmon surface. This can be partially overcome by achieving very thin layer coatings. For the polymerisations necessary in molecular imprinting, one method available is the immobilisation of initiator to the surface before the pre-polymerisation mixture is added;[lxv] this has the effect of confining the reaction to the vicinity of the surface. Using this technique together with common MIP monomers, Piacham *et al*. were able to limit film thicknesses to less than 50 nm.[lxvi] A special case of this is the use of molecular imprinting to form Langmuir monolayers arranged on a sensor surface. This is especially useful when a target molecule can have multiple binding sites, such as with concanavalin *A*. [lxvii] Vikholm-Lundin *et al*. demonstrated protein binding on imprinted monolayers that was equivalent to immobilised antibodies.[lxviii]

While SPR relies on changes in material properties which are more or less localised at the interface between liquid and surface, in the case of a bulk hydrogel we can also measure the binding through changes in conductance of the medium. Zhang *et al*. have demonstrated that impedance analysis and piezoelectricity can be used to measure binding of imprinted biomolecules on quartz electrodes.[lxix] Their study showed that both methods could be used for analyte detection in the ng/ml range, and, importantly were effective in polar solvent near physiological temperatures and pH. As with other detection methods involving MIPs, the nature of the solvent is key, and properties such as pH and ionic strength will have considerable effect – which can be heightened with working with very small polymer amounts. This was demonstrated by the changes seen in lysosyme-MIP selectivity with NaCl buffer concentrations in the report from Matsunaga *et al*.[lxx]

A 1.3 Reflectometric Interference Spectroscopy

An underused and simple real-time analytical technique is reflectometric interference spectroscopy (RIfS). This method combines the sensitivity of optical

signal changes, as in SPR, but is considerably simpler and requires less equipment and expertise. Importantly, it is also label-free and non-destructive, meaning it can easily be adapted for use on different samples, the test/detection surfaces can be reused, and no additional functionalisation steps are required.

The principles of RIfS are depicted in Figure A-3. Unlike the refractive changes measured in SPR, the shift involved is based on physical thickness changes at the interface. The detection in RIfS arises from the interference of two polarised light beams, which are partially reflected as they cross the interface of materials with two different refractive indices. Assuming no absorption of the radiation, the white light passed through these surfaces will travel at different optical paths, and have a specific phase difference. When the optical path length through these layers is less than the coherence wavelength, they will produce an interference spectrum. This pattern is based upon the wavelength of light used, the optical thickness (given by the product of the refractive index of the layer and its physical thickness), the incident angle, and the refractive index of the surrounding medium. All other properties remaining constant, the change in optical thickness (such as would occur from a binding event) will produce a spectral shift that is detectable by the instrument. For the case of perpendicular incidence, a non-absorbing layer, and low reflectances, the reflectance, R, is given by:[lxxi]

$$R = R_1 + R_2 + 2\sqrt{R_1 R_2}\cos(4\pi nd/\lambda) \qquad \textbf{[A-9]}$$

where R_1 and R_2 denote the Fresnel reflectance at the two interfaces, n is the refractive index of the intermediate film layer, d is the thickness of the film, and λ the wavelength of the incident light. When an analyte binds to the surface, it causes a shift in the wavelength of the interference pattern. Measurement of the binding signal is usually done via shifting of an extremum in the intensity over time.

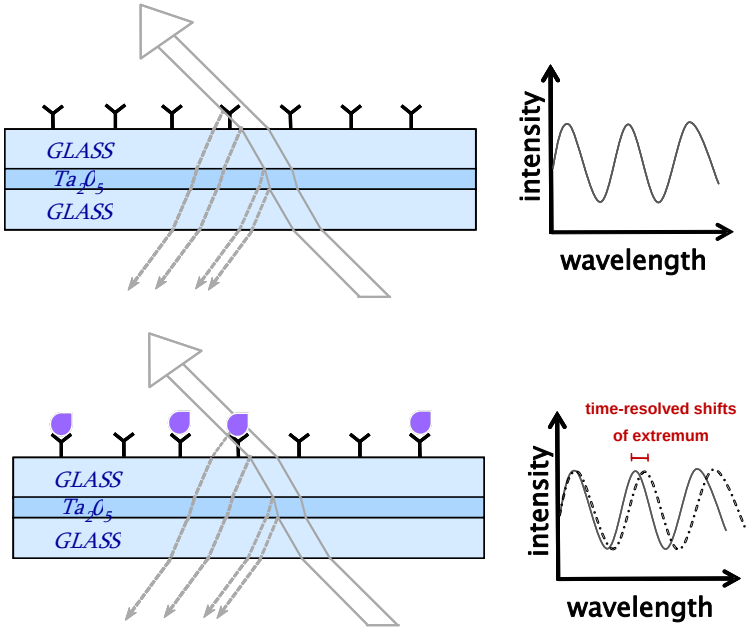


Figure A-3. Schematic illustration of the principles for RIfS detection, depicting shift in the interference pattern as an analyte binds to the functionalised test surface.

By this fashion, RIfS detection can be used for both gaseous and liquid-phase analyses. It is extremely useful in particular for monitoring biological events, since it is a direct measure of localised binding, e.g. when a target molecule is 'captured' by the surface. As a real-time surface immobilisation technique, measurement of analyte binding enables kinetic studies to be undertaken. The depth of penetration offered by RIfS is an improvement over SPR, covering a detection region up to 40 μm (compared with a maximum depth of 300 nm in advanced SPR), depending on the wavelength of light used.[lxxii] This allows for a wider range of study, especially for a series of complex surface reactions, 'sandwich' chemistries, and large biomolecules. Another major advantage over SPR is a much lower sensitivity to fluctuations in temperature. Whereas rigid temperature control of the environment and surfaces creates a great problem for SPR analyses, since the refractive index is highly temperature dependent, in RIfS this is counterbalanced by a change in the physical thickness. For example, as temperature increases, the Clausius–Mossotti relation shows that the thermal expansion, and hence the density, will cause the refractive index, n,

to decrease.[lxxiii] Concurrently, thermal expansion of the biopolymer layer with elevated temperature will cause an increase in the thickness, d. As the optical thickness is given by the product of these two values, the temperature influence remains low.[lxxi] RIfS-based biosensors have been shown to be stable over time and regeneratable.[lxxiv]

By its simple nature and strong applicability to measure surface binding of molecules, RIfS has been applied in many biomolecule detection systems, including high-throughput screening [lxxv] and the potential for clinical diagnostics.[lxxvi] It can be used easily for label-free detection of proteins without the need for complex chemistries or detection systems.[lxxvii] Kumpf and Gauglitz have shown that it can be integrated together with electrophoresis, to combine strong detection and analytical separation of compounds of interest, with an assay of its biological function or activity.[lxxviii] Kurihara *et al*. recently reported on the use of RIfS with a microfluidic system to observe specific protein-protein interactions[lxxix] and antibody-antigen binding.[lxxx] Reflectometric systems have also been used for cell studies: Merkl *et al*. have observed cell binding with detection of *Legionella pneumophila*,[lxxxi] while Möhrle *et al*. tested preferential cell adhesion on functionalised surfaces.[lxxxii] Kumeria *et al*. incorporated a nanoporous aluminium oxide layer into a RIfS system for the detection of circulating tumor cells in a PBS stream.[lxxxiii]

At the present time, only two reports have been made with the use of reflectometric interference and chitosan. A 2001 study by Lü *et al*. contains a simple kinetic study of protein adsorption onto spun-coated, dehydrated chitosan films.[lxxxiv] Just recently, a second study has been conducted wherein microwells filled with chitosan hydrogel were constructed as part of a microfluidic sensor. These gels acted as a pH sensor, responding with a volume shift that was detectable by RIfS (affecting the thickness and refractive index). Using this microfluidic device, the authors were able to monitor bacterial metabolism by

relating it to environmental pH changes, and estimate the susceptibility to antibiotics.[lxxxv]

Just as few groups have examined the replacement of the recognition element in RIfS with molecularly imprinted polymers. Kolarov *et al*. have applied immobilised acrylic MIP nanospheres on a modified glass slide to measure the selective and specific capture of template molecules.[lxxxvi] Belmont *et al*. investigated MIP using both nanoparticles prepared by miniemulsion polymerisation and spun-coated films, on the transducer surfaces.[lxxxvii] While these tests done in toluene showed an improved response from imprinting effect, the detection reproducibility needs to be improved upon, and no examination was done on binding of analogous molecules. A final study by Nopper *et al*. showed an improved separation factor of enantiomeric templates, as proof-of-concept towards chiral chemical separation and detection using typical MIPs in aqueous media, however requires further optimisation to improve on selectivity.[lxxxviii] The use of a chitosan film, together with imprinting for protein recognition as suggested here, represents a new step in the development of future biosensors.

Appendix B: Statistical Design of Experiments

In order to maximise the amount of information gathered from the experimental runs in this investigation, it was elected to apply statistical design methods to plan out and observe effects for the bulk imprinting study. Such experimental design can provide researchers with massive advantages in conducting studies. Whilst traditionally limited in application to the industrial sector, DOE can be an invaluable tool in the pocket of any user involved in experimentation. The value of such multi-conditional methods to investigate experimental effects was first established in the 18th century for identification of scurvy,[lxxxix] and in subsequent work in the 20th century the foundation was laid for its true potential to divulge statistically relevant data in an efficient approach.[xc, xci] Since that time, such experimental design methods have continued to be developed as useful tools for practical scientists, in both process planning and optimisation.

Proper experimental setup is advantageous in four key ways: 1. it reduces the overall amount of experiments necessary to achieve a desired set of information, 2. it allows for the construction of a model representing the system being investigated, 3. it provides information on the effect different setup parameters have on a measured result, and 4. it can tell the user the degree to which components of the system interact with each other. It can be particularly useful in investigation of complex systems, such as molecular imprinting, where many simultaneous factors contribute to an end condition. Many different techniques have been developed to approach experimental design, each with its own set of advantages (e.g information yielded) and disadvantages (e.g. required assumptions), and proper selection of an appropriate design is a vital step to obtain relevant information from a set of experiments.[xcii]

Statistical experimental design is often described via orthogonality of the parameters being studies, i.e. through mathematically independent ('orthogonal') assessments of the effects. A full factorial design refers to a set of experimental

designs that examines k factors in a series of n observations. When, as is often the case, an experimental unit is set up for each of these factors at two discrete levels (e.g. a high and low value), it is termed a 2-level factorial design.[xciii] The number of observations necessary to describe this system is determined by

$$n_e = 2^k \qquad \textbf{[B-1]}$$

where n_e is the number of experimental observations, and k is the number of factors being examined. For example, in an experiment looking at three factors, this would equate to eight observations, or 'treatment conditions.' As this is a factorial relationship, that number increases rapidly with the more factors being considered. In the case of a five factor experiment, it would necessitate 32 separate observations. Thus the desired determination of effects must be carefully balanced by the required number of individual experiments.

As the instance of a 2-level design refers to a simplified special case of the experimental setup, more complexities arise when the examination of factors is desired at multiple levels. In many cases, simply examining a factor at two extrema does not provide enough information to adequately characterise its effects. Situations where three or more levels might be desirable include: when the effect of the factor is considered to be non-linear in the range of interest, if the purpose of the study is to find an optimum level for the particular factor, or under circumstances when it is important to include the current level of the factor as well as the low and high values in analysis.[xciv]

Multi-level designs are of course able to provide a significant amount of information to the user; however, as the number of levels (i.e. the base used in the factorial computation) is increased, the number of experiments drastically goes up. A simple three-level design of the same three factors described above would necessitate 27 observations, rather than just 8; for five factors the number balloons to 243. As discussed earlier, the purpose of DOE is to gather the information required for quantitative description of the system in the most efficient fashion.

Conducting the large number of test runs necessary for fully describing a model at the levels desired is not always practical. However, the cost in the increase of treatment combinations for examining an extra level is sometimes a necessary condition, as not all experimental functions will follow a linear trend; thus it may be necessary to consider this relationship in certain circumstances where knowledge of the process suggests that the factors may be non-linear, or where insufficient information is available otherwise for optimisation. Clearly having prior knowledge of the process becomes an essential ingredient for successful design of experiments.[xciii]

It is evident that in most common situations, it may easily become impractical, or even impossible, to implement a full multi-level design by the factorial method. The process is costly and inefficient, even with a minimisation of experimental runs; however, scientists would still wish to attain the results from these experiments for the purposes of gathering process information and optimisation. In general, statistical design allows one to investigate a polynomial relationship an order of one less than the numbers of levels in the factor, e.g. in the case of the two-level design presented above, it is possible to investigate the linear, or first order effects. A three-level design allows inferences about a second order, or quadratic, relationship. The control response for the second order function is modeled by the expression

$$Y = b_1 X_1 + b_{1,1} X_1^{\,2} \qquad \textbf{[B-2]}$$

where b is the slope and X is the control factor.[xciii]

It becomes possible herein to use confounding of factors to reduce a full factorial design to a fractional factorial design. These have the notation (for two level designs) 2^{k-p}, where p is the fractionalisation element (also referred to as the number of generators) and reflects effects being confounded together. A 2^{k-p} design requires only 2^{k-p} experiments, and 2^p effects are confounded together, i.e. grouped together such that they cannot be estimate independently of each other.

This has a considerable effect on reducing the experimental cost, as a 2^{k-1} requires half as many treatment combinations, and a 2^{k-2} only one quarter the amount, and so forth.

It is important here to discuss the degrees of freedom (*df*), which are measured to provide the information in an experimental design. By expanding the number of levels used, we are also increasing the amount of interaction terms present, which have a significant cost in the *df* as the design size is expanded. This can also be discussed in terms of the resolution of an experimental design. The resolution is a feature that describes how estimated main effects are confounded (or aliased) with the estimated two-, three-, and higher level interactions. In general, the resolution of a design is one more than the smallest order interaction that some main effect is confounded with.[xcii] For example, *Resolution III* designs would have main effects that are confounded with two-factor interactions, *Resolution IV* designs would have no main effects confounded with two-factor interactions, but two-factor interactions would be aliased with each other, and *Resolution V* designs would contain no aliasing of main effects or two-factor interactions with either any other main effect or two-factor interaction, but two-factor interactions would be aliased with three-factor interactions. In the special case of designs possessing orthogonality, wherein the estimation of main effects and interactions are independent of one another, it is considered 'infinite resolution.'

In practice, we find that the more complex interaction cases, those of the quadratic form and above, generally do not occur to such an extent that it has a significant effect on the response,[xcv] or wherein the coefficient falls within the error of the experiment.[xcii] The discrimination of less serious effects and avoidance of missed real effects can be achieved through proper design procedures and subset selection for blocking and aliasing.[xcvi] Through neglecting these higher order interactions and properly designing the experiment, we can maintain sufficient degrees of freedom for the system, and characterise it in a

minimum number of treatment combinations. One of the methods of achieving this is using a centre point value (e.g. at zero) and two extrema for each factor. These are called 'composite designs;' the simplest composite design would be a 2^k factorial with a centre point.[xciv] Envisioning rotation of the design about this centre point, we can see that the parameters are simplified to an approximation of only a 2^2 factorial design. The geometry of such a composite design is shown schematically in Figure B-2.

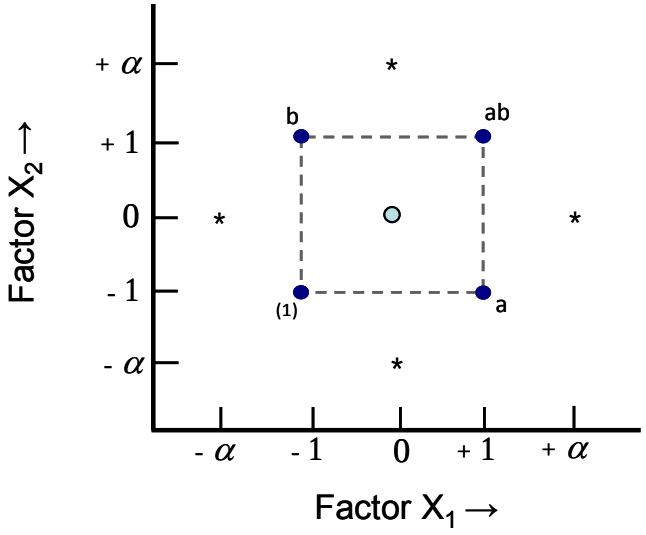


Figure B-1. Geometric outline of a two-factor multilevel design.

This method of centralising the experimental design symmetrically is key to many of the developed models for higher level system designs. While the same information of the standard 2^k design remains present, instead of the original three levels, we now have each factor examined at five levels, which enables investigations up to fourth-order (quartic) relationships. While we may still negate the contribution of higher order effects, the expanded volume of the design allows curvilinear relationships to be determined, in lower treatment combinations than necessary for a full or partial factorial design, and without the accompanying sacrifice of any degrees of freedom/loss of essential information for the system. The above is an example of a central composite design (CCD). Figure B-3 provides a visualisation of a CCD for three factors. The CCD is important as each

level remains mathematically independent, however, appropriate level choice remains a critical parameter of the model.

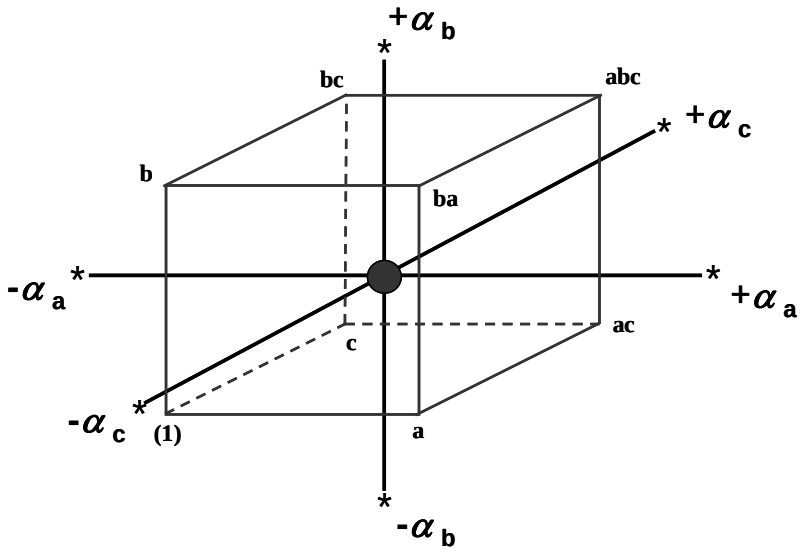


Figure B-2. Diagram of schematic representation for a 3-factor Central Composite Design.

It may not be possible in all cases to determine the full five levels necessary for such a setup. A modification of the parameter structure used for the CCD design can be made by taking experimental factors in pairs, and to then use these to build several 2^2 factorial designs while holding the other factors at the center point. This is called the Box-Behnken (B-B) design. It is considered an economical alternative to the CCD, in that each factor needs only be evaluated at three levels (i.e. $-\alpha$, 0, $+\alpha$).[xcii] While the B-B designs require more treatment combinations, and therefore are not as efficient as the CCD and have a more limited capability for orthogonal blocking, they are a useful resource to fill a gap between simple 2^{k-p} and multi-level full factorial designs. The information sacrificed in the analysis are higher order interactions (three factor or greater interactions, or quadratic interactions) that in many cases are not likely to occur.[xciii]

Appendix C: Additional Results and Discussion

As a result of the inherent difficulty in both achieving molecular recognition and accurately measuring any imprint effect, this project involved several areas of study which did not produce positive results, but will be presented here for the sake of completeness.

C 1.1 Liposomal System

As part of the system improvement, it was desired to establish a method of producing particles of a controlled diameter and limited size distribution. As more and more research fields in biomedicine are turning to solutions on a nano-ordered scale, hydrogel nanoparticles provide an opportunity to explore another scope of this imprinted material. While generally nanoparticles are easy to form, given the available knowledge base on the subject, hydrogels as nanomaterials are not as widely reported. Techniques for forming them can be chemical or physical,[xcvii] including precipitation,[xcviii] core-shell synthesis,[xcix] inverse and emulsion polymerisation,[c, ci] and even microfluidic assembly.[cii] It was postulated that molecularly imprinted nanogels of chitosan could be later incorporated into macro-delivery vehicles such as polymersomes.[ciii]

Taking inspiration from a paper by Kazakov *et al*., it was decided to investigate the possibility of making chitosan microspheres in the inner hydrophilic chamber of a liposome.[civ] The authors used encapsulation with an *L*-α-phosphatidylcholine bilayer over an inner aqueous solution of acrylamides, and applied UV polymerisation of this medium in the liposomal microreactor. The lipid bilayer was then solubilised by detergent and the phospholipids removed by dialysis. This had the effect of producing crosslinked polyacrylamide nanogels. A similar report was later made by An *et al*. for the templated creation of PEG nanogels, which emphasised the uniformity of spheres fabricated by this method.[cv] As this process successfully produced hydrogels in the confined space of a liposome using similar photopolymerisation, it was resolved to explore a

similar technique using the modified glycol chitosan. While chitosan nanogels have been formed polyion micelles via genipin crosslinking,[cvi] this methodology requires the synthesis of complex polymers and relies on a crosslinking agent with some level of cytotoxicity.

While many methods are available for the formation of liposomes,[cvii] the lipid vesicles used in many drug loading and encapsulation studies are generally formed via the lipid hydration method, or freeze-thaw method. Thin-film lipid hydration was chosen for its simplicity and efficiency in forming multilamellar liposomes (MLV). Liposomes were prepared according to a procedure adapted from New.[cviii] Briefly, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, Genzyme Pharmaceuticals, Switzerland, 100 mg), cholesterol (26.3 mg), and dimethyldioctadecylammonium bromide (DDAB, 5.2 mg) as a cationic surfactant were used to create the lipid layer. These were dissolved in a mixed solution of 4:1 by volume of chloroform:methanol. The organic components were gradually removed from the lipid phase by low heat and vacuum on a rotary evaporator in a 250 ml round bottom flask. This produced a thin homogenous lipid film inside the reaction vessel. The resultant bilayer was then rehydrated with a solution of methacrylated glycol chitosan in high purity water, and vortexed thoroughly to form large unilamellar vesicles (LUVs).

To make the nano-sized liposomes, the vortexed suspension was transferred into a mini extruder (Avanti Polar Lipids, Alabaster, AL) and then the solution was extruded by successively passing (20x) through two 200 nm pore size 19-mm polycarbonate filters (GE Osmonics) membranes at a temperature of 60^{o}C. The extruded suspension of vesicles was collected in 4 ml glass vials. Photocrosslinking of the chitosan was done in a similar fashion to previously described, using the Dymax light curing box. After brief exposure to the ultraviolet light (~ 10 min) the inner chitosan core of the vesicles became crosslinked. Digestion of the outer lipid layer was done in a fashion similar to that used bacterial cell wall solubilisation.[cix] Triton-X 100 can be used as a common

lysis buffer for digestion of liposomes,[cx] when not crosslinked for extra stability.[cxi] A 0.5 % v/v solution of Triton-X was used together with agitation of the mixture to break up the liposomes. Lipids were separated from the particles by filtration and washing with excess PBS. Collected nanospheres of chitosan gel were maintained in solution until subsequent studies.

Interactions between chitosan and liposomes have been reported by Henricksen *et al*.,[cxii, cxiii] and chitosan has been used as part of a coating material for liposomal delivery vehicles,[cxiv] and as supporting matrix,[cxv] but no report of creating chitosan particles in the fashion has been made. The nearest study was a report from Alamelu and Panduranga Rao from 1991, wherein carboxymethyl chitosan was incorporated into the inner chamber of PC liposomes for investigation of its effects.[cxvi] Because such a method has not been used specifically for chitosan encapsulation, or for creating nano-sized chitosan hydrogels, the process provides a unique new strategy for gel sphere formation.

Chitosan microspheres were characterised used a Malvern ZetaPALS particle sizer. 1 ml of suspension containing approximately 0.05 g/ml particles was placed in a measurement cuvette. Readings were taken at a scan rate of 0.2 s^{-1} and the numbers reported are the mean values from 10 readings.

C 1.2 Particle Characterisation

Additional studies were performed to assess the morphology of gel particles produced by varying methods. As a measure of success and usefulness of the techniques, the particles were characterised for average size and homogeneity. Depending on the scale of the particles, they were examined visually by either light microscopy, scanning electron microscopy (SEM), or tunnelling electron microscopy (TEM). It might also provide the observation of differences, if any, in the surface characteristics of gels due to the presence of template during crosslinking.

C 1.2.1 Light Microscopy

As a method of forming microspheres, a sample protocol was developed to examine the formation of particles from acrylamide, as this is a well-studied and controllable polymerisation system. Particles were homogenised at three different speeds (3000, 7000, and 11000 rpm) to observe the changes in particle size. Sample microspheres are shown in Figure C-1. As expected, increasing the homogenisation speed resulted in finer particles. It was also evident that, while there were some large particles evident, the distribution of particle size was fairly uniform. The gelled particles produced via this method ranged in the size of 5-10 μm.

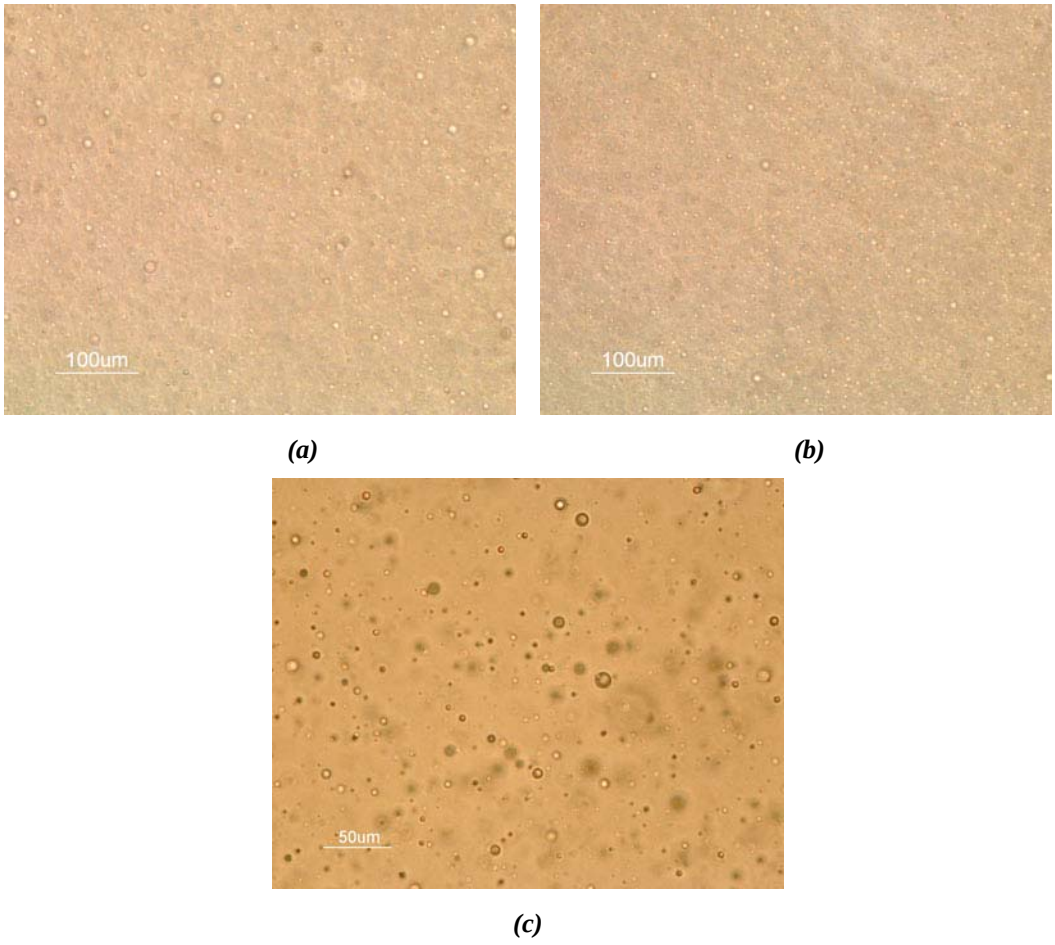
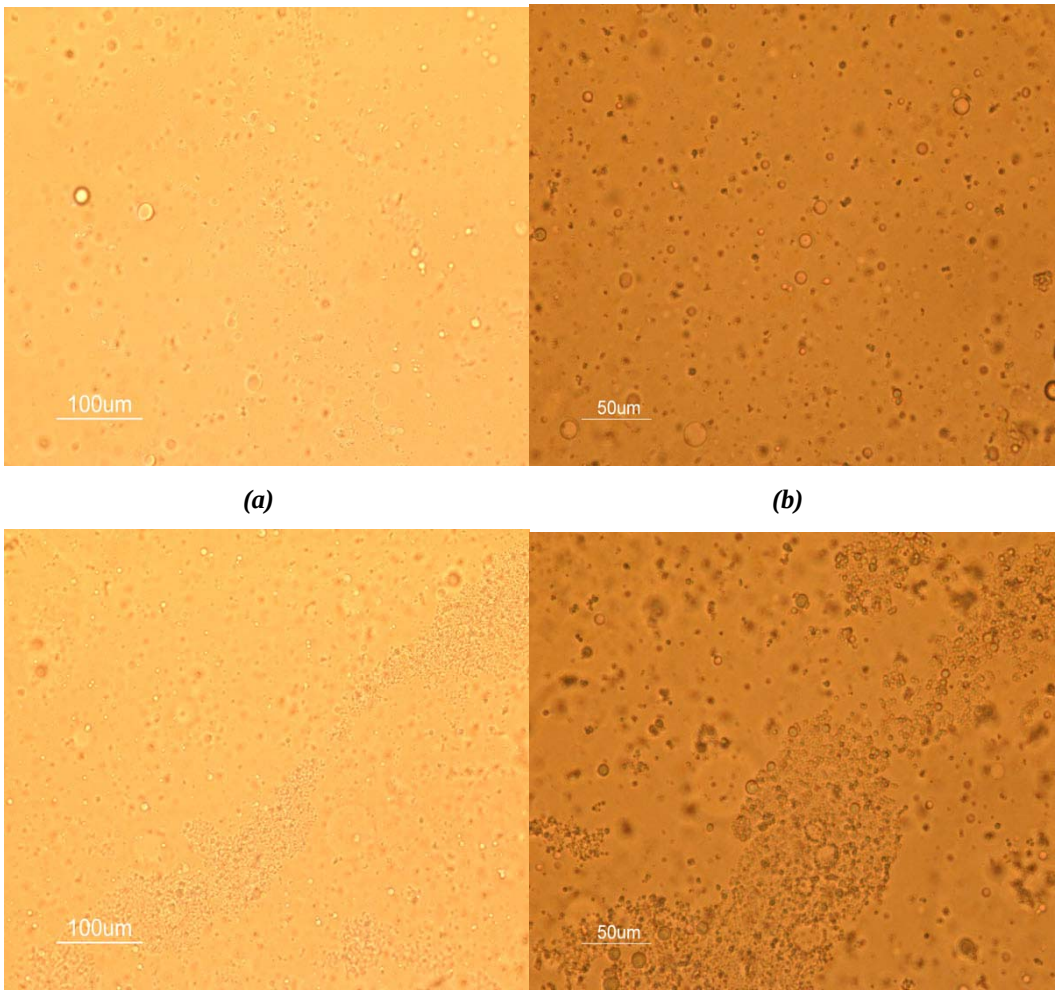


Figure C-1. Acrylamide particles produced in emulsion by homogenisation at (a) 3000 rpm, (b) 7000 rpm, and (c) 11000 rpm.

Next, preparation of particles by similar procedure was examined with the modified chitosan polymer. In this case the emulsion system is slightly modified as the polymerisation reaction is initiated by UV exposure. Because of this extra stage, requiring removal of solution to place inside the curing chamber, the produced emulsion is much more prone to aggregation of particles before the crosslinking can take place. From Figure C-2 we can see that production of the microspheres by this method was much less successful compared against the previous acrylamide trials. The distribution of particle size is a good deal larger, as the produced solution of gels is very heterogeneous. It also appears there is a significant amount of gel debris, which is unexpected and unusual, since this is usually an effect associated with gelation in solution during homogenisation, as a result of gel fracture from the force of mixing. There were also some areas of loose clustering of the particles noticeable in some of the samples (see Figure C-2 *(c)* and *(d)*). This is likely due to not maintain the complete dispersion when transferring the homogenised solution to be photocrosslinked.



(a) *(b)*

(c) *(d)*

Figure C-2. Chitosan microspheres produced by homogenisation in water-in-oil emulsion.

C 1.3 Binding Studies

C 1.3.1 QCM

Quartz crystal microbalance measurement with dissipation (QCM-D) was performed with a Q-Sense E4 unit and QE 401-1168 controller (Västra Frölunda, Sweden). This particular model is equipped with four individual sensors, that can be connected to four separate streams in parallel for simultaneous monitoring, or in series for sequential analyses. The sensors are contained in an isolated temperature controlled chamber, and flow into and out of the QCM device was controlled by an Ismatec ISM 596B peristaltic pump. Changes in frequency (Δf)

and energy dissipation (ΔD) were measured at the fundamental frequency of the crystal (f = 5 MHz) as well as at the third, fifth, and seventh overtones (15, 25, and 35 MHz, respectively). Data collection was done using the QTools software.

Gold coated QCM sensors were first prepared by cleaning in a 5:1:1 by volume solution of water, 30% H_2O_2, and 30% ammonium hydroxide. Following this, the sensor chips were washed excessively with water and dried under a stream of nitrogen gas. Next they were treated with UV/ozone (BioForce Nanosciences, Ames, IA) for ten minutes. Spin coating on the surface was done using a Laurell WS-400B-6NPP/Lite spin coater under an inert nitrogen atmosphere, above which was place a Fiber Lite MI-150 High Intensity Illuminator (Dolan-Jenner Industries, Boxborough, MA) UV spot curer, so that photocrosslinking could take place simultaneously with spinning of the pre-polymer solution. After optimisation trials, the following spin conditions were found to produce a thin, homogenous coating of chitosan: ramp up speed of 500 rpm for 10 s, full spin cycle at 5000 rpm for 50 s, and a slowdown period of 300 rpm for 15 s. 40 µl of concentrated functionalised and initiated chitosan solution was added to the sensor chip surface, the lamp turned on for a 10 s UV burst for initial exposure, and subsequently crosslinked for a total of 20 min.

When operating the QCM, samples were firmly sealed inside sensor electrodes. Before each run, the unit was equilibrated with the sample in air to reach a stable reading. Sensor temperature was maintained at 25^{o}C. The system was operated under a continuous flow of 1x PBS at a rate of 0.2 ml/min.

C 1.3.2 SPR

To facilitate examination of imprinting effect by SPR, thin films of chitosan gel were adhered onto gold-coated glass chips (Horiba, France). The chips were, on average, 1 cm^2. The slides were prepared by cleaning in a solution of piranha (sulfuric acid and 30% hydrogen peroxide, mixed in a 3:1 ratio by volume) at

70^{o}C for 10 min. The slides were then rinsed thoroughly with water and dried under N_2 stream. This process removed any residual debris and organic matter from the surface. It also renders the surface more hydrophilic, by hydroxylating it. Spin-coating and photocrosslinking of chitosan layers was done in a similar fashion as to that described previously for QCM chips. A UV spot curing lamp was set at a distance of 26 mm above the surface of the glass slides to offer sufficient exposure to the coating area and intensity for radical formation. The spinning program used consisted of an acceleration stage at 500 rpm for 10 sec, a maintained max spin speed of 4500 rpm for 40 sec, finishing with a deceleration stage of 300 rpm for 30 sec. 200 μl of initiated chitosan solution (with and without protein template) was added to the surface of the freshly cleaned SPR slides, which was sufficient to completely cover the surface area. An initial burst exposure of 10 sec was used to start the crosslinking reaction before the spinning was begun. Total UV exposure time was 40 min. Template leaching was done by soaking each slide in a 5 ml solution of water, changing the solution regularly.

Analysis of binding was done using an SPRi biodetection instrument (model SPRi-Lab+, GenOptics, France). The SPRi Lab apparatus was equipped with an 800 nm LED source, CCD camera, and a microfluidic cell. The entire analytical construct was placed in a temperature-controlled incubator (Memmert Peltier, Rose Scientific, Canada) for all readings. A peristaltic pump circulated phosphate buffer saline (PBS, pH 7.4) through the fluidic system at 100 μl/min and was used to fill the fluidic cell. Sample injection was accomplished by way of a control valve with 100 μL injection loop. Measurements were obtained using SPRi-lab GenOptics software (Horiba Scientific, Kyoto, Japan).

C 1.3.3 RIfS

The starting surface used was silicon nitride chips (of approximate dimensions: *L* 9 mm × *W* 9 mm × *H* 0.725 mm). These were prepared by initial surface washing with ethanol and water and drying under a compressed nitrogen stream. To

facilitate binding reactions, the surfaces of the chips were activated according to a procedure adapted from Choi *et al.*[cxvii] To increase surface reactivity and prepare for silylation, the SiN chips were bombarded with UV/ozone for 15 min inside a PC440 chamber equipped with a mercury vapor lamp, (BioForce Nanosciences, Inc., Ames, USA). Following cleaning, the chips were places inside glass vials, which were subsequently sealed with a septum and purged repeated with nitrogen gas.

A reaction solution was separately prepared in an anaerobic atmosphere from trimethylsilyl chloride (TMS) and toluene, to obtain a 1% (v/v) solution. Approximately 5 ml of the solution was added to the chip-containing vial to full immerse the surface of the SiN. After a ten minute incubation at room temperature, the chips were removed from solution, rinsed with fresh toluene, followed by distilled water, and dried under a low flow of N_2. To transform the modified residues, TMS coated SiN chips were subsequently treated again by UV-O_3 cleaner for 15 min to transform surface groups into crosslinked silanol/siloxane moieties (see Figure C-3).

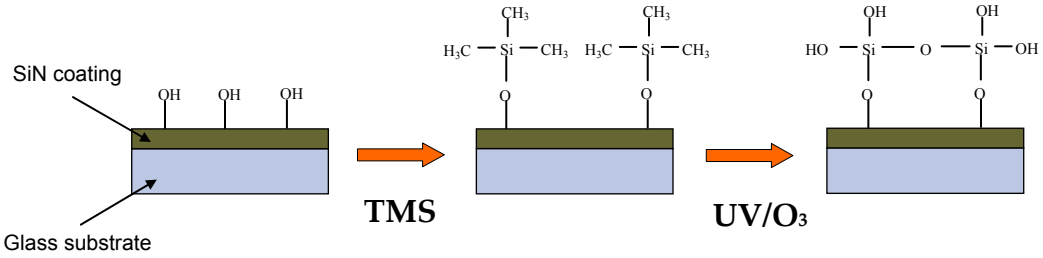


Figure C-3. Surface modification of SiN substrates by silylation.

Two different types of silane modification were attempted to add surface functionality to the analysis chips. Initial trials suffered from silane oligomerisation in solution, preventing successful surface modification. The optimal conditions were found to be with a 2% (v/v) solution of active silane, 3-methacryloxypropyltrimethoxysilane (MPTS), in a solution of 95% (v/v) of

ethanol in water. To initiate the hydrolysation of methoxy groups at condensation reaction to the surface at low pH, 1% (v/v) of acetic acid was added to the solution. Surface reaction proceeded at room temperature for 8 hours. Functionalised chips were then washed with excess water and dried in an oven at 110^{o}C for 30 min. The resultant chips possessed methacryl-functionality to enable radical crosslinking with double bonds in modified chitosan.

Surface-silanated SiN chips were used to attach the layer of chitosan hydrogel to the base for RIfS analysis. Several different conditions and methods were experimented with for laying down a chitosan gel on the surface, and the following represents a general procedure. Concentrated solutions of a methacrylated glycol chitosan were mixed with photoinitiator (I2959). Approximately 100 μl of initiated pre-polymer solution was added to the surface of functionalised SiN chips. The solutions were allowed to pre-soak on the chips (~1-2 min) to fully wet the underside and aid in solubilising the functional groups. An Omron ZUV-90 LED light was used at a height of 400 mm above the chip surface to initiate photopolymerisation. After an initial burst of 15 sec, the samples were spun coated with 1H-DX II spin coater (Mikasa Co. Ltd., Japan) while simultaneously having UV light exposure centred on the chip. Typical spin conditions involved a program with a 3 sec ramp up time, continuous speeds between 2000 and 4500 rpm, followed by a 3 sec slowdown period. Total photocrosslinking time for samples was 10 min.

A second investigation examined the used of an immobilised photoinitiator on the surface of the RIfS chips. This was done in order to facilitate strong binding to the underlying SiN surface (i.e. prevent delamination of the gel) and to create a very thin layer of crosslinked chitosan, by limiting reaction and propagating radicals locally to areas near the target surface. The procedure was adapted from one by Sunayama *et al.*[cxviii] Firstly, a 0.5% (v/v) solution of aminopropyltriethoxy silane was made in 95% (v/v) ethanol/water. This was mixed thoroughly for 1 h to fully hydrolyze ethoxy groups. Fresh SiN chips were

prepared as previously, by surface washing, drying with nitrogen, and UV/ozone treatment for 15 min. They were then immersed in silane solution, and allowed to react for 1 h. After rinsing, the surface was cured at 80^{o}C on a hot plate for 30 min. Initiator immobilisation was achieved using a solution comprised of 1 mM 4,4-azobis-(4-cyanovaleric acid) (ACVA, photoinitiator) and 1.2 mM 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride n-hydrate (DMT-MM, coupling agent). These were dissolved in 30 ml methanol, to which the silanated RIfS chips were added. The petri dish was protected from light as the reaction was allowed to proceed for 1 h. Grafted chips were washed with MeOH and dried under vacuum at RT for 20 min. Addition and gelation of chitosan layers to these surfaces was as previously described above.

Another aspect explored in this thin film/RIfS system, was creation of a thin layer MIP using chitosan as the binding matrix, and acrylamide polymer as the recognition medium. Ideally, the template molecule would adhere to the chitosan network due to specific interactions with its functionality, and this would be surrounded by a more dense network of polyacrylamide that would act to 'capture' the template. This is demonstrated schematically in Figure C-4. Theoretically, the two types of hydrogel could either be formed one on top of another in discrete layers, where there would be some soluble degree of binding between the two, or they could be formed as an interpenetrating matrix. To simplify construction and have a comparable system to the other gel samples, the former was chosen as the initial model.

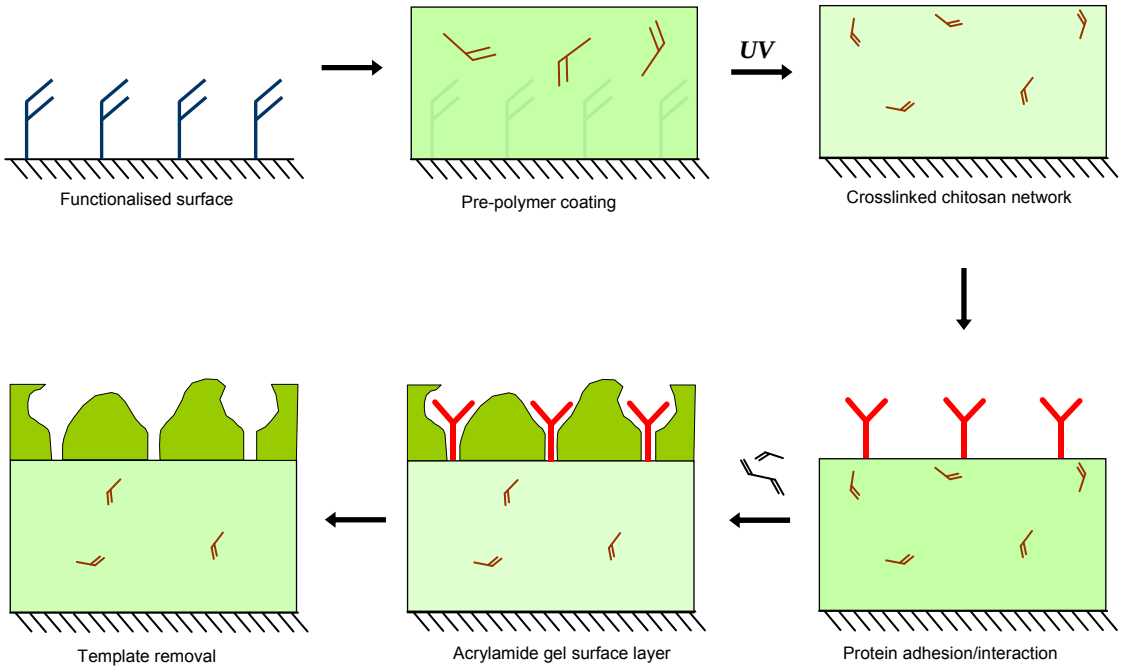


Figure C-4. Schematic demonstration of conceptual bilayered MIP construct consisting of chitosan base on a functionalised surface, as support for upper layer of imprinted acrylamide.

As shown above, following a coating of chitosan onto the RIfS chip, a template is allowed to interact and adhere to the chitosan gel. In this study, the proteins chosen as model templates were *β*-lactoglobulin (-'ve charge, small template), human serum albumin (-'ve charge, large template), and avidin (+'ve charge, large molecule). These were selected to assess the effects of electrostatic interaction with the chitosan matrix and size of the molecule. Protein solutions (1 mg/ml in 1x PBS) were incubated on the surface of spin-coated chitosan for 20 h. Following a gentle rinse, the upper coating was achieved by brief soaking in a mixture of 70 mg/ml acrylamide, 7 mg/ml *N, N'*-methylenebisacrylamide, 0.08% (v/v) TEMED, and 0.08% ammonium persulphate, prepared in 0.01 M sodium dihydrogen phosphate at 4^{o}C.

Studies were also completed attempting simultaneous gelation of the acrylamide and chitosan together. Since the modified chitosan and acrylamide monomer and crosslinker all contain double-bonds and the non-specific free

radical reaction remains uncontrolled, it is conceivable this method would produce a type of chitosan-*co*-acrylamide, wherein the chains acted as an IPC but there is also some degree of co-polymerisation between them. For these trials, acrylamide and methylenebisacrylamide were added in a 10:1 v/v ratio, respectively, alongside 0.05% and 0.1% (of acrylamide solution volumes) of TEMED and APS, respectively. Modified chitosan (in water) and acrylamide pre-solution in $(NH_4)_2S_2O_8$ buffer were combined and mixed just prior to spin-coating. UV exposure was conducted simultaneously to spinning, as before.

For binding measurements, a RIfS-based molecular interaction analyzer (Fluidware MI Affinity LCR-01 system) was used. Continuous flow of buffer was achieved with a Harvard Apparatus Econoflow syringe pump, equipped with a 25 ml polypropylene syringe and operating a flow rate of 0.02 ml/min. Sample injection was done by a Gilson 234 Autoinjector. The system was operated by V937 MI affinity collection software, v. 0.8.2.3, and Gilson Unipoint ver. 3.5 for the autosampler. Integration of the SiN chips into the RIfS detector was accomplished with a PDMS microfluidic cell (*L* 5 mm × *W* 1 mm × *H* 0.0.2 mm, total cell volume: 1 μL, Konica Minolta Opto, Inc., Tokyo, Japan) overlayed on top of the sample chip. The PDMS flowcell consisted of a single linear chamber path, with inlet and outlet ports corresponding to injection and waste streams on the RIfS unit.

C 1.4 Supplementary Surface Binding Results

C 1.4.1 QCM

Thin layers of imprinted chitosan gel were examined for template capture using a quartz crystal microbalance. The system allowed for simultaneous monitoring of an MIP-coated QCM chip alongside control samples (i.e. blank and non-imprinted polymer). Several different protein solutions we used to determine the limit of detection and saturation level for binding. These ranged in value from 5 μg/ml to 20 mg/ml. Samples were injected (0.2 ml/min) in solution using Tygon tubing

(Ismatec), taking care each time to backflush and avoid and air bubbles in the feed stream.

While control samples showed a steady signal expected of unmodified chips, the sensors coated with chitosan had difficulty locking on to a stable frequency. This was likely due to the thickness of the hydrogel coating, which increases considerably after swelling. The relatively large mass of the polymer layer acted too heavily against the oscillations of the sensor chip, and this damping effect impeded any possible measurement of the response for many of the samples. To correct this effect, modifications were done to the spin coating procedure to try and improve to get a very thin layer of hydrogel. This included using lower concentration of functionalised chitosan, faster speeds, and longer spin times. However, in order to balance these with the ability to still maintain enough solvent and time for complete crosslinking of the hydrogel, rather than simple evaporative casting of the chitosan, there were limiting values that could be used.

When a fitting signal could be locked onto with the instrument, it was attempted to observe the signal change as a result f mass increase from protein binding on the surface. If any imprint effect exists in the MIP, it should result in a higher degree of adsorption/capture from solution and so be visible as a distinct increase in signal on the QCM-D. Through several trials and repeated runs, it was not possible to detect any change on the surface when the protein was injected. This was common across multiple sample of imprinted gels, and increasing the protein concentration in solution. The procedure was modified to ‘incubate’ the surface in the presence of the protein solution, allowing it to be exposed to the gel for a period of 10 mins with no flow of the mobile phase. However, it was found that there was still no detectable change as a result of protein binding. This was an unexpected result, as even with standard unmodified surfaces it should be possible see some level of non-specific adsorption to the surface. Some degree of fouling due to protein settling and adsorption is common when working with biofluids.

Considering the functionality and porosity of the chitosan hydrogel, one would expect this effect to be only increased, even in the absence of any molecular imprinting. A possible explanation for this may is that, as exemplified by the difficulty on locking onto the characteristic frequencies, the chitosan layer is impeding proper oscillation of the QCM sensors, thus small changes are not being accurately measured, or are masked by the presence of the hydrogen. The lack of a change in the signal may be attributable to overdamping of the quartz chip, in which case any changes or accurate measurements are not possible, or that the thickness of the hydrogel and its elasticity is causing any adsorbed protein to not contribute any appreciable effect the mass (force) seen at the sensor surface, or a combination of these factors.

C 1.4.2 SPR

Slides coated in imprinted chitosan polymer were examined with an SPRi instrument. After placing the modified chips into the unit, it was secured in place with the prism, and flow of PBS begun. The entire surface of the chip was visualised during the angular scan, and five locations were selected at random that gave representative samples of the surface. These imaged areas were chose to monitor the binding interactions. Unfortunately, it was not possible to determine a characteristic angle at which to initiate the surface plasmon. Several attempted scans using different areas and changing the samples produced similar results.

It remains unclear whether the inability to effectively get coupling with the surface plasmon was due to the thickness of the chitosan layer, in a similar way that a thick coating hindered measurements with the QCM, or could be related to an intrinsic property of the chitosan itself. In the described system, the system was setup for analytical observations near the surface of modified SPR chips, on a molecular scale, e.g. grafting functionalisation or antibody binding. While the depth of penetration for SPR should allow some observation of at least the regional response of the chitosan gel, it is possible that the hydrogel coating did

not maintain a uniform secure bond with the glass substrate, hence impeding effective plasmon coupling.

Another possible source of difficulty that may have contributed to a hindered degree of mensuration is that the refractive index of the chitosan hydrogel did fall within the analytical range of the set-up used. To uniformly bind and create an optical seal of similar degree between the SPR prism and the test slides, an optical solution is used. This gel serves to match the refractive indices of the sandwiched layers and reduce the Fresnel reflection at the surface of an element. In this case a methylene iodide formulation/chlorofluorocarbon solution was used for refractive index matching (Cargille Labs, Cedar Grove, NJ). This liquid covers an approximate refractive index range near to n_D =1.7250. The refractive index of chitosan films is estimated to be approximately 1.5.[cxix] To some degree this can be affected by the individual properties of the chitosan, such as DA and MW,[cxx] however we expect negligible variation between samples prepared from the same batch in a similar fashion. Proper detection can also be affected by the wavelengths used for the source emissions. As demonstrated by Zacher and Wischerhoff, the type of light used for the SPR on hydrogel coatings (e.g. dextran) can affect the sensitivity and penetration of plasmon measurements.[cxxi] However, it is also possible that the degree of swelling and temperature fluctuations are leading to signification changes in the index of reflection, such as has been demonstrated for spin-coated chitosan hydrogels by Murray and Dutcher.[cxxii]

The strong hydrophilicity and swelling of chitosan polymers not only changes the physical arrangement, but can also lead to strong water-coupling, a factor that can have a large effect on both SPR and QCM measurements.[cxxiii] To correctly measure these types of films by the SPRi instrument on hand may require modification of the setup and optimisation of the physical parameters to determine the proper conditions for coupling a plasmon wave to this chitosan hydrogel. This can include changing of the light source, using a different optical

gel and prism pairing, or using incident angles currently outside the capabilities. It may also be possible to use different coating methods to improve on the uniformity and lower the thickness of the chitosan on top of the chip. Lastly, a surface modification could be used to directly bond (e.g. via thiol-chemistry based covalent linkage) the MIP to the gold surfaces, to ensure thorough union between layers and maximise uniform plasmon penetration into the hydrogel.

C 1.4.3 RIfS

C 1.4.3.1 Measurement of Chitosan Layer Response

As a first step in using the RIfS instrument for MIP binding analysis, it was important to establish a baseline response of the chitosan hydrogel. Unfortunately, this proved challenging as the signal suffered from continuous drift, making control readings difficult to gauge. Sample profiles of various test chips with chitosan coatings are shown in Figure C-5.

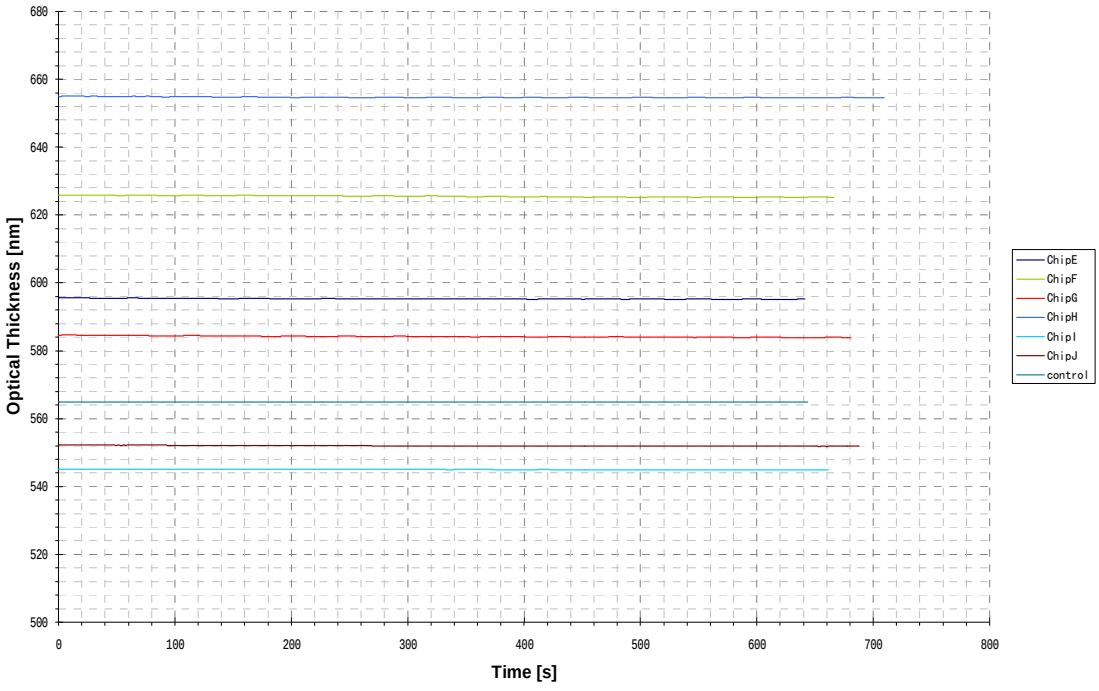


Figure C-5. RIfS response of chitosan-coated SiN chips in air.

As can be seen above, the samples provided consistent response over time, as would be expected, and show some difference in optical thickness between chips, corresponding to varying spin conditions used to coat them. When the samples were wetted by PBS, there was a notable signal change due to swelling of the chitosan hydrogel, as shown in Figure C-6. However, as can bee seen in the figure, there occurred for several samples a point where the signal would 'drop off' and change to considerably different optical thickness value. It was not clear why this occurred, and visible examination of the sensor chips did not note any particular delamination of the hydrogel. Because of this, it was difficult to gauge a starting point or base value at which to begin binding experiments. It also created difficulty when a reading was ongoing, and the signal would experience such a shift. It can also be noted that the system suffered from lack of repeatability between samples, as different runs of the same chip did not result in close overlap of signal, nor the unexpected response behaviour.

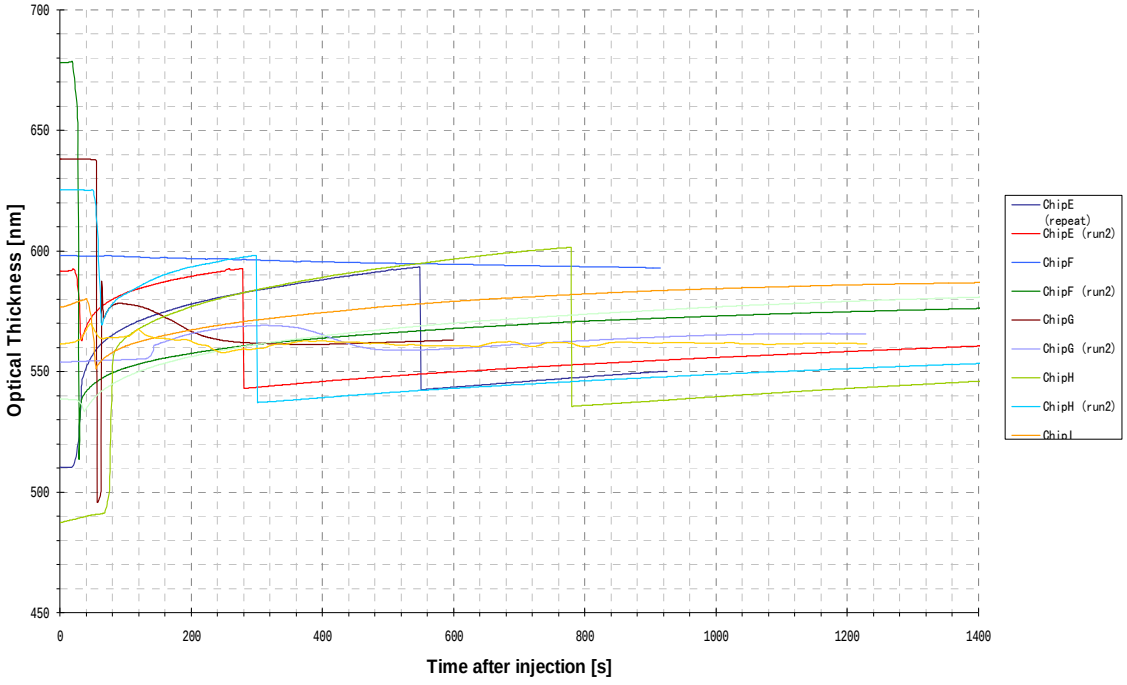
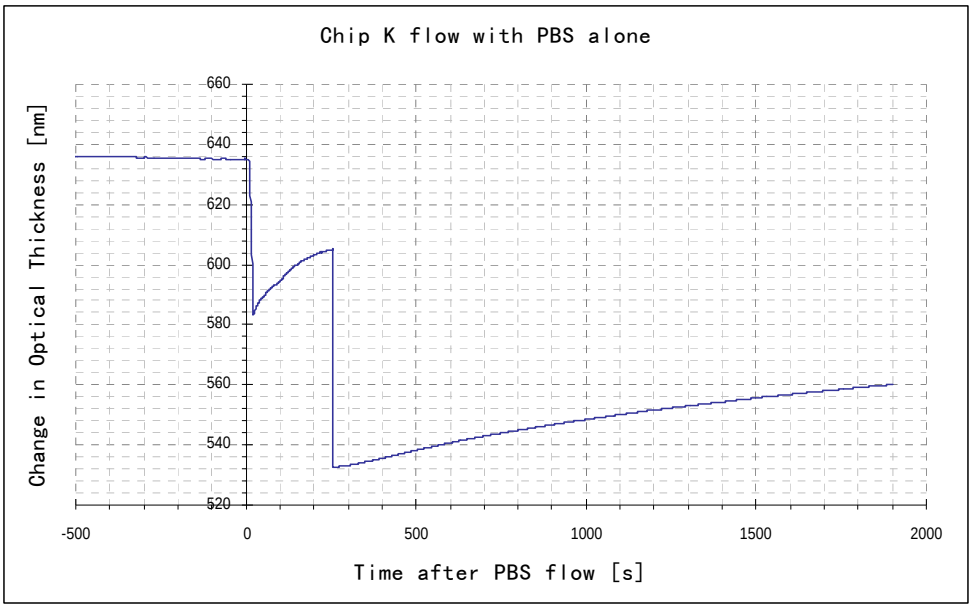


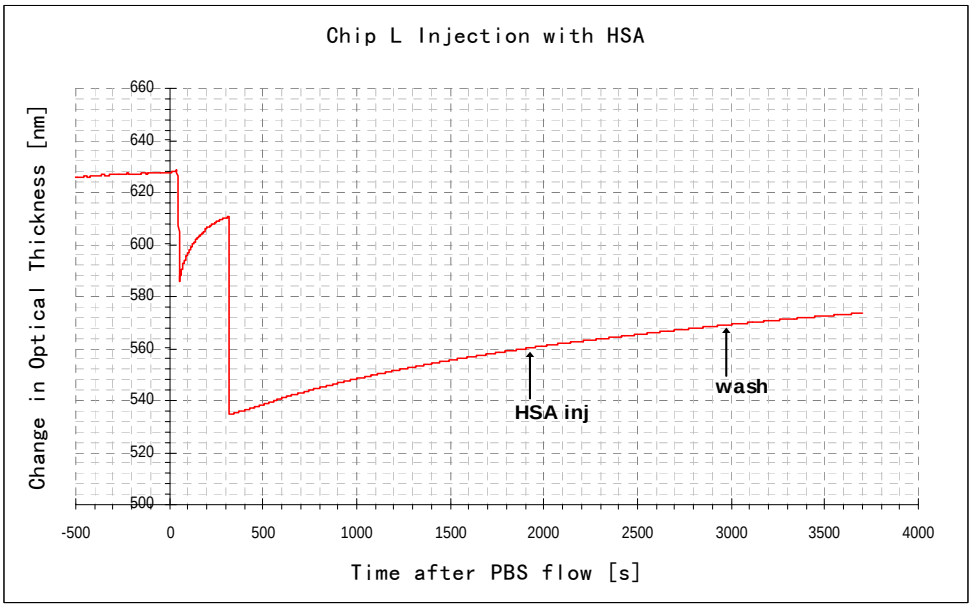
Figure C-6. Swelling behaviour as measured by RIfS of the chitosan films with flow of 1x PBS.

C 1.4.3.2 Protein Binding

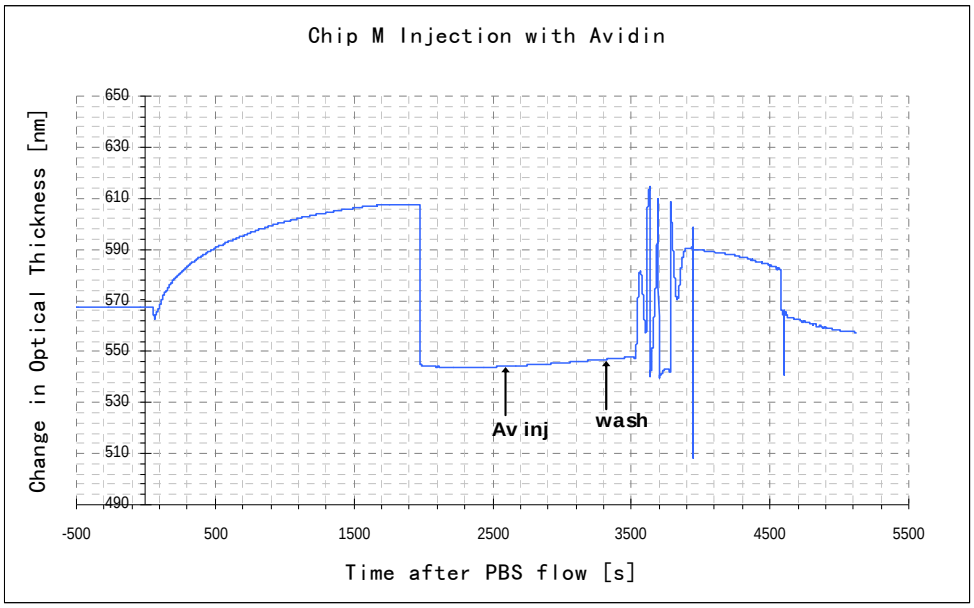
Next the interaction of proteins with the base chitosan layer was examined so that a general reading of protein capture could be determined. Several different model proteins were chosen to observe the effects of varying molecular size and charge. Representative samples of the reflectometric profiles following protein injection are shown in Figure C-7.



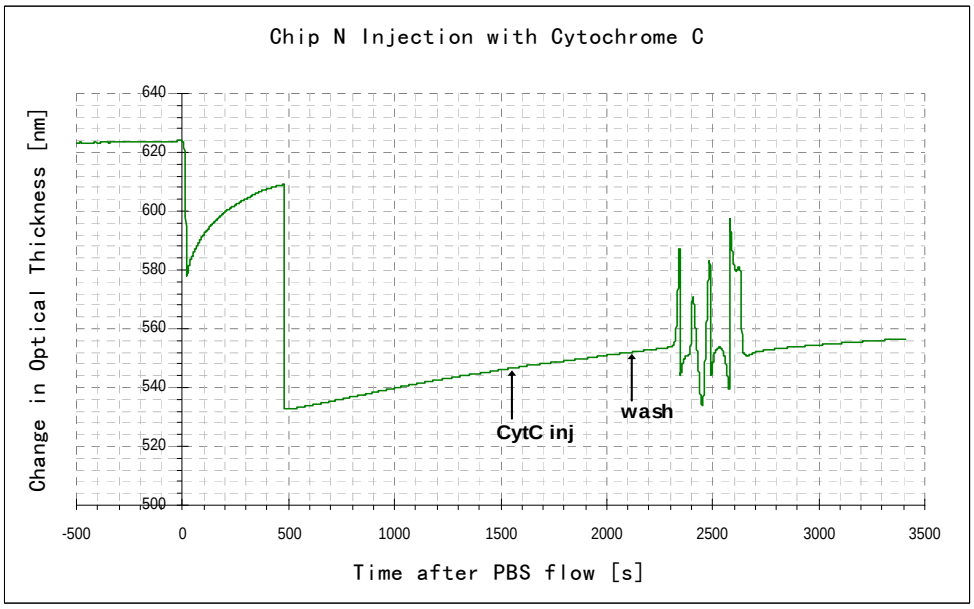
(a)



(b)



(c)



(d)

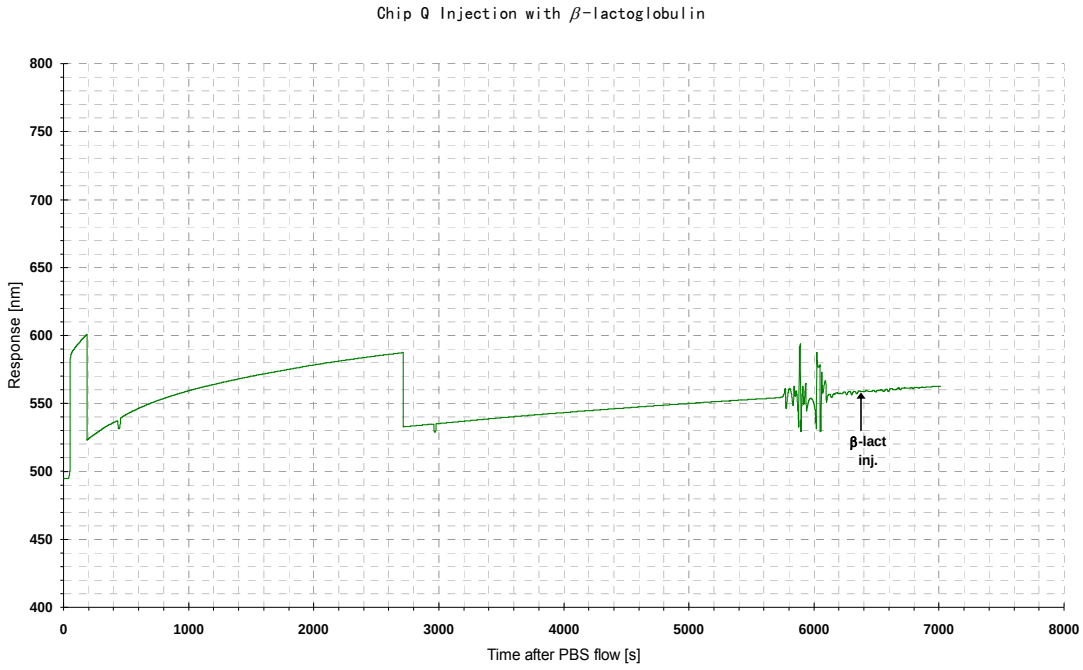
Figure C-7. Response in RIfS signal of chitosan films to injection of various model proteins. Injection of (a) PBS solution, (b) human serum albumin, (c) avidin, and (d) cytochrome *C*.

Evident from these graphs is that in each case there was once again a dropping off of the signal at some point during the reading. It was attempted to have the sample injection be at a time when the system was at steady-state,

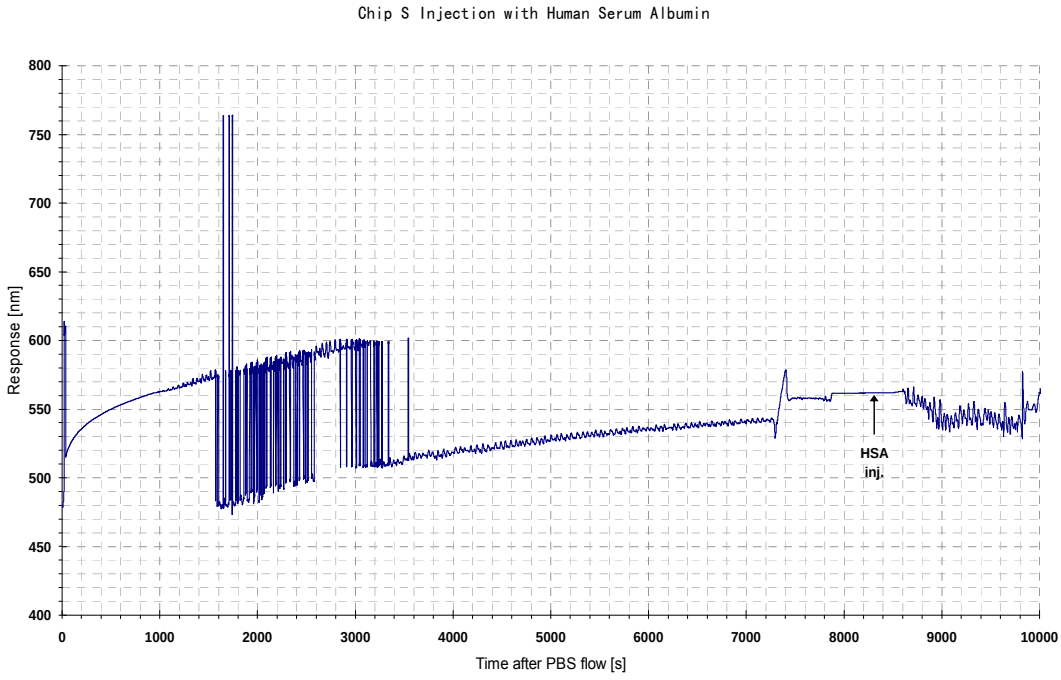
however as in some cases there was a continuous drift of the signal, this could not always be achieved. Consistently between samples, no change could be detected with the protein binding. A close inspection of the area where one would have expected an interaction with the surface to result in change in the signal pattern, none could be detected. A wash step using a solution of SDS/glycine HCl was used to regenerate the surfaces, and this had a noticeable effect visible in the detection spectrum. It is also worthwhile noting that following the wash with stripping solution, the baseline did not always return to its previous value prior to the introduction of protein or washing 'noise.'

C 1.4.3.3 Capture of Template on MIPs

While no direct protein adsorption was able to be detected on the chitosan films alone, it was still desired to examine if an imprinted matrix could be created using the hydrogel for protein support. Chips coated in a bilayer of chitosan and acrylamide were subjected to analysis by RIfS with protein solutions of the corresponding template. Sample spectra are shown in Figure C-8.


Chip Q Injection with β-lactoglobulin
Response [nm]
Time after PBS flow [s]
β-lact inj.

(a)


Chip S Injection with Human Serum Albumin
Response [nm]
Time after PBS flow [s]
HSA inj.

(b)

(c)

Figure C-8. Protein binding on MIP chitosan-acrylamide films: (a) *β*-lactoglobulin, (b) human serum albumin, and (c) a close-up of the two responses near the injection time.

Once again the collected data made it difficult to draw conclusive results from. The observations of signal drop off continued, and in some cases worsened, making very long (e.g. up to 24 h) equilibration times necessary, and estimated for overall baseline stability based on local patterns. After a steady-state was reached, there still seemed to be no detectable change on the surface as a result of protein binding. It is not clear if this result was due to the lack of any interaction with the surface, or due to non-detectability of the binding events. One would expect at least some level of protein binding to occur, as even some will adhere to the surface non-specifically. However, due to the proven sensitivity of RIfS detection, we would also expect to be able to easily detect even small changes in the optical thickness of these polymer layers. It is also evident that the noise level can be very high, as seen in the above figures, and this may cause significant masking of the signal changes, as the general fluctuation may overlap or in the

very least make it difficult to judge any variation in signal due to actual changes taking place on the sensor surface.

Supplementary References

[i] Lu, C., Czanderna, A.W. Applications of piezoelectric quartz crystal microbalances. *Methods and Phenomena, Vol. 7*. Elsevier Science Publishers. pp 1-393. 1984.

[ii] O'Sullivan, C. K., Guilbault, G. G. "Commercial quartz crystal microbalances – theory and applications." Biosensors and Bioelectronics. 14:8-9. pp 663-670. 1999.

[iii] Sauerbrey, Günter. "Verwendung von Schwingquarzen zur Wägung dünner Schichten und zur Mikrowägung." Zeitschrift für Physik. 155:2. pp 206-222. 1959.

[iv] Lu, C.-S., Lewis, O. "Investigation of film-thickness determination by oscillating quartz resonators with large mass load." Journal of Applied Physics. 43:11. pp 4385-4390. 1972.

[v] "QCM-D Technology." *http://www.q-sense.com/qcm-d-technology*. Q Sense AB. Västra Frölunda, Sweden. (accessed 28 January 2013)

[vi] Sittel, K., Rouse, Jr, P.E., Bailey, E.D. "Method for Determining the Viscoelastic Properties of Dilute Polymer Solutions at Audio-Frequencies." Journal of Applied Physics. 25:10. pp 1312-1320. 1954.

[vii] Hirao, M., Ogi, H., Fukuoka, H. "Resonance EMAT system for acoustoelastic stress measurement in sheet metals." Review of Scientific Instruments. 64:11. pp 3198-3205. 1993.

[viii] Rodahl, M., Kasemo, B. "A simple setup to simultaneously measure the resonant frequency and the absolute dissipation factor of a quartz crystal microbalance." Review of Scientific Instruments. 67:9. pp 3238-3241. 1996.

[ix] Bruckenstein, S., Shay, M. "Experimental aspects of use of the quartz crystal microbalance in solution." Electrochimica Acta. 30:10. pp 295-1300. 1985.

[x] Ward, Michael D., Buttry, Daniel A. "In Situ Interfacial Mass Detection with Piezoelectric Transducers." Science. 249:4972. pp 1000-1007. 1990.

[xi] Lee, M.-H., Thomas, J.L., Tseng, H.-Y., Lin, W.-C., Liu, B.-D., Lin, H.-Y. "Sensing of Digestive Proteins in Saliva with a Molecularly Imprinted Poly(ethylene-co-vinyl alcohol) Thin Film Coated Quartz Crystal Microbalance Sensor." ACS Applied Materials & Interfaces. 3:8. pp 3064-3071. 2011.

[xii] Reddy, S.M., Phan, Q.T., El-Sharif, H., Govada, L., Stevenson, D., Chayen, N.E. "Protein Crystallization and Biosensor Applications of Hydrogel-Based Molecularly Imprinted Polymers." Biomacromolecules. 13:12. pp 3959-3965. 2012.

[xiii] Matsuguchi, M., Uno, T. "Molecular imprinting strategy for solvent molecules and its application for QCM-based VOC vapor sensing." Sensors and Actuators B: Chemical. 113:1. pp 94-99. 2006.

[xiv] Kriz, D., Ramström, O., Mosbach, K. "Peer Reviewed: Molecular Imprinting: New Possibilities for Sensor Technology." Analytical Chemistry. 69:11. pp 345A-349A. 1997.

[xv] Malitesta, C., Losito, I., Zambonin, P.G. "Molecularly Imprinted Electrosynthesized Polymers: New Materials for Biomimetic Sensors." Analytical Chemistry. 71:7. pp 1366-1370. 1999.

[xvi] Haupt, K., Noworyta, K., Kutner, W. "Imprinted polymer-based enantioselective acoustic sensor using a quartz crystal microbalance." Analytical Communications. 36:11-12. pp 391-393. 1999.

[xvii] Friggeri, A., Kobayashi, H., Shinkai, S., Reinhoudt, D.N. "From Solutions to Surfaces: A Novel Molecular Imprinting Method Based on the Conformational Changes of Boronic-Acid-Appended Poly(L-lysine)." Angewandte Chemie. 113:24. pp 4865-4867. 2001.

[xviii] Liu, F., Liu, X., Ng, S.-C., Chan, H.S.-O. "Enantioselective molecular imprinting polymer coated QCM for the recognition of l-tryptophan." Sensors and Actuators B: Chemical. 113:1. pp 234-240. 2006.

[xix] Kanekiyo, Y., Inoue, K., Ono, Y., Sano, M., Shinkai, S., Reinhoudt, D.N. "'Molecular-imprinting' of AMP utilising the polyion complex formation process as detected by a QCM system." Journal of the Chemical Society, Perkin Transactions 2. 0:12. pp 2719-2722. 1999.

[xx] Kanekiyo, Y., Sano, M., Iguchi, R., Shinkai, S. "Novel nucleotide-responsive hydrogels designed from copolymers of boronic acid and cationic units and their applications as a QCM resonator system to nucleotide sensing." Journal of Polymer Science Part A: Polymer Chemistry. 38:8. pp 1302-1310. 2000.

[xxi] Dickert, F., Achatz, P., Halikias, K. "Double molecular imprinting - a new sensor concept for improving selectivity in the detection of polycyclic aromatic hydrocarbons (PAHs) in water." Fresenius' Journal of Analytical Chemistry. 371:1. pp 11-15. 2001.

[xxii] Wu, A.-H., Syu, M.-J. "Synthesis of bilirubin imprinted polymer thin film for the continuous detection of bilirubin in an MIP/QCM/FIA system." Biosensors and Bioelectronics. 21:12. pp 2345-2353. 2006.

[xxiii] Apodaca, D.C., Pernites, R.B., Ponnapati, R.R., Del Mundo, F.R., Advincula, R.C. "Electropolymerized Molecularly Imprinted Polymer Films of a Bis-Terthiophene Dendron: Folic Acid Quartz Crystal Microbalance Sensing." ACS Applied Materials & Interfaces. 3:2. pp 191-203. 2010.

[xxiv] Reimhult, K., Yoshimatsu, K., Risveden, K., Chen, S., Ye, L., Krozer, A. "Characterization of QCM sensor surfaces coated with molecularly imprinted nanoparticles." Biosensors and Bioelectronics. 23:12. pp 1908-1914. 2008.

[xxv] Iqbal, N., Lieberzeit, P.A. "Chapter 9 - Artificial Receptors for Mass-Sensitive Sensors: Targeting Analytes from Surfaces, Nanoparticles, and Bioanalytes by Molecular Imprinting." *in* Molecularly Imprinted Sensors. Eds. Li, S., Ge, Y., Piletsky, S.A., Lunec, J. pp 195-235. Elsevier. Amsterdam, NL. 2012.

[xxvi] Hirayama, K., Sakai, Y., Kameoka, K. "Synthesis of polymer particles with specific lysozyme recognition sites by a molecular imprinting technique." Journal of Applied Polymer Science. 81:14. pp 3378-3387. 2001.

[xxvii] Liu, S., Zhou, D., Guo, T. "Construction of a novel macroporous imprinted biosensor based on quartz crystal microbalance for ribonuclease A detection." Biosensors and Bioelectronics. 42:0. pp 80-86. 2013.

[xxviii] Kugimiya, A., Takeuchi, T. "Molecularly Imprinted Polymer-Coated Quartz Crystal Microbalance for Detection of Biological Hormone." Electroanalysis. 11:15. 1158-1160. 1999.

[xxix] Hayden, O., Dickert, F.L. "Selective Microorganism Detection with Cell Surface Imprinted Polymers." Advanced Materials. 13:19. pp 1480-1483. 2001.

[xxx] Dickert, F.L., Hayden, O., Halikias, K.P. "Synthetic receptors as sensor coatings for molecules and living cells." The Analyst. 126:6. pp 766-771. 2001.

[xxxi] Dickert, F. L., Hayden, O. "Bioimprinting of Polymers and Sol−Gel Phases. Selective Detection of Yeasts with Imprinted Polymers." Analytical Chemistry. 74:6. pp 1302-1306. 2002.

[xxxii] Hayden, O., Bindeus, R., Haderspöck, C., Mann, K.-J., Wirl, B., Dickert, F.L. "Mass-sensitive detection of cells, viruses and enzymes with artificial receptors." Sensors and Actuators B: Chemical. 91:1-3. pp 316-319. 2003.

[xxxiii] Dickert, F.L., Hayden, O., Bindeus, R., Mann, K.-J., Blaas, D., Waigmann, E. "Bioimprinted QCM sensors for virus detection—screening of plant sap." Analytical and Bioanalytical Chemistry. 378:8. pp 1929- 1934. 2004.

[xxxiv] Jenik, M., Schirhagl, R., Schirk, C., Hayden, O., Lieberzeit, P., Blaas, D., Paul, G., Dickert, F.L. "Sensing Picornaviruses Using Molecular Imprinting Techniques on a Quartz Crystal Microbalance." Analytical Chemistry. 81:13. pp 5320-5326. 2009.

[xxxv] Lin, T.-Y., Hu, C.-H., Chou, T.-C. "Determination of albumin concentration by MIP-QCM sensor." Biosensors and Bioelectronics. 20:1. pp 75-81. 2004.

[xxxvi] Percival, C. J., Stanley, S., Braithwaite, A., Newton, M. I., McHale, G. "Molecular imprinted polymer coated QCM for the detection of nandrolone." Analyst. 127:8. pp 1024-1026. 2002.

[xxxvii] Ersöz, A., Denizli, A., Özcan, A., Say, R. "Molecularly imprinted ligand-exchange recognition assay of glucose by quartz crystal microbalance." Biosensors and Bioelectronics. 20:11. pp 2197-2202. 2005.

[xxxviii] Lubbers, D. W., Opitz, N. "Eine neue pCO2-bzw: pO2-Messonde zur Messung des pCO2 oder pO2 von Gasen und Flüssigkeiten." Zeitschrift Für Naturforschung C. 30:4. 532-533. 1975.

[xxxix] Brecht, A., Gauglitz, G. "Optical probes and transducers." Biosensors and Bioelectronics. 10:9-10. pp 923-936. 1995.

[xl] Homola, J., Yee, S.S., Gauglitz, G. "Surface plasmon resonance sensors: review." Sensors and Actuators B: Chemical. 54:1-2. pp 3-15. 1999.

[xli] Wood, R. W. "XLII. On a remarkable case of uneven distribution of light in a diffraction grating spectrum." Philosophical Magazine Series 6. 4:21. pp 396-402. 1902.

[xlii] Kretschmann, E., Raether, H. "Radiative decay of non radiative surface plasmons excited by light." Zeitschrift fuer Naturforschung, Teil A: Astrophysik, Physik und Physikalische Chemie. 23:12. pp 2135-2136. 1968.

[xliii] Otto, A. "Excitation of nonradiative surface plasma waves in silver by the method of frustrated total reflection." Zeitschrift für Physik A Hadrons and Nuclei. 216:4. pp 398-410. 1968.

[xliv] Gordon II, J. G., Ernst, S. "Surface plasmons as a probe of the electrochemical interface." Surface Science. 101:1-3. pp 499-506. 1980.

[xlv] Pockrand, I., Swalen, J. D., Gordon Ii, J. G., Philpott, M. R. "Surface plasmon spectroscopy of organic monolayer assemblies." Surface Science. 74:1. pp 237-244. 1978.

[xlvi] Pattnaik, P. "Surface plasmon resonance - Applications in understanding receptor-ligand interaction." Applied Biochemistry and Biotechnology. 126:2. pp 79-92. 2005.

[xlvii] Kretschmann, E. "Die Bestimmung optischer Konstanten von Metallen durch Anregung von Oberflächenplasmaschwingungen." Zeitschrift für Physik A Hadrons and Nuclei. 241:4. pp 313-324. 1971.

[xlviii] Lai, E.P.C., Fafara, A., VanderNoot, V.A., Kono, M., Polsky, B. "Surface plasmon resonance sensors using molecularly imprinted polymers for sorbent assay of theophylline, caffeine, and xanthine." Canadian Journal of Chemistry. 76:3. pp 265-273. 1998.

[xlix] Slinchenko, O., Rachkov, A., Miyachi, H., Ogiso, M., Minoura, N. "Imprinted polymer layer for recognising double-stranded DNA." Biosensors and Bioelectronics. 20:6. pp 1091-1097. 2004.

[l] Diltemiz, S.E., Denizli, A., Say, R. "Molecularly imprinted ligand-exchange recognition assay of DNA by SPR system using guanosine and guanine recognition sites of DNA." Sensors and Actuators B. 133:2. pp 484-488. 2008.

[li] Wu, W., Shen, J., Li, Y., Zhu, H., Banerjee, P., Zhou, S. "Specific glucose-to-SPR signal transduction at physiological pH by molecularly imprinted responsive hybrid microgels." Biomaterials. 33:29. pp 7115-7125. 2012.

[lii] Taguchi, Y., Takano, E., Takeuchi, T. "SPR Sensing of Bisphenol A Using Molecularly Imprinted Nanoparticles Immobilized on Slab Optical Waveguide with Consecutive Parallel Au and Ag Deposition Bands Coexistent with Bisphenol A-Immobilized Au Nanoparticles." Langmuir. 28:17. pp 7083-7088. 2012.

[liii] Sener, G., Uzun, L., Say, R., Denizli, A. "Use of molecular imprinted nanoparticles as biorecognition element on surface plasmon resonance sensor." Sensors and Actuators B: Chemical. 160:1. pp 791-799. 2011.

[liv] Huang, S.-C., Lee, G.-B., Chien, F.-C., Chen, S.-J., Chen, W.-J., Yang, M.-C. "A microfluidic system with integrated molecular imprinting polymer films for surface Plasmon resonance detection." Journal of Micromechanics and Microengineering." 16:7. pp 1251-1257. 2006.

[lv] Zheng, H.F., Du, X.Z. "Enhanced Binding and Biosensing of Carbohydrate-Functionalized Monolayers to Target Proteins by Surface Molecular Imprinting."Journal of Physical Chemistry B. 113:32. pp 11330-11337. 2009.

[lvi] Lautner, G., Kaev, J., Reut, J., Öpik, A.. Rappich, J., Syritski, V., Gyurcsányi, R.E. "Selective Artificial Receptors Based on Micropatterned Surface-Imprinted Polymers for Label-Free Detection of Proteins by SPR Imaging." Advanced Functional Materials. 21:3. pp 591-597. 2011.

[lvii] Tokareva, I,m Tokarev, I., Minko, S., Hutter, E., Fendler, J.H. "Ultrathin molecularly imprinted polymer sensors employing enhanced transmission surface plasmon resonance spectroscopy." Chemical Communications. 0:31. pp 3343-3346. 2006.

[lviii] Matsui, J., Takayose, M., Akamatsu, K., Nawafune, H., Tamaki, K., Sugimoto, N. "Molecularly imprinted nanocomposites for highly sensitive SPR detection of a non-aqueous atrazine sample." Analyst. 134:1. pp 80-86. 2009.

[lix] Matsui, J., Akamatsu, K., Nishiguchi, S., Miyoshi, D., Nawafune, H., Tamaki, K., Sugimoto, N. "Composite of Au Nanoparticles and Molecularly Imprinted Polymer as a Sensing Material." Analytical Chemistry. 76:5. pp 1310-1315. 2004.

[lx] Riskin, M., Tel-Vered, R., Frasconi, M., Yavo, N., Willner, I. "Stereoselective and Chiroselective Surface Plasmon Resonance (SPR) Analysis of Amino Acids by Molecularly Imprinted Au-Nanoparticle Composites." Chemistry – A European Journal. 16:24. pp 7114-7120. 2010.

[lxi] Zhang, W., He, X.-W., Chen, Y., Li, W.-Y., Zhang, Y.-K. "Composite of CdTe quantum dots and molecularly imprinted polymer as a sensing material for cytochrome c." Biosensors and Bioelectronics. 26:5. pp 2553-2558. 2011.

[lxii] Kim, Y., Jeon, J.B., Chang, J.Y. "CdSe quantum dot-encapsulated molecularly imprinted mesoporous silica particles for fluorescent sensing of bisphenol A." Journal of Materials Chemistry. 22:45. pp 24075-24080. 2012.

[lxiii] Biteen, J.S., Lewis, N.S., Atwater, H.A., Mertens, H., Polman, A. "Spectral tuning of plasmon-enhanced silicon quantum dot luminescence." Applied Physics Letters. 88:13. pp 131109-1-131109-3. 2006.

[lxiv] Hoa, X.D., Kirk, A.G., Tabrizian, M. "Towards integrated and sensitive surface plasmon resonance biosensors: A review of recent progress." Biosensors and Bioelectronics. 23:2. pp 151-160. 2007.

[lxv] Lotierzo, M., Henry, O. Y. F., Piletsky, S., Tothill, I., Cullen, D., Kania, M., Hock, B.. Turner, A. P. F. "Surface plasmon resonance sensor for domoic acid based on grafted imprinted polymer." Biosensors and Bioelectronics. 20:2. pp 145-152. 2004.

[lxvi] Piacham, T., Josell, Å., Arwin, H., Prachayasittikul, V., Ye, L. "Molecularly imprinted polymer thin films on quartz crystal microbalance using a surface bound photo-radical initiator." Analytica Chimica Acta. 536:1-2. pp 191-196. 2005.

[lxvii] Zheng, H., Du, X. "Enhanced Binding and Biosensing of Carbohydrate-Functionalized Monolayers to Target Proteins by Surface Molecular Imprinting." The Journal of Physical Chemistry B. 113:32. pp 11330-11337. 2009.

[lxviii] Vikholm-Lundin, I., Pulli, T., Albers, W.M., Tappura, K. "A comparative evaluation of molecular recognition by monolayers composed of synthetic receptors or oriented antibodies." Biosensors and Bioelectronics. 24:4. pp 1036-1038. 2008.

[lxix] Zhang, Z., Long Y., Nie, L., Yao, S. "Molecularly imprinted thin film self-assembled on piezoelectric quartz crystal surface by the sol–gel process for protein recognition." Biosensors and Bioelectronics. 21:7. pp 1244-1251. 2006.

[lxx] Matsunaga, T., Hishiya, T., Takeuchi, T. "Surface plasmon resonance sensor for lysozyme based on molecularly imprinted thin films." Analytica Chimica Acta. 591:1. pp 63-67. 2007.

[lxxi] Proll, G., Markovic, G., Steinle, L., Gauglitz, G. "Reflectometric Interference Spectroscopy." *in* Biosensors and Biodetection. Methods and Protocols Vol. 503 – Optical Based Sensors. Eds. Rasooly, A., Herold, K.E. *Methods in Molecular Biology*. pp 167-178. Humana Press. New York. 2009.

[lxxii] Hecht, E. Optics. 2nd Edition. Ed. Zając, A. Addison-Wesley. Reading, MA. 1987.

[lxxiii] Bosman, A. J., Havinga, E. E. "Temperature Dependence of Dielectric Constants of Cubic Ionic Compounds." Physical Review. 129:4. pp 1593-1600. 1963.

[lxxiv] Kröger, K., Jung, A., Reder, S., Gauglitz, G. "Versatile biosensor surface based on peptide nucleic acid with label free and total internal reflection fluorescence detection for quantification of endocrine disruptors." Analytica Chimica Acta. 469:1. pp 37-48. 2002.

[lxxv] Birkert, O., Gauglitz, G. "Development of an assay for label-free high-throughput screening of thrombin inhibitors by use of reflectometric interference spectroscopy." Analytical and Bioanalytical Chemistry. 372:1. pp 141-147. 2002.

[lxxvi] Albrecht, C., Fechner, P., Honcharenko, D., Baltzer, L., Gauglitz, G. "A new assay design for clinical diagnostics based on alternative recognition elements." Biosensors and Bioelectronics. 25:10. pp 2302-2308. 2010.

[lxxvii] Choi, H.W., Sakata, Y., Kurihara, Y., Ooya, T., Takeuchi, T. "Label-free detection of C-reactive protein using reflectometric interference spectroscopy-based sensing system." Analytica Chimica Acta. 728:0. pp 64-68. 2012.

[lxxviii] Kumpf, M., Gauglitz, G. "Biomolecular interaction analysis under electrophoretic flow conditions." Analytical & Bioanalytical Chemistry. 384:5. pp 1129-1133. 2006.

[lxxix] Kurihara, Y., Takama, M., Masubuchi, M., Ooya, T., Takeuchi, T. "Microfluidic reflectometric interference spectroscopy-based sensing for exploration of protein–protein interaction conditions." Biosensors and Bioelectronics. 40:1. pp 247-251. 2013.

[lxxx] Kurihara, Y., Takama, M., Sekiya, T., Yoshihara, Y., Ooya, T., Takeuchi, T. "Fabrication of Carboxylated Silicon Nitride Sensor Chips for Detection of Antigen–Antibody Reaction Using Microfluidic Reflectometric Interference Spectroscopy." Langmuir. 28:38. pp 13609-13615. 2012.

[lxxxi] Merkl, S., Vornicescu, D., Dassinger, N., Kehrel, M., Harpel, S., Keusgen, M. "Approaches to the detection of whole cells using reflectometric interference spectroscopy." physica status solidi (a). 209:5. pp 864-870. 2012.

[lxxxii] Möhrle, B.P., Köhler, K., Jaehrling, J., Brock, R., Gauglitz, G. "Label-free characterization of cell adhesion using reflectometric interference spectroscopy (RIfS)." Analytical & Bioanalytical Chemistry. 384:2. pp 407-413. 2006.

[lxxxiii] Kumeria, T., Kurkuri, M.D., Diener, K.R., Parkinson, L., Losic, D. "Label-free reflectometric interference microchip biosensor based on nanoporous alumina for detection of circulating tumour cells." Biosensors and Bioelectronics. 35:1. pp 167-173. 2012.

[lxxxiv] Lü, X., Huang, Y., Ma, C. "Evaluation of Protein Adsorption on Chitosan Surfaces with Reflectometry Interference Spectroscopy." Sensors. 1:5. pp 148-160. 2001.

[lxxxv] Tang, Y., Zhen, L., Liu, J., Wu, J. "Rapid Antibiotic Susceptibility Testing in a Microfluidic pH Sensor." Analytical Chemistry. 85:5. pp 2787-2794. 2013.

[lxxxvi] Kolarov, F., Niedergall, K., Bach, M., Tovar, G., Gauglitz, G. "Optical sensors with molecularly imprinted nanospheres: a promising approach for robust and label-free detection of small molecules." Analytical and Bioanalytical Chemistry. 401:10. pp 3245-3252. 2012.

[lxxxvii] Belmont, A.-S., Jaeger, S., Knopp, D., Niessner, R., Gauglitz, G., Haupt, K. "Molecularly imprinted polymer films for reflectometric interference spectroscopic sensors." Biosensors and Bioelectronics. 22:12. pp 3267-3272. 2007.

[lxxxviii] Nopper, D., Lammershop, O., Wulff, G., Gauglitz, G. "Amidine-based molecularly imprinted polymers—new sensitive elements for chiral chemosensors." Analytical & Bioanalytical Chemistry. 377:4. pp 608-613. 2003.

[lxxxix] Dunn, P.M. "Perinatal lessons from the past: James Lind (1716-94) of Edinburgh and the treatment of scurvy." Archives of Disease in Childhood - Fetal and Neonatal Edition. 76:1. pp F64-F65. 1997.

[xc] Fisher, R.A. "The Arrangement of Field Experiments." Journal of the Ministry of Agriculture of Great Britain. 33. pp 503-513. 1926.

[xci] Fisher, R.A. The Design of Experiments. Hafner Pub. Co. New York, NY. 1935.

[xcii] Singh, B., Kumar, R., Ahuja, N. "Optimizing Drug Delivery Systems Using Systematic "Design of Experiments." Part I: Fundamental Aspects." Critical Reviews in Therapeutic Drug Carrier Systems. 22:1. pp 27–105. 2004.

[xciii] Barker, T.B. Quality by Experimental Design. *Quality and Reliability, Vol. 43*. Eds. Schilling, E.G., Bingham, Jr, R.S., Rabinowitz, L., Witt, T. Marcel Dekker, Inc. New York, NY. 1994.

[xciv] Moen, R.D., Nolan, T.W., Provost, L.P. Quality Improvement Through Planned Experimentation. Second Edition. McGraw-Hill. Toronto, ON. 1999.

[xcv] Liyana-Pathirana, C., Shahadi, F. "Optimization of extraction of phenolic compounds from wheat using response surface methodology." Food Chemistry. 93:1. pp 47-56. 2005.

[xcvi] Hamada, M., Wu, C.F.J. "Analysis of designed experiments with complex aliasing." Journal of Quality Technology. 24:3. pp 130-137. 1992.

[xcvii] Amamoto, Y., Otsuka, H., Takahara, A. "Synthesis and Characterization of Polymeric Nanogels." *in* Nanotechnologies for the Life Sciences. Ed. Kumar, C. Wiley-VCH Verlag GmbH & Co. KGaA. Weinheim, DE. 2007.

[xcviii] Andreasen, S.Ø., Chong, S.-F., Wohl, B.M., Goldie, K.N., Zelikin, A.N. "Poly(vinyl alcohol) Physical Hydrogel Nanoparticles, Not Polymer Solutions, Exert Inhibition of Nitric Oxide Synthesis in Cultured Macrophages." Biomacromolecules. 14:5. pp 1687-1695. 2013.

[xcix] Saleem, Q., Liu, B., Gradinaru, C.C., Macdonald, P.M. "Lipogels: Single-Lipid-Bilayer-Enclosed Hydrogel Spheres." Biomacromolecules. 12:6. pp 2364-2374. 2011.

[c] Guerrero-Ramírez, L.G., Nuño-Donlucas, S.M., Cesteros, L.C., Katime, I. "Smart copolymeric nanohydrogels: Synthesis, characterization and properties." Materials Chemistry and Physics. 112:3. pp 1088-1092. 2008.

[ci] Lu, X., Sun, M., Barron, A.E. "Non-ionic, thermo-responsive DEA/DMA nanogels: Synthesis, characterization, and use for DNA separations by microchip electrophoresis." Journal of Colloid and Interface Science. 357:2. pp 345-353. 2011.

[cii] Hong, J.S., Stavis, S.M., DePaoli Lacerda, S.H., Locascio, L.E., Raghavan, S.R., Gaitan, M. "Microfluidic Directed Self-Assembly of Liposome−Hydrogel Hybrid Nanoparticles." Langmuir. 26:13. pp 11581-11588. 2010.

[ciii] Marguet, M., Sandre, O., Lecommandoux, S. "Polymersomes in "Gelly" Polymersomes: Toward Structural Cell Mimicry." Langmuir. 28:4. pp 2035-2043. 2011.

[civ] Kazakov, S., Kaholek, M., Teraoka, I., Levon, K. "UV-Induced Gelation on Nanometer Scale Using Liposome Reactor." Macromolecules. 35:5. pp 1911-1920. 2002.

[cv] An, S.Y., Bui, M.-P.N., Nam, Y.J., Han, K.N., Li, C.A., Choo, J., Lee, E.K., Katoh, S., Kumada, Y., Seong, G.H. "Preparation of monodisperse and size-controlled poly(ethylene glycol) hydrogel nanoparticles using liposome templates." Journal of Colloid and Interface Science. 331:1. pp 98-103. 2009.

[cvi] Maggi, F., Ciccarelli, S., Diociaiuti, M., Casciardi, S., Masci, G. "Chitosan Nanogels by Template Chemical Cross-Linking in Polyion Complex Micelle Nanoreactors." Biomacromolecules. 12:10. pp 3499-3507. 2011.

[cvii] Riaz, M. "Liposomes preparation methods." Pakistan Journal of Pharmaceutical Sciences. 9:1. pp 65-77. 1996.

[cviii] New, R.R.C. "Preparation of Liposomes." Liposomes: A Practical Approach. Ed. New, R.R.C. pp. 98-99. IRL Press. Oxford, UK. 1990.

[cix] Hoischen, C., Fritsche, C., Gumpert, J., Westermann, M., Gura, K., Fahnert, B. "Novel Bacterial Membrane Surface Display System Using Cell Wall-Less L-Forms of Proteus mirabilis and Escherichia coli." Applied and Environmental Microbiology. 68:2. pp 525-531. 2002.

[cx] Wichelecki, D.J., McNew, T.M., Aygun, A., Torrey, K., Stephenson, L.D. "Detection of Liposome Lysis Utilizing an Enzyme-Substrate System." Applied Biochemistry and Biotechnology. 165:2. pp 548-558. 2011.

[cxi] Zhou, F., Neutra, M.R. "Antigen Delivery to Mucosa-Associated Lymphoid Tissues Using Liposomes as a Carrier." Bioscience Reports. 22:2. pp 355-369. 2002.

[cxii] Henriksen, I., Smistad, G., Karlsen, J. "Interactions between liposomes and chitosan." International Journal of Pharmaceutics. 101:3. pp 227-236. 1994.

[cxiii] Henriksen, I., Våagen, S.R., Sande, S.A., Smistad, G., Karlsen, J. "Interactions between liposomes and chitosan II: Effect of selected parameters on aggregation and leakage." International Journal of Pharmaceutics. 146:2. pp 193-203. 1997.

[cxiv] Manca, M.L., Manconi, M., Valenti, D., Lai, F., Loy, G., Matricardi, P., Fadda, A.M. "Liposomes coated with chitosan–xanthan gum (chitosomes) as potential carriers for pulmonary delivery of rifampicin." Journal of Pharmaceutical Sciences." 101:2. pp 566-575. 2012.

[cxv] Alamelu, S., Panduranga Rao, K. "Liposomes sequestered in chitosan gel as a delivery device for dapsone." Carbohydrate Polymers. 24:3. pp 215-221. 1994.

[cxvi] Alamelu, S., Panduranga Rao, K.P. "Studies on the carboxymethyl chitosan-containing liposomes for their stability and controlled release of Dapsone." Journal of Microencapsulation. 8:4. pp 505-519. 1991.

[cxvii] Choi, H.W., Sakata, Y., Kurihara, Y., Ooya, T., Takeuchi, T. "Label-free detection of C-reactive protein using reflectometric interference spectroscopy-based sensing system." Analytica Chimica Acta. 728:0. pp 64-68. 2012.

[cxviii] Sunayama, H., Ooya, T., Takeuchi, T. "Fluorescent protein recognition polymer thin films capable of selective signal transduction of target binding events prepared by molecular imprinting with a post-imprinting treatment." Biosensors and Bioelectronics. 26:2. pp 458-462. 2010.

[cxix] Jiang, H., Su, W., Caracci, S., Bunning, T. J., Cooper, T., Adams, W. W. "Optical waveguiding and morphology of chitosan thin films." Journal of Applied Polymer Science. 61:7. pp 1163-1171. 1996.

[cxx] Koralewski, M., Bodek, K.H., Marczewska, K. "Optical Properties of Chitosan in Aqueous Solution." Polish Chitin Society. Monograph XI. pp 29-39. 2006.

[cxxi] Zacher, T., Wischerhoff, E. "Real-Time Two-Wavelength Surface Plasmon Resonance as a Tool for the Vertical Resolution of Binding Processes in Biosensing Hydrogels." Langmuir. 18:5. pp 1748-1759. 2002.

[cxxii] Murray, C.A., Dutcher, J.R. "Effect of Changes in Relative Humidity and Temperature on Ultrathin Chitosan Films." Biomacromolecules. 7:12. pp 3460-3465. 2006.

[cxxiii] Orelma, H., Filpponen, I., Johansson, L.-S., Laine, J., Rojas, O.J. "Modification of Cellulose Films by Adsorption of CMC and Chitosan for Controlled Attachment of Biomolecules." Biomacromolecules. 12:12. pp 4311-4318. 2011.