Development of protein-based hydrogels as encapsulation matrices for *Lactobacillus casei* ATCC 393

BY

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April 2015

A thesis submitted to McGill University

in partial fulfillment of the requirements for the degree of Master of Science

Abstract

Bio-based polymers have been a topic of high interest in the past decades due to their biocompatibility, biodegradability and their relative low toxicity. Proteins, polysaccharides and lipids are the main biological materials utilized for their synthesis. The diversity of the functional groups of proteins allows for a wide variety of chemical and physical linkages and structures. The subject of this thesis focuses on the synthesis and characterization of physically crosslinked protein-based hydrogels. These hydrogels have been utilized in the biomedical and food industry as they generally exhibit excellent biocompatibility and therefore can be used for applications such as tissue engineering and drug delivery. Moreover due to their high nutritional value and ability to form gels, protein-based hydrogels are highly used in the food industry.

As a part of this thesis, the synthesis and characterization of physically crosslinked pea protein isolates-alginate (PPI-alginate) and canola protein isolates-alginate (CPI-alginate) hydrogels microspheres for encapsulation applications were performed. In addition, the effect of extra polyelectrolyte complexation (PEC) with another biopolymer, chitosan, was also studied. The thermogravimetric analysis and the differential scanning calorimetry results showed that CPI microspheres have a similar thermal stability as PPI microspheres. The swelling tests showed that in general, CPI microspheres have a higher swelling capacity than PPI microspheres in all swelling media (distilled water, simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)). Furthermore, degradation test were performed in simulated gastric fluid and simulated intestinal fluid media with enzymes to explore the effect of PEC on the stability of hydrogels. The results indicated that PEC had no influence on the stability of hydrogels in SGF but significantly delayed the degradation of hydrogels under SIF conditions. These findings suggest that PEC could be utilized to modify protein-alginate hydrogels to create matrices for prolonged release applications.

The PPI-alginate hydrogel matrices were utilized in a subsequent study to encapsulate *Lactobacillus casei* ATCC 393, an acid sensitive probiotic strain. The goal of this study was to target applications related to the delivery of probiotics to the human gut. The PPI-alginate hydrogels were selected not only because the high nutritional value of PPI but also the due to the compatibility of PPI with the specific probiotic strain. A high encapsulation yield (85.69% ± 4.82) was achieved by using this matrix. Moreover, the *L. casei* loaded PPI-alginate hydrogel microspheres were freeze dried and tested for long term storage. A robust number (59.9 % ± 17.4) of viable cells was detected after 84 days of storage at -15 ℃. Both the fresh microspheres and stored hydrogel microspheres have been tested in simulated gastrointestinal to verify the probiotics' survival rate as well as their release rate. The *L.casei* in stored hydrogel microspheres were more sensitive to the acidic gastric conditions. However, both types of microspheres displayed significant protective effects. The release tests showed a rapid release of bacteria in the first 20 min of digestion. The complete release of *L. casei* from the matrices was observed 60 min after their contact with the SIF media.

Résumé

Les polymères bio-sourcés ont été un sujet de grand intérêt au cours des dernières décennies en raison de leur biocompatibilité, biodégradabilité et de leur faible toxicité. Les protéines, les polysaccharides ainsi que les lipides sont les principaux matériaux biologiques utilisés pour leur synthèse. La diversité des groupes fonctionnels des protéines permet une grande variété de liens physiques et chimiques permettant la formation d'une vaste gamme de structure. L'objet de cette thèse implique l'utilisation d'isolats de protéines dans la synthèse d'hydrogels réticulés physiquement. Les hydrogels dont la composante principale constitue les protéines sont utilisés dans l'industrie biomédicale puisqu'ils présentent généralement une excellente biocompatibilité et peuvent donc être utilisés pour des applications telles que l'ingénierie tissulaire ainsi qu'agent de libération contrôlé. De plus, en raison de leur haute valeur nutritive et de leur capacité à former des gels, ils sont abondamment utilisés dans l'industrie alimentaire.

Dans le cadre de cette étude, des hydrogels micro-sphériques réticulés physiquement à base d'isolats de protéines de pois et d'alginate (IPP-alginate) ainsi que d'isolats de protéines de canola et d'alginate (IPC-alginate) furent synthétisés dans le but de les utiliser afin d'encapsuler des bactéries probiotiques. De plus, l'effet de la complexation polyélectrolyte (CPE) avec un autre biopolymère, la chitosane, a également été étudié. Les résultats obtenus par l'analyse thermogravimétrique ainsi que l'analyse calorimétrique différentielle à balayage ont montrés que les hydrogels synthétisés à partir de protéines de pois ont une stabilité thermique similaire à ceux ayant été synthétisés avec des protéines de canola. Par contre, les hydrogels synthétisés à partir de protéines de canola ont une capacité de gonflement à l'équilibre plus élevée que les hydrogels synthétisés à partir de protéines de pois et ce, dans tous les médias testés (eau distillée, liquide gastrique simulé (LGS) et liquide intestinal simulé (LIS)). De plus, des enzymes furent ajoutés au LGS ainsi qu'au LIS afin d'effectuer des tests de dégradation. Lors de ces tests, il fut démontré que l'effet de la CPE est nul lors des

essais dans le LIS mais retarde la dégradation dans le LGS. Ces résultats suggèrent que la CPE peut être utilisée afin de modifier les hydrogels à base de protéines et d'alginates afin de créer des matrices pour des applications nécessitant de la libération contrôlée.

Des hydrogels à base de protéines et d'alginate ont été utilisées afin d'encapsuler des probiotiques (*Lactobacillus casei* ATCC 393). Les hydrogels IPP-alginate ont été choisis dus à la valeur nutritive des protéines de pois ainsi qu'à leur compatibilité élevée avec *L.casei*. Un rendement élevé d'encapsulation (85,69 % ± 4.82) a été réalisé à l'aide de ces matrices. Après l'étape d'encapsulation, les matrices furent lyophilisées et entreposées afin de vérifier leur stabilité lors de l'entreposage à long terme. Un bon nombre de cellules viables (59,9 % ± 17,4) a été détecté après 84 jours de stockage à -15 °C.

Les hydrogels non-lyophilisés ainsi que les hydrogels lyophilisés contenant des probiotiques ont été testés dans le liquide gastro-intestinal simulé pour vérifier le taux de survie des probiotiques ainsi que leur vitesse de libération. Les résultats ont montrés que le taux de survie de *L.casei* encapsulé dans les matrices lyophilisées est moindre dans le liquide gastrique simulé que lorsqu'encapsulé dans les matrices non-lyophilisées. Toutefois, les deux types d'hydrogels ont des effets protecteurs significatifs par rapport aux probiotiques non encapsulés. Les tests de libération contrôlés ont montré une libération rapide des bactéries dans les 20 premières minutes de digestion. La libération complète de *L. casei* par les matrices a été observée après 60 minutes après leur contact initial avec le LIF.

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ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my supervisor Dr. Marie-Josée Dumont for giving me the opportunity to work in her lab and for her invaluable guidance. Thank you for your unwavering support, motivation and encouragement throughout the duration of this research.

I am also grateful to Dr. Michael Ngadi, Dr. Valérie Orsat and Dr. Suha Jabaji-Hare for their permission to access their laboratory equipment.

I would like to thank Mr. Yvan Gariépy, Mr. François Gagné-Bourque, Mrs. Mamta Rani and Miss Kiruba Krishnaswamy for their technical expertise. You have provided an invaluable help associated with the use of state-of-the-art techniques such as scanning electron microscopy, Fourier transform infrared spectroscopy, freeze-drying and microbiological tests.

I would also like to thank the following colleagues and friends: Agneev Mukherjee, Cheng Lu, Gordon Gong, Parsley Li, Peter Olusola Adewale, Ogan Mba, Shenqiang Hu, Sunli Chen, Weida Shi and Yang Li. Thank you for your suggestions and support throughout my graduate life.

The McGill start-up fund and the NSERC Discovery Grant are gratefully acknowledged for the financial support.

Last but not the least, I would like to express my gratefulness to my family, thank you for your unconditional love and support.

CONTRIBUTION OF AUTHORS

This thesis is submitted in the form of original papers published in international and peer-reviewed scientific journals. The candidate, Meng Xu, was responsible for designing and conducting the experiments, handling the data analysis, as well as the preparation of the manuscripts and the thesis. Marie-Josée Dumont, supervisor of the candidate and Assistant Professor in the Department of Bioresource Engineering of McGill University, contributed to all aspects of this research work. She provided assistance, including planning and directing the research, editing and reviewing the thesis and the manuscripts before submission for publication. Dr. Suha Jabaji, Associate Professor in the Department of Plant Science, provided partial supervisory suggestions related to the encapsulation of *Lactobacillus casei* ATCC 393. Mr. François Gagné-Bourque, PhD candidate under the supervision of Dr. Jabaji, provided technical help related to microbiological experiments and provided suggestions on data analysis (chapter 4). The details of the manuscripts that have been published are provided below:

1. Xu, Meng and Dumont, Marie-Josée, 2015, Evaluation of the stability of pea and canola protein-based hydrogels in simulated gastrointestinal fluids, *Journal of Food Engineering*, 165(0), 52-59.

2. Xu, Meng, , Gagné-Bourque, François, Dumont, Marie-Josée and Jabaji, Suha, 2015, Encapsulation of *Lactobacillus casei* ATCC 393 and evaluation of survival after freeze-drying, storage and in simulated gastrointestinal conditions, *Journal of Food Engineering*, 168(0), 52-59

CHAPTER 1

INTRODUCTION

1.1 General introduction

Hydrogels are crosslinked polymeric networks which are composed of hydrophilic polymer chains. Hydrogel networks are formed via physical or chemical crosslinking [1]. Once the network formed, these materials are able to swell and retain a large amount of water without losing their structure. In 1960, Wichterle and Lim [2] were the first researchers to suggest the utilization of hydrogels for uses in the biomedical field. Because of their hydrophilic and biocompatible character, these matrices have been used as water absorbent, for drug delivery, cell encapsulation and tissue engineering applications.

Hydrogels can be classified based on the nature of their backbones, being natural or synthetic in origin [3]. Synthetic hydrogels are attractive due to their long service life and precise chemical structure [4]. However, long service life also implies low biodegradability which can lead to deleterious environmental consequences. In 2013, a paper reported the high level of pollution of the Laurentian Great lakes caused by non-biodegradable microplastic beads [5]. This is a small example of why the development of bio-based polymers which are biodegradable is of interest to replace synthetic polymers for short life commodity applications.

Bio-based polymers are normally converted from biomass [6]. They can be derived from plant co-products such as oil, starch, proteins, cellulose and lignin as well as animal processing wastes like chicken feather and fish skin. These polymers can be degraded via enzymatic hydrolysis within living organisms or through microbial

degradation in soil. It is believed that replacing synthetic polymers with bio-based polymers will reduce the CO_2 emissions and the pollution caused by their fragmentation [6].

Since 1980, the influential work of Lim and Sun demonstrated the suitability of a bio-based hydrogel system composed of an alginate-calcium (alginate/Ca^{2+}) complex for cell encapsulation [7]. Alginate/Ca^{2+} systems have also been extensively studied for biomedical uses and, more recently, for food applications [8]. Alginate is an anionic polysaccharide extracted from brown algae. It is a linear polymer with blocks of α-D-gluronic (G) and β-D-Mannuronic (M). In 1973, Morris *et al.* proposed the 'egg-box' model which explains the crosslink of alginate chains by calcium ions [9]. The calcium induced gelation of alginate is called ionotropic gelation. The formation of an 'egg-box' structure can be achieved without additional heating. Such a mild gelation process makes alginate/Ca^{2+} hydrogels suitable for the encapsulation of living cells and bioactive compounds. As an anionic polysaccharide, alginate can form gels with positively charged polymers. Mixing of aqueous solutions of two oppositely charged polymers can lead to the aggregation of these polymeric chains due to the electrostatic force. This process is named polyelectrolyte complexation (PEC) [10]. Chitosan is a linear polysaccharide which is composed of (1-4)-linked D-glucosamine and *N*-acetyl-D-glucosamine. It is the product of N-deacetylation of chitin. The pKa of the amino group in chitosan is 6.5, thus chitosan is protonated in acidic solutions [11]. Chitosan is a nontoxic, biocompatible and biodegradable natural polymer which has been widely studied for food, medical and pharmaceutical applications [12]. It has been reported that mixing alginate with chitosan help to reduce the leaching of encapsulated drug during processing [13]. The polyelectrolyte complexation with chitosan is an common method to strengthen the alginate particles [10].

Apart from polysaccharides, proteins are also important members of natural polymers. Recent work performed in our laboratory [14, 15] demonstrated that low

commercial value proteins (e.g. canola proteins) can be used for the production of bio-based polymeric materials. Canola proteins are extracted from canola meal, which is the by-product of vegetable oil refining industry. In 2013, around 4.0 million tons of canola meal was produced as by-product in Canada [16]. Due to their abundance and potential as bio-based polymers, canola proteins are of interest to this study for the development of hydrogels as wall materials for encapsulation. However, their use for food applications has been mostly limited to animal feed.

Another food protein which is of interest due to its abundance in Canada is pea proteins extracted from field peas (*P. sativum ssp. arvense*). These proteins are traditionally used for animal feed. However recently, due to their high nutritional value, digestibility, bioavailability and long term health benefits [17], pea proteins have been used as additives to enrich the protein content of food products [18]. Moreover, it has been reported that pea proteins can be used as wall material to synthize hydrogels for probiotic encapsulation [19].

Probiotics are microorganisms living in the human intestinal tract which confer health benefits to the host [20]. It has been reported that probiotics contribute to the balance of the ecosystem in the human gut [21]. These health benefits include antagonistic and immune effects as well as the alleviation of lactose intolerance [22]. Addition of probiotics in food products is an excellent way to produce functional foods [23]. However, loss of bioactivity of probiotics due to stressors (e.g. low pH, high temperature, oxygen and light) has to be avoided during processing. Several strategies have been developed to protect these microorganisms [22, 24]. Among these methodologies, encapsulation is believed to have less effect on the biological properties of probiotics [25].

To the best of our knowledge, no study has investigated the incorporation of PEC with protein-alginate. Thus, chapter 3 is providing new knowledge of the effect of

additional PEC on the stability of protein-alginate hydrogels throughout the digestion process. Chapter 4 firstly investigated the encapsulation and dry storage of a model probiotic (*L. casei* ATCC 393) in Pea protein isolate-alginate hydrogel capsules.

1.2 Study objectives

The aim of this study was to explore the potential of using natural polymers to synthesize hydrogels. Two proteins (pea proteins and canola proteins) and two polysaccharides (alginate and chitosan) were used for the purpose. The formation of hydrogels based on these polymers was done via ionotropic gelation and polyelectrolyte complexation (with the presence of both alginate and chitosan). The detailed objectives are listed below.

Objectives:

I. To review the current knowledge related to the utilization of proteins as feedstock for the production of hydrogels as superabsorbent material, for tissue engineering, and microencapsulation (Chapter 2).

II. To synthesize hydrogels based via ionotropic gelation of protein-alginate solution and to assess the effects of protein to alginate (P:A) ratio, protein source and addition of chitosan on the properties of hydrogels. The thermal stability, morphology, and stability in simulated gastrointestinal fluid of the resulting hydrogels have been tested (Chapter 3).

III. To utilize the hydrogels synthesized in Chapter 3 to encapsulate a model probiotic: *Lactobacillus Casei* ATCC 393. The effects of encapsulation on the survival of bacteria through freeze-drying and subsequent storage were studied. Finally, the protective effects of hydrogels against SGF and their release capacity in SIF were determined (Chapter 4).

CHAPTER 2

LITERATURE REVIEW: Protein-based hydrogels

2.1 Introduction

Hydrogels are highly crosslinked polymeric networks which can undergo a change in volume (swelling/shrinkage) based on changes in environmental conditions, such as temperature, pH, ionic strength and the nature of the solvent. These spontaneous physical changes in response to the variations of the environment conferred them the title of smart materials. They are recognized for their hygienic applications (e.g. diapers) but are also widely used in the biomedical and the food industries.

According to the types of crosslinks, hydrogels can be classified as chemical or physical gels. In the case of chemical crosslinking, functional groups from the polymer backbone will form covalent bonds with a crosslinking agent. Physical crosslinking arise from physical interactions such as polymer chain entanglements, hydrogen bonding, ionic interactions and hydrophobic interactions [26]. Physically crosslinked hydrogels have attracted increasing interest in recent years since the utilization of toxic chemical crosslinkers can be avoided. Depending on the polymerization technique, hydrogels can be found in the state of microspheres, powders, coatings and films.

Due to their hydrophilic nature, hydrogels are commonly used as water absorbent materials. It is of current knowledge that hydrogels can absorb water in a range from 20% to 100,000% (weight basis) based on their crosslinking degree. The first water-absorbent polymer based on acrylic acid (AA) and divinylbenzene was synthesized in 1938 [27] and the first commercial superabsorbent polymer (SAP) based on starch-graft-polyacrylonitrile was launched in the 1970s [28]. Due to their high water retention ability, hydrogels hold a degree of flexibility which is similar to natural tissues [29]. In

1960, Wichterle and Lim [2] were the first researchers to suggested the biocompatible nature of hydrogels. They synthesized poly(2-hydroxyethyl methacrylate) hydrogels which have been used for several biomedical applications such as soft contact lenses [30]. Afterwards, hydrogels have been extensively explored for biomedical applications. However, although synthetic materials have controllable properties, the toxicity of residual monomers or crosslinkers limits their application in the biomedical field [31]. Yet, several natural polymers such as polysaccharides and polypeptides can be degraded by human enzymes. Moreover, they share similar properties and structures to human tissues [32]. This is the reason why natural polymers have been widely utilized in the biomedical field.

Proteins are one of the most important natural polymers that can be used as structural elements for the synthesis of hydrogels. The primary structure of proteins consists of a linear sequence of polypeptide which may contain up to 20 different amino acids. These amino acids possess different functional groups which can be chemically modified. Several studies reported that the structure and the properties of proteins can be manipulated by protein engineering [33-36]. Moreover, the side chains of the amino acids have different pKa which renders protein-based hydrogels sensitive to pH variations. This is of high interest for biosensors or control-release applications [34, 37-41] such as encapsulation and delivery of nutraceutical along the gastrointestinal tract [1].

Already, proteins such as collagen, keratin and elastin have been explored for the development of artificial extracellular matrices for tissue engineering. The resulting matrices displayed a high biocompatibility and showed efficient degradation in the human body [3]. Moreover, food protein-based hydrogels have also been used as coating or microencapsulation materials for the protection or delivery of nutraceutical products [1]. Table 2.1 gives a few examples of the types of proteins used for the mentioned applications.

Table 2.1: Protein-based hydrogels and their applications

Application	**Protein**	**References**
Water or aqueous absorbent material	Soy protein, fish protein, cottonseed protein, collagen, feather protein, ovalbumin protein	[42-58]
Tissue engineering	Collagen, gelatin, elastin, fibroin	[35, 59]
Microencapsulation, delivery and controlled release	Gelatin, soy protein, whey protein	[60-63]

This review focuses on the current knowledge and recent developments related to the synthesis of hydrogels based on proteins. In this chapter, the hydrogels reviewed are categorized according to their applications namely superabsorbent polymers, tissue engineering and microencapsulation. The diversity of the polymerization techniques, protein sources and their influences on the properties of resulting hydrogels are reviewed.

2.2 Water absorbent material: Protein-based superabsorbent polymers (SAPs)

Due to their hydrophilic character, hydrogels are excellent water absorbent materials. On average, hydrogels retain > 20% (weight basis) of water within their polymeric network [27, 64]. However, the water absorbing capacity of hydrogels varies depending on the monomers/polymers and crosslinkers used. Some hydrogels are defined as superabsorbent polymers (SAPs) due to their ultrahigh water absorption capacity [29, 64]. These SAPs have been widely commercially utilized, especially for hygienic applications [28]. The synthesis of most commercial SAPs is based on

synthetic polymers such as polyacrylic acid and polyacrylamide (PAA and PAAm) [29, 50]. Currently, there has been an increasing interest regarding the utilization of bio-monomers for their synthesis (e.g. protein and starch-based) [50, 65] because of their biodegradable nature, their low toxicity, and their biocompatibility [32].

2.2.1 SAP hydrogels based on Ethylenediaminetetraacetic dianhydride (EDTAD)-modified protein

In 1996, Hwang and Damodaran were the first scientists to modify proteins (soy protein isolate (SPI) and fish proteins (FP)) with EDTAD to synthesize SAPs [43, 45, 66]. This method was quickly extended to several other proteins such as keratin [58], gelatin [57], ovalbumin [51, 56] and hydrolyzed cottonseed proteins [50]. The equilibrium swelling abilities of these hydrogels are shown in Table 2.2.

Table 2.2: Swelling capacity of selected EDTAD modified protein-based SAPs

Polymers	Crosslinker	Post-gelation treatment	Swelling capacity (g/g)	Ref
Protein-based				
EDTAD-SPI*	Glutaraldehyde	N.A	105	[45]
EDTAD-SPI	Glutaraldehyde	N.A	~300	[66]
EDTAD-FP*	Glutaraldehyde	ethanol	425	[48]
EDTAD-ovalbumin	Glutaraldehyde	N.A	296	[51]
Protein-synthetic material-based				
hydrolyzed collagen-*g*-PAA*	MBA* (KPS*)	ethanol	920	[53]
hydrolyzed cotton seed protein-*g*-PAA	MBA(KPS)	ethanol	890	[50]
hydrolyzed collagen-*g*-PAA/HEA*	MBA(APS*)	ethanol	364	[54]
kC*/gelatin-*g*-PAAm*	MBA(APS)	ethanol	3310	[52]
collagen-*g*-poly(AMPS*)	MBA(APS)	ethanol	268	[39]
CFP-g-PKA*/PVA*	MBA(KPS)	N.A	714	[67]

AMPS*: 2-acrylamido-2-methylpropanesulfonic acid
APS*: ammonium persulfate
CFP*: chicken feather protein
FP*: fish protein
HEA: 2-hydroxyethyl acrylate
kC*: kappa-carrageenan
KPS*: potassium persulfate
MBA*: N,N'-methylene bisacrylamide
PAA*: poly (acrylic acid)
PAAm*: poly (acrylamide)
PKA*: poly (potassium acrylate)
PVA*: polyvinyl alcohol
SPI*: soy protein isolate

The EDTAD-modification targets the lysine residues on the peptide chains (Fig. 2.1). The method is initiated by the denaturation of proteins which unfold the proteins and expose their functional groups. The EDTAD-modification is usually conducted by the acylation of proteins in an alkaline environment. The acylation step permits the introduction of carboxylate anions within the structure. This causes conformational changes to the protein and leads to an extensive unfolding which increases the flexibility and therefore the expansion of the hydrogel network [66, 68].

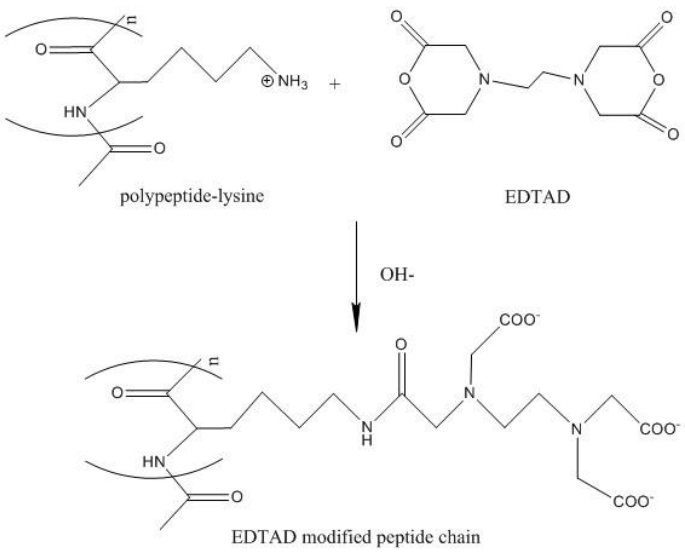

Fig. 2.1. EDTAD modification of lysyl group on peptide chain. (Adapted from Hwang *et al.* [66])

In order to achieve gelation, the remaining amine groups must be crosslinked. For example, Rathna and Damodaran crosslinked the remaining amine groups with glutaraldehyde to achieve gelation [48]. However, when the gels were conditioned at pH 9, proteins regained some degree of secondary structure. The recovery of the secondary structure is against the relaxation of the network which decreases the amount of water retained and therefore the swelling ratio. To increase the flexibility of the network, the crosslinked gels were treated with ethanol to prevent the recovery of the secondary structure [48]. The water swelling equilibrium of the treated hydrogels reached 425g/g which is almost twofold higher than the untreated gels [48].

Recently, the utilization of *Rhizomucor pusillus* proteins to produce SAP hydrogels was reported by Majdejabbari *et al.* [51, 69]. Fungal proteins were extracted from *Rhizomucor pusillus* and further modified with EDTAD. The proteins were then crosslinked with 40 mM glutaraldehyde to form the hydrogels. The matrices showed a swelling capacity of 87.6 g/g. This study demonstrated the possibility of utilizing proteins from microorganisms to produce SAP materials.

2.2.2 SAP hydrogels based on graft copolymerization of protein-synthetic material blends

Graft copolymerization of vinylic monomers onto the backbones of proteins is another efficient method to modify proteins for the synthesis of SAPs [53]. Hydrophilic monomers are introduced to the side chain of proteins to increase the water swelling ability of the network. This modification is firstly initiated by the transformation of protein to a radical state. In order to do so, initiators such as potassium persulfate (KPS) and ammonium persulfate (APS) are decomposed to form sulfate anion radicals (i.e. HSO_4^-, SO_4^{2-}) under heating [70]. These radicals attract protons from the functional groups of the protein side chains and transform the peptides to macro-radicals [53]. Acrylic acid (AA), acrylamide (AAm), and mathacrylic acid (MAA) are common monomers that are grafted onto the proteins. AA is usually neutralized before grafting onto the initiated proteins. N,N'-methylene bisacrylamide (MBA) has been widely used as a crosslinker to connect the graft peptide chains to form a three-dimensional networks.

In 2006, Pourjavadi *et al.* [53] reported the synthesis of a SAP using collagen. The AA was grafted onto the hydrolyzed collagen backbone and KPS was used as the initiator. The hydrogel network was crosslinked by MBA. The influence of the concentration of the initiator on the swelling ability in water was studied. The highest maximum absorbency (920 g/g) was achieved at low initiator concentration (<2.6mM). This is probably due because at high initiator concentration, partial destruction of the network occurs because of an increase in the formation of radicals. However, when the initiator concentration exceeded 2.6mM, the crosslinking density also increased which counteracted the relaxation-expansion behavior of hydrogel networks. This could be due to a phenomenon called “self-crosslinking” as reported by Aoki *et al*.[71]

In 2008, Pourjavadi and Salimi reported the sythesis of hydrolyzed collagen-g-

poly(sodium acrylate-co-2-hydroxyethylacrylate) hydrogels [54]. AA and 2-hydroxyethylacrylate (HEA) were grafted onto the protein side-chains. The influence of the monomer ratio (AA:HEA) on the water uptake was studied. The AA:HEA ratios studied were: 3:1, 1:1 and 1:3. The highest water capacity (364g/g) was achieved with a 3:1 AA:HEA ratio. The authors pointed out that this could result from the increasing number of ionic groups from AA. Compared to other graft polymerized hydrogels, hydrolyzed collagen-*g*-PAA/HEA hydrogels showed low sensitivity to salt. The non-ionizable OH group from HEA may have contributed to this behavior.

In 2010, Bardajee *et al.* [52] reported an highly swelling nanoporous hydrogel based on kappa-carageenan/gelatin (kC-gelatin) hybrid backbone. The maximum swelling capacity was reported to reach 3310 g/g. Similarly to previous studies, AA monomers were graphed onto the kC-gelatin backbone. However, after the gelation and the drying steps, the hydrogels were hydrolyzed by treatment with 2M NaOH at 85 ℃. The hydrolysis led to the breakage of amide bonds, and converted the amide groups to amine and carboxylic acid groups. After hydrolysis, the hydrogel network gained more flexibility and the water capacity increased due to the increased number of carboxylic groups.

More recently, keratin extracted from chicken feathers (CFP) grafted to poly (potassium acrylate)/polyvinyl alcohol (CFP-*g*-PKA/PVA) semi-interpenetrating network (IPN) superabsorbent polymers were synthesized by Li *et al*. [67]. AA was neutralized with 60 mg/L KOH solution before the AA was grafted onto the CFP. PVA and modified CFP were crosslinked with MBA to achieve the gelation. The maximum swelling capacity (714g/g) was reached at pH 6.

In summary, several efforts have been made to produce protein-based superabsorbent hydrogels. Proteins recovered from agricultural or food processing wastes have been mainly used for such purpose. In order to improve the hydrophilicity

of proteins, polypeptides can be modified with EDTAD or via graft copolymerization with synthetic polymers. In general, hydrogels from graft copolymerization have a higher swelling capacity than hydrogels based on EDTAD-modification. However, due to its semi-synthetic nature, graft copolymerized gels do have major disadvantages. For example, toxic residues from the crosslinkers can remain in the gels.

2.3 Protein-based hydrogels as scaffolds for tissue engineering

Hydrogels have been studied as artificial scaffolds and delivery matrices for tissue engineering [35] due to their high water retention which mimic the human tissues. It was found that the introduction of cell-implanted hydrogels improve the regeneration of tissues such as cartilages [72, 73], bones [74, 75], spinal cord [76] and brain [77]. Hydrogels have also been used as delivery matrices for growth factors such as insulin-like growth factor (IGF-1) [78, 79], fibroblast growth factor (FGF) [80] and transforming growth factor (TGF) [81].

To design hydrogels for tissue engineering, the extracellular matrix (ECM) is an important reference since it is composed of extracellular molecules secreted by cells which provides structural and biochemical support to the surrounding cells. ECM supports cell adhesion, proliferation and differentiation. Therefore introducing proteins present in the ECM in the formulation of hydrogels increases the biocompatibility of the matrices [3]. Four of these commonly used proteins for such purpose namely collagen, gelatin, elastin and fibroin are reviewed [35].

2.3.1 Gelatin and collagen

Collagen is the major protein component in ECM [3]. The structural element of collagen is the triple α-helix (Fig 2.2a). Each α-helix is composed of amino acid repeats of -Gly-X-Y- , where X and Y usually represent proline and hydroxyproline (Fig. 2.2b) [82]. Due to different amino acid profiles, these α-helices have variable conformations.

There are twenty nine known types of collagen where twelve of them are the most common (type I – type VII). These types of collagen have different secondary protein structure and they are present in different tissues [83].

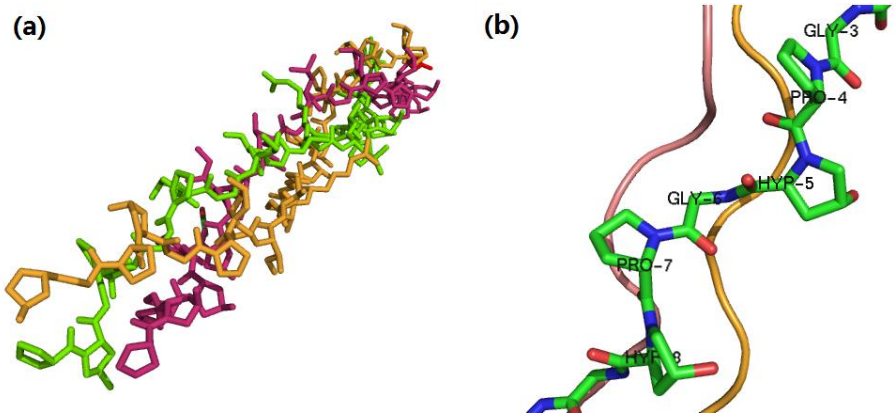

Fig. 2.2. (a) The triple helix structure of human collagen. (b) GLY-PRO-HYP repeats (adapted from Kramer *et al.*[84] , PDB code: 1BKV)

Collagen has been extensively studied for biomedical applications [83, 85-90]. Porous collagen scaffolds for engineered hard tissues are normally produced by lyophilisation. Further dehydrothermal treatment [87, 91] or chemical crosslinkers [92] can be used to crosslink the resulting gels. Different strategies can be employed to increase the mechanical properties of the tissues. For example, calcium phosphate is usually incorporated to collagen scaffolds to increase their biocompatibility and their mechanical stiffness [93-95]. Hirata *et al.* [96] reported that collagen-based scaffolds coated with multi-layers of carbon nanotubes showed similar mechanical properties to bones and an excellent cell adhesion. Moreover, electrospun fibrous scaffolds based on collagen are normally incorporated with synthetic polymers. For example, poly-caprolactone (PCL), poly-lactic acid (PLA) and poly-glycolic acid (PGA) are commonly blended with collagen to increase the tensile properties of the resulting nanofibers [88, 90, 97-99].

Gelatin is the hydrolytic degradation product of collagen. Gelation of gelatin is easily achieved due to its low sol-gel transition temperature (around 30 ℃) [35].

Several methods have been applied to stabilize the gels via means of chemical, physical and enzymatic crosslinking (Table 2.3). Moreover, several methods have been developed to modify gelatin to increase the biocompatibility of the resulting gels. The modification of gelatin is usually conducted via grafting of functional groups to the lysine and hydroxylysine residues [100]. Li and co-workers [101] reported the formation of amphiphilically-modified gelatin by grafting hexanoyl anhydrides to the amino groups. The cellular uptake test showed that this modification increased the cytocompatibility and the cell uptake of the resulting hydrogels. For example, the encapsulation efficiency of a model molecule (camptothecin) increased from 43.3% to 70.1% as the hydrophobic substitution ratio increased from 69.6% to 97.7% [101]. Moreover, the modification of gelatin can also help to mimic the physical properties of native tissues. Chondroitin sulfate-modified gelatin hydrogels designed to target the corneal stromal tissues were engineered by Lai and co-workers [102]. Chondroitin sulfate belongs to the family of glycosaminoglycan and holds the property of increasing the hydration of the cornea [103] and its incorporation in hydrogels resulted in a material with similar hydrophilic and mechanical strength than native cornea [102].

Table 2.3: Protein-based hydrogels for tissue engineering applications

Hydrogels	Gelation agent/method	Application	Ref.
chitosan-gelatin/nanohydroxyapatite (nHA)	Glutaraldehyde	N/A	[104]
chitosan-gelatin/hydroxyapatite (HA)	EDC*, NHS*	Bone	[105]
gelatin-hyaluronic acid	EDC	soft tissue	[106]
chitosan-agarose-gelatin	Glutaraldehyde	cartilage	[107]
chitosan-gelatin	Glutaraldehyde	Bone	[108]
PMMA*-gelatin	Glutaraldehyde	Bone	[109]
PCL*-gelatin	Genipin	muscle	[110]
Gelatin	Genipin	N/A	[111]
collagen-gelatin-HA	Genipin	Bone	[112]
gelatin-HA	Lyophilisation	Bone	[113]
chitosan-gelatin-PCL	Lyophilisation	Heart	[114]
Tecophilic*-gelatin	Electrospining	blood vessel	[115]
poly(ε-Caprolactone)-gelatin	Electrospining	N/A	[116]
PLLA*-cationized gelatin	Electrospining	cartilage	[117]

EDC*: 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide

NHS*: N-hydroxy-succinimide

PCL*: polycaprolactone

PEO*: polyethylene oxide

PLLA*: poly-lactic acid

PMMA*: polymethylmethacrylate

PPO*: polypropylene oxide

Tecophilic*: Tecophilic™ thermoplastic polyurethanes

The structure and the physicochemical properties of hydrogels can be tuned to mimic variety of tissues. Depending on their applications, hydrogels can be processed into different physical forms. Freeze-drying is usually involved in the development of gelatin-based or collagen-based hydrogels for hard tissue engineering (e.g. bone and cartilage, bone and cartilage substitutes and implants). After lyophilisation, the polymer solution becomes a cryogel [113]. Cryogels possess a highly porous structure which resembles to the structure of bones [113]. Moreover, to further improve the mechanical and biological similarity of resulting hydrogels to natural bones, hydroxyapatite have been incorporated in gelatin-based hydrogels for hard tissue engineering [104, 105, 112, 113]. Hydroxyapatite ceramics exhibit physicochemical properties which are similar to bone minerals. It is a brittle material which is hard to be processed and maintained in patients' defect sites. However, crosslinking hydroxyapatite into gelatin-based

hydrogels can increase the biocompatibility and reduces the difficulties to handle this material [113].

Another method to produce gelatin-based hydrogels is by electrospinning. The resulting gelatin films are excellent candidates for soft tissue engineering [115-117]. The standard laboratory setup for electrospinning consists in a syringe with a needle connected to a high voltage power (5 to 50 kV) supply, a syringe pump and a flat collector. Trifluoroethanol, formic acid, acetic acid and water are common solvents used for gelatin electrospinning [118]. Gelatin solution is normally filled in the syringe and pumped at a constant rate. The liquid is then charged due to a high voltage power supply. Once the polymer solution is pumped out of the needle, a stream of polymer solution is formed due to the electrostatic forces. The electrostatic repulsions stretch the polymer solution to form a stream which reaches the rotating collector and forms the fibers.

2.3.2 Elastin

Elastin is an abundant protein which exists in elastic tissues such as tendon, blood vessel, skin and elastic cartilage [119]. Elastin is highly insoluble in water due to its uncommon amino acid composition (75% of hydrophobic residues) and its extensive number of inter-chain crosslinks [120]. The crosslinking of elastin has been achieved thermally [121], chemically by the use of glutaraldehyde [122-124] or 1,6-hexamethylene diisocyanate (HMDI) [123], and enzymatically using bacterial transglutaminase [125]. Due to its hydrophobicity and inter-chain crosslinks, only a few researchers utilized native elastin for tissue engineering applications.

Crosslinking elastin onto PCL scaffolds was reported by Annabi *et al.* [126, 127]. They used pressurized CO_2 as foaming agent to produce highly porous PCL scaffolds. The scaffolds were then impregnated in an elastin solution and crosslinked with glutaraldehyde under vacuum. The results indicated that the addition of elastin into the

matrix dramatically increased the adhesion of chondrocyte to PCL scaffolds [127]. Thus, the gas foaming technique could be used for the surface modification of PCL scaffolds and promote the incorporation of elastin into the scaffolds. In 2013, Palumbo *et al.* [128] reported a graft copolymerized elastin-based hydrogel. Ethylenediamine (EDA) was used to modify hyaluronic acid. The activated EDA-hyaluronic acid was grafted onto the gelatin backbone and crosslinked with ethylene glycol diglycidyl ether. The resulting gel showed good water affinity, adhesion and viability of human umbilical artery smooth muscle cells. This hydrogel was also biodegradable via the hydrolysis of hyaluronidase.

Numerous genetically recombinant elastin-like polypeptides (ELP) were synthesized [123-125, 129-131]. These materials were produced by introducing synthetic elastin DNA coding into microbes such as *Escherichia coli* (E.coli) [121, 130]. The ELPs were expressed by E.coli and purified. ELPs have a lower critical solution temperature (LCST) of around 32 ˚C [121]. When the temperature reaches above the LCST, hydrophobic force induces the aggregation of ELPs [35]. Based on this feature, ELPs can be isolated and purified by thermal precipitation. Recombinant ELPs are coded by synthetic genes; therefore their amino acid sequence and molecular weight can be controlled. Thus, the highly tunable structure and relatively easy purification process make ELPs an excellent candidate for tissue engineering.

2.3.3 Fibroin

Silk fibroin and sericin are two major proteins produced by silk worm (*bombyx mori*). Silk fibroin is a favorable material for tissue engineering due to its biocompatibility and excellent mechanical properties [132]. Sericin is a gum-like protein which coats the silk fibers. Silk sericin has been reported to be responsible for immune response [133]. Therefore, natural silk is usually purified to remove the sericin when used for biomedical applications [134]. The gelation of silk fibroin results from a conformational change from a random coil to an ordered β-sheet structure [132, 134,

135]. The gelation rate is influenced by several factors. For example, reducing the electrostatic repulsion by lowering the pH [136, 137] or adding Ca^{2+} ions [136] can decrease the gelation time. The gelation rate can be accelerated by increasing the temperature [135] since it increases the hydrophobic interactions of fibroin and lead to higher gel strength (e.g. 0.03 N at 37 ℃ and 0.05 N at 50 ℃) and lower porosity.

Physical methods such as ultrasonication have been used to induce an intermediate gelation of fibroin. This technique permits the acceleration of the formation of β-sheets which accelerate the gelation of fibroin. Hu *et al.* [138] reported the synthesis of crosslinked fibroin-hyaluronic acid hydrogels by ultrasonication. After the treatment, mesenchymal stem cells encapsulated within this gel retained their viability and proliferated in the culture.

Interpenetrating polymer network (IPN) based on gelatin and fibroin were reported by Xiao *et al.* [139]. The gelatin was modified with methacrylate and mixed with a fibroin solution. The gelatin-fibroin mixture was first exposed to UV radiation to crosslink the gelatin-methacrylate. The semi-IPN was further treated with 70% methanol to induce the crystallization of fibroin. During crystallization, the dehydration of fibroin caused the formation of β-sheet structures which formed the hydrogels. The resulting IPN showed a high compressive modulus and a low degradation rate. Kundu *et al.* [140] reported a fibroin/PVA-based semi-IPN. PVA was first reacted with 2-isocyanatoethyl methacrylate (2-ICEMA) to produce polymerizable PVA methacrylate macromonomers. The PVA within the scaffold was crosslinked by UV radiation. The fibroin became physically entangled with the PVA network to form the fibroin/PVA semi-IPN. Since the fibroin was not chemically crosslinked to the network, the PVA/fibroin gels showed a rapid weight loss after 1 day of incubation in phosphate buffered saline (PBS). However, these matrices could still be a good candidate for control-delivery purposes as successful release tests were performed using a model compound (dextran).

Genetically engineered silk-elastin-like protein polymers have been generated. These are normally produced by transgenic silkworm. Asakura *et al.* [141, 142] designed a recombinant silk fibroin which possesses partial collagen sequences. The resulting recombinant silk with the sequence $[TGRGDSPAS]_8$ showed 6 times higher cell adhesion activity than the wild type (silk fibroin). The recombinant silk was used to engineer vascular grafts, but due to the limited amount of recombinant silk used, the resulting scaffold did not show significant difference on cell adhesion and proliferation. Nagano *et al.* [143] designed a transgenic silk fibroin containing a calcium binding sequence $[(AGSGAG)_4E_8AS]_4$. The introduced poly-glutamic acid domains increased the calcium binding affinity of the silk fibroin. The resulting gels promoted early bone mineralization and bone restoration.

Similar to elastin, the structure of synthetic silk fibroin can be manipulated by genetic engineering to form desirable biomaterials. Tissue engineering requires precise structural control of the feedstock to achieve homogenous scaffolds. Compared to native elastin and fibroin, synthetic proteins seem more likely to meet this requirement.

To summarize, depending on the techniques used during the gelation processes, the resulting hydrogels can be present in different physical forms (e.g. films, spongy cryogels, solid molded form and injectable gels). Incorporation of biocompatible materials (e.g. HA and chondroitin sulfate) within the hydrogel networks have been explored to enhance mechanical and biological similarities of hydrogels to native tissues. Moreover, artificial polypeptides have been recently reported. The genetically modified polypeptides displayed desired properties for tissue engineering applications. The original properties of native protein remain in some degrees. However, they have more precise chemical structure which ease the purification and processing of these polypeptides.

2.4 Protein-based hydrogels as wall materials for microencapsulation

The porous structure of hydrogels can be adjusted by controlling the crosslinking density and the choice of monomers. This unique property makes hydrogel an excellent wall material for encapsulation and control-release of bioactive components. Similar to the principle of gel permeation chromatography, the retention time of drugs or cells varies depending on their molecular weight and size [144]. Thus, adjusting the pore size of hydrogels permits the loading of bioactive components and the subsequent control-release [145]. Hydrogel-based drug delivery systems have been widely used in the pharmaceutical field [146]. Recently, the utilization of hydrogels as encapsulation matrices for nutraceutical compounds attracts more and more attention [1, 20, 147]. Nutraceutical compounds are purified or isolated from food. Vitamins, probiotics, enzymes, antioxidants are common nutraceuticals. These products are believed to hold physiological benefits for human health. However, these products denature or degrade easily during processing and storage [1]. The encapsulation of these products in hydrogel reduces their exposure to oxygen, light and gastro-intestinal environment and thus lowers the loss of their bioactivity during processing and storage.

2.4.1Encapsulation of probiotics

Probiotics are microorganisms which confer physiological benefits to the host [148]. The potential health benefits of probiotics are numerous and may include anti-carcinogenic actions [149], an immune system stimulation [150, 151], serum cholesterol reduction [152, 153], and the relief of lactose intolerance [154]. Overall, their metabolism have positive effects on the gut environment and the immune system [155]. Nowadays, dairy products containing probiotics are increasing in popularity. During processing, storage and digestion, several stressors (e.g. oxygen, low temperature, low pH in gastrointestinal tract and bile salts) lead to the loss of viability of probiotics [20]. Strategies to protect these microorganisms include the incorporation

of protectants (e.g. sorbitol and trehalose [156], glycerine [157]), the manipulation of starter cultures [158, 159] and the encapsulation. Among these methods, encapsulation has less effect on the biological properties of the probiotics [25]. Several food proteins have been utilized to develop hydrogels for the encapsulation of probiotics (Table 2.4).

Table 2.4: Protein-based hydrogels for probiotics encapsulation

Hydrogels	**Gelation agent/method**	**Probiotics**	**Ref.**
Gelatin-alginate	Genipin	*Bifidobacterium pseudocatenulatum*	[160]
Gelatin-alginate	Genipin	*Bifidobacterium adolescentis*	[161]
Casein-whey protein	Chymosin enzyme	*Lactobacillus rhamnosus*	[162]
Whey protein	pH induced gelation	*Lactobacillus rhamnosus*	[163]
Legumin proteins-alginate	Ca^{2+}	*Bifidobacterium adolescent*	[164]
Pectin-whey protein	Ca^{2+}	*Lactobacillus rhamnosus*	[165]
Casein	Transglutaminase	*Lactobacillus paracasei*, *Bifidobacterium lactis*	[166]
Whey protein	Ttransglutaminase	*Bifidobacterium bifidum*	[167]
Soy protein- high methoxy pectin	pH induced gelation	*Lactobacillus delbrueckii*	[168]
Chickpea protein/alginate/κ-carrageenan	Genipin, Ca^{2+}	*Bifidobacterium adolescentis*	[169]

Gelatin has been widely used as a gelling agent in the food industry. Annan *et al.* [161] reported a gelatin-based hydrogel with an alginate coating. The gelatin core was crosslinked with genipin and subsequently coated with alginate. The coating was achieved by 'internal' or 'external' Ca^{2+} source methods. Briefly, the internal method consists in mixing Ca^{2+} ions with gelatin cores before immersing the cores in an alginate solution. The external method consists in immersing the gelatin cores in an alginate solution before being transferred and crosslinked with Ca^{2+} in an oil-water emulsion. The 'external' Ca^{2+} source method showed a lower encapsulation yield than the 'internal' method and uncoated control. However, the alginate coating by 'external' method showed better protection (240 min of disintegration time) for pepsin-induced degradation in simulated gastric fluid than those by 'internal' method (120 min) and uncoated control (45 min). The survival test of *B. adolescentis* was conducted in

simulated gastric fluid. Compared to the 3.45 log CFU/ml loss of viability for free cells, both gelatin encapsulation matrices without coating and with alginate coating showed a lower loss of 2.55 log CFU/ml and 1.21 log CFU/ml, respectively.

Whey proteins, another widely studied food protein, is a byproduct of cheese production [170]. Whey protein gels are normally achieved by thermal gelation which involves the thermal denaturation of native proteins and the intramolecular aggregation of globular proteins [171]. However, the heating process needed to produce these gels limits their application for heat-sensitive ingredients. Cold-set gels have been developed to fill this gap. The cold gelation of globular proteins is normally achieved by adding salt (e.g. Ca^{2+}) into pre-denatured protein solutions [1]. Ca^{2+} induced gelation of whey protein and the encapsulation effects were studied by Doherty *et al.* [172]. *L. rhamnosus* was encapsulated with Ca^{2+}-alginate hydrogel beads synthesized by the extrusion method. The LIVE/DEAD microscopy staining test showed that the high pressure applied during extrusion had no detrimental effect on the cells' viability. Further simulated gastro-intestinal studies showed that whey protein hydrogels were stable in an acidic environment and had a protective effect on *L. rhamnosus* in simulated gastric fluid. However, the rapid disintegration of the gel and the release of probiotics were triggered by intestinal enzymes. Gerez *et al.*[165] reported the synthesis of pectin-whey protein-based cold-set hydrogels. Ca^{2+} ions were used to induce the gelation. The authors also pointed out that the gelation process occurred at pH 4 which is lower than the isoelectric point (pI) of whey proteins (pH 5.2-5.4). Thus the protonated $–NH_3$ groups from whey proteins formed electrostatic interactions with the carboxyl groups of pectin. This interaction increased the crosslinking density and thus resulted in a better protection of *L. rhamnosus* in simulated gastric fluid.

The gelation of casein-whey protein matrices induced by proteolysis and subsequent self-aggregation of κ-casein was reported [162]. Chymosin enzymes were used for the hydrolysis of κ-casein in milk. The hydrolysis led to the release of

hydrophilic groups and reduced the repulsive forces which induced micelle aggregation. In this study, two sets of gels were synthesized with native and denatured whey proteins, respectively. An atomic force microscopy study was conducted to understand the interactions between two strains of lactobacillus (*L. rhamnosus* GG and *L. rhamnosus* GR-1) and two types of whey proteins (native and denatured). The highest adhesion force was observed between *L. rhamnosus* GG and the denatured whey proteins. In further microbiological tests, the denatured whey protein-casein gels showed a higher encapsulation yield of 97% as compared to 88% for native whey protein gels. The denatured whey protein gels also had a better protection of *L. rhamnosus* GG against simulated gastric conditions. This can be explained by the specific interactions between the strain and the denatured whey protein [173]. The high adhesion force could explain the higher retention of bacteria during the encapsulation and the better protection against simulated gastrointestinal environment. This study indicated that the interaction between probiotics and wall materials may play an important role in microencapsulation.

Enzymatic crosslinking of food proteins has been explored as well. Zou *et al.* [167] reported the enzymatic crosslinking of whey proteins with transglutaminase. The resulting gels were freeze-dried and spray-dried, and their protective effects on *B. bifidum* F-35 were compared. The results showed that the exposure to heat during spray-drying resulted in a higher loss of viable cells. It is noteworthy to mention that the authors also produced sucrose co-freeze-dried beads. The addition of sucrose caused approximately 2 log (CFU/ml) increase in the encapsulation yield of *B. bifidum* F-35. Heidebach *et al.* [166] reported the synthesis of casein-based microcapsules crosslinked with transglutaminase and their protective effects towards two freeze-dried bacteria strains (*L. paracasei* F19 and *B. lactis* Bb12) during storage. The results showed that the microcapsules effectively increased the survival of *B. lactis* Bb12 during 90 days of storage. The *B. lactis* Bb12 is an anaerobic strain while *L. paracasei* F19 is facultative anaerobic. The detrimental oxidative changes on the cell membrane during storage may

lead to the reduction of viability of *B. lactis* Bb12. Thus, entrapment of *B. lactis* Bb12 decreased their exposure to oxygen and led to a higher survival rate during storage.

Cold-set gels have been synthesized using other proteins. Speroni and Añón reported a cold-set gel based on soy proteins treated by high pressure [174]. Under high pressure, the denaturation of soy proteins requires lower temperature than conventional methods and therefore may be of interest for the encapsulation of heat-labile compounds or probiotics. Ca^{2+} ion induced gelation of alginate-legumin proteins blends (including chickpea, faba, lentil or pea protein isolates) was reported by Khan *et al.* [164]. The alginate-legumin hydrogels demonstrated excellent protective attributes against simulated gastric fluid. Once encapsulated, *B. madolescenti* showed at least 6.2 log CFU/g survival after 2 hours of exposure as compared to <1.0 log without encapsulation. The subsequent exposure to simulated intestinal fluids led to the immediate release of the bacteria (~5.2 log CFU/mL).

2.4.2 Encapsulation of nutraceutical compounds

Apart from probiotics, nutraceutical products also include bioactive molecules such as vitamins, enzymes, and antioxidants. Although these bioactive compounds do not have a living cell structure such as probiotic, their bioactivity also decreases during the extraction, processing and storage [1, 175]. Food proteins are excellent candidates as encapsulation material for nutraceutical molecules. In addition to their nutritional values, proteins contain diverse functional groups which provide enhanced interactions between proteins and the targeted nutraceutical molecules [176]. Binding nutraceutical molecules to protein-based hydrogels not only stabilizes the hydrogels but also increases the encapsulation efficiency and the protective effects of these molecules. Some recent studies are summarized in Table 2.5.

Table 2.5: Food protein-based hydrogels for encapsulation of nutraceutical molecules

Hydrogels	**Gelation agent/method**	**Nutraceutical molecules**	**Ref.**
Whey protein	Thermal gelation	Bilberry anthocyanins	[177]
Whey protein	Thermal gelation	Bilberry anthocyanins	[175]
Alginate-whey protein	Ca^{2+}	Riboflavin	[178]
Soy protein	Ca^{2+}	Riboflavin	[179]
β-lactoglobulin	Thermal gelation	(-)-Epigallocatechin-3-gallate	[180]
β-lactoglobulin-low methoxyl pectin	Polyelectrolyte complexation	Docosahexaenoic acid (DHA)	[181]
Casein	Hydrophobic aggregation/Ca^{2+}	Docosahexaenoic acid (DHA)	[182]

Several factors such as pH, temperature, crosslinking methods, selection of wall materials, and interactions between the wall material and the nutraceutical compounds can influence the encapsulation yield and protective effects of hydrogels. Betz *et al.* [175, 177] encapsulated anthocyanins, an antioxidative phenolic compound using whey protein-based thermal gels. The gelation of whey proteins was achieved by heating the mixture to 80 ℃ at a low pH (pH 1.5). The most stable form of anthocyanins occurs at low pH (pH< 3) where they are found as flavylium cations [183]. Thus, working at low pH can reduce the thermally-induced degradation of anthocyanin.

The protective effects of the native and denatured β-lactoglobulin proteins were compared by Shpigelman *et al.* [180]. They reported the encapsulation of (-)-Epigallocatechin-3-gallate (EGCG) with β-lactoglobulin thermal gels. The hydrogels based on denatured proteins (preheated at 80 ℃) not only showed a more stable structure but also had a better protective effect on EGCG. This result can be explained by the interactions between EGCG and β-lactoglobulin. It was previously reported that EGCG could bind to a wide variety of proteins via hydrophobic interactions and hydrogen bonds [184]. The preheated proteins had an unfolded structure which improved the interactions between EGCG and the protein network.

Lipophilic compounds like ω-3 polyunsaturated fatty acids which have reduced

water solubility and a high sensitivity to oxidation have also been studied. In 2009, Zimet and Livney [181] reported the encapsulation of docosahexaenoic acid (DHA), a common ω-3 fatty acid, with a β-lactoglobulin-pectin hydrogel. The hydrogel matrix was formed by polyelectrolyte complexation between β-lactoglobulin and pectin which is negatively charged. They studied the binding affinity between DHA and β-lactoglobulin. The binding constant (K_b= (6.75±1.38) ×10^5 M^{-1}) of DHA to β-lactoglobulin was defined by a spectrofluorometric titration method. The resulting gels could load ~166 times the concentration of DHA than the surrounding serum. In 2010, Zimet, Rosenberge and Livney [182] studied the interactions between DHA and casein. A relatively higher affinity (K_b= (8.38±3.12) ×10^6 M^{-1}) was reported. This was probably due to the formation of nanoparticles upon the addition of DHA (pre-dissolved in ethanol) to the casein solution. They hypothesized that this aggregation was caused by the hydrophobic interactions between the DHA and the casein and was further evidenced by the formation of smaller aggregates at low temperature (4 ℃). The low temperature weakened the hydrophobic interactions which led to smaller size aggregates [185]. Although the stability test showed a ~38.7% loss of DHA during the encapsulation process, the significant protective effect (20 fold slower degradation rate than free DHA) of casein was observed.

These studies demonstrated the robust interactions between nutraceutical molecules and the protein backbones of hydrogels. The interactions lead to an improved encapsulation yield and protective effect on nutraceutical compounds. Food proteins showed the potential to interact with a variety of lipophilic compounds via hydrophobic or electrostatic interactions [176]. This feature could become a focus for future research directions.

2.5 Conclusion

Generally, protein-based hydrogels are a new generation of smart materials which have

the following advantages:

- They can compatible to the human body and can mimic the native tissues,
- A wide variety of agents, especially enzymes can crosslink proteins,
- They can undergo changes in volume, structure, swelling ability and stability in response to environmental stimulus,
- They are biodegradable and have some degree of controllable degradation.

This review showed that protein-based superabsorbent polymers are likely to be used for personal hygiene and for agricultural applications. They are rarely expected to interact directly with the human digestive system or tissues. Thus, there are few restrictions on the selection of the protein sources. Recently, the utilization of microbial proteins [69] and by-product proteins [15] (e.g. canola proteins from oil refining industry) for superabsorbent hydrogels have been reported. Since several studies have been conducted using synthetic materials-based superabsorbent polymers to change the water or fertilizer retention of soil [186, 187], processing these low value proteins into SAPs could increase their value. It has to been pointed out that the shelf life, service life and degradation products of SAPs have an impacts on their application [188]. Therefore, studies on the decomposition and related degradation products of protein-based SAPs could be of interest for future researches.

This review also showed the potential of protein-based hydrogels as scaffolds for tissue engineering applications. The properties of these hydrogels can be tuned to mimic the native tissues. Incorporation of native materials and growth factors within protein based-hydrogels [79, 99, 112] showed an improved regeneration of tissues. Recent studies which reported the production of artificial polypeptides by recombinant DNA technology in microorganisms [121] and plants [189] demonstrated the new perspectives for large-scale production of recombinant proteins. These engineered proteins were reported to have controllable molecular weight and amino acid sequences [121, 125, 129]. The combination of a variety of gelation methods with these novel

artificial polypeptides could become popular amongst the scientific community for the synthesis of hydrogels for tissue engineering.

Finally the review covered the emerging field of encapsulation of dietary nutraceutical products. For the purpose, food proteins are usually utilized since they are part of the human diet.

Connecting statement

The literature review (Chapter 2) provided information regarding the potential of proteins as backbones for the synthesis of bio-based hydrogels. In Chapter 3, two protein sources (canola and pea protein isolates) were utilized as feedstock for the synthesis of protein-alginate hydrogel microspheres. The resulting hydrogel microspheres were freeze-dried and characterized. The effects of polyelectrolyte complexation with chitosan, protein to alginate ratio and protein sources on the physicochemical properties of resulting hydrogels have been studied.

Chapter 3 has been accepted and published in the Journal of Food Engineering.

CHAPTER 3

Evaluation of the stability of pea and canola protein-based hydrogels in simulated gastrointestinal fluids

3.1 Abstract

This work aims to investigate the effect of polyelectrolyte complexation (PEC) on the stability of protein-alginate hydrogels in simulated gastrointestinal fluids. The properties of two types of hydrogels based on pea protein isolates (PPI) and on canola protein isolates (CPI) were compared. The molecular structure of hydrogels was assessed by FTIR and their thermal stability was tested by DSC and TGA. The swelling capacity of hydrogels was tested in distilled water, simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) without enzymes. CPI beads showed higher swelling capacity than PPI beads in all swelling media. In SIF, relatively higher swelling, an increase in volume and partial degradation were observed for all hydrogel beads. In addition, the swelling of beads in SIF was found to be dependent on PEC with chitosan. Moreover, degradation tests in SGF and SIF media with enzymes were performed to assess the effect of PEC on the stability of hydrogels. The results indicated that PEC had no influence on the stability of hydrogels in SGF but significantly delayed the degradation of hydrogel under SIF conditions. These findings suggest that PEC could be utilized to modify protein-alginate hydrogels to create matrices for prolonged release applications.

3.2 Introduction

Alginate/Ca^{2+} hydrogel systems and their applications in the biomedical and the food industries have been extensively investigated due to their biocompatibility, biodegradability and low toxicity [4]. Alginate is a linear biopolymer composed of α-

L-gluronic and β-D-mannuronic acids. The negatively charged carboxylate side chains of alginate can be crosslinked with divalent cations to allow for the sol-gel transition. This process is called ionotropic gelation which is usually achieved in aqueous solution at room temperature. Due to the mild gelation conditions involved, alginate/Ca^{2+} systems have been studied as delivery matrices for bioactive components such as functional proteins, lipids [190, 191], probiotics [192] and human cells [193]. As a delivery matrix, the control of the porosity is a key factor because it influences the diffusion of media through the matrix and the release of bioactive components [10]. One of the limitations of alginate/Ca^{2+} systems is the difficulty in controlling the porosity since it depends on several factors such as the pH, the crosslinking density, and the hardening time [194]. Therefore, co-encapsulation with food proteins has been explored in order to overcome these challenges. Food proteins such as whey protein [195], a by-product of the cheese industry, and a variety of legume proteins [164] have been utilized in the synthesis of hydrogels because of their nutritional value, their biocompatibility, low toxicity and their ability to form gels. It was reported that the incorporation of whey proteins into alginate/Ca^{2+} matrix resulted in solid gels that delayed the release of encapsulated bioactive compounds [196]. Pea protein-alginate based hydrogels are known for their ability to protect probiotics against simulated gastric fluid and for their control-release ability in simulated intestinal conditions [19].

Apart from the co-encapsulation with proteins, polyelectrolyte complexation (PEC) based on alginate-chitosan has been extensively studied for the modification of alginate/ Ca^{2+} systems [197, 198]. The process of two oppositely charged polymers interacting to form a complex is termed PEC. Chitosan-alginate is one of the most common polyelectrolyte complexes. Chitosan is a linear polysaccharide comprised of β-(1-4)-linked D-glucosamine and *N*-acetyl-D-glucosamine. The pK_a of the amino groups in chitosan is around 6.5. Thus, in acidic solution, chitosan is protonated which makes it a positively charged polymer [11]. It has been suggested that PEC influences the pore size and the network complexity of the resulting alginate network. As a result, it could

reduce the leaching of bioactive components during preparation [199]. To the best of our knowledge, no research has been conducted to investigate the effect of PEC with chitosan on the stability of protein-alginate hydrogels in simulated gastrointestinal conditions.

In this study, canola protein isolates (CPI) and pea protein isolates (PPI) were utilized to synthesize protein-alginate matrices followed by PEC with chitosan. Canola and peas are important crops in Canada. In 2013, approximately 9.0 million tons of canola was processed to produce 3.0 million tons of canola oil [16]. Meanwhile, 4.0 million tons of canola meal was produced as a by-product. CPI is extracted from canola meal which is currently used as source of proteins in low-value animal feed [18]. Only a few studies have investigated the potential of using CPI as feedstock in the synthesis of bio-based polymers. The production of field pea in Canada is the highest in the world and reached 3.8 million tonnes in 2013 [200]. Pea proteins have an excellent amino acid profile due to their high content of lysine, an essential amino acid. Recently pea proteins have attracted increasing interest because of its relatively high digestibility, bioavailability and long term health benefits [17].

Chitosan was added to the formulation of hydrogels because it can provide additional crosslinking and therefore is expected to increase the stability of the resulting hydrogels in a simulated gastrointestinal environment. Thus, the aim of this study was to assess the effect of PEC with chitosan on the physicochemical properties of protein-alginate hydrogels and the stability of the hydrogels against the enzyme activity in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF).

3.3 Methodology

3.3.1 Materials

Canola protein isolates (CPI) (Isolexx®, 95 % protein, 5 % moisture, 0.4 % fat and 1 % ash) were provided by BioExx Specialty Proteins Ltd. (Toronto, ON, Canada). Pea protein isolates (PPI) (Propulse N™, 81.73 % protein, < 10.3 % sugars, < 0.7 % starch, 3.40 % moisture, < 0.5% fat and < 4.0% ash) were obtained from Nutri-Pea Ltd. (Portage la Prairie, MB, Canada). Alginic acid sodium salt with low viscosity (viscosity of 1% aq. solution: < 300 cps) and high molecular weight chitosan (Cat. No. 150597) (degree of deacetylation > 90%; 3000 cp viscosity) were purchased from MP Biomedicals, LLC (Solon, OH). Calcium chloride dehydrate, sodium chloride and hydrochloric acid were purchased from Fisher Scientific (Fair Lawn, NJ). Sodium hydroxide was purchased from EMD (Damstadt, Germany). Monobasic potassium phosphate (KH_2PO_4), pepsin from porcine gastric mucosa (powder, ≥250 units/mg solid) and pancreatin from porcine pancreas (4 × USP specifications) were purchased from Sigma-Aldrich (St. Louis, MO).

3.3.2 Preparation of the hydrogel beads

The protein solution was prepared by dissolving 9 g of protein in 0.1M NaOH solution (pH 12.5). The solution was heated to 80 ℃ and stirred for 30 minutes to denature the proteins. Thereafter, it was cooled to room temperature (21 ± 2 ℃). After neutralization to pH 7.0 with 1.0 M HCl, 4.5g of alginate sodium salt was added. The solution was then reheated to 80 ℃ and stirred for 60 minutes. The solution was then filtered to remove solids and the temperature was reduced and maintained at 40 ± 2 ℃. PEC hydrogel beads were formed by extruding the protein-alginate solution into the gelation solution containing 0.5% (w/v) chitosan and 0.1 M $CaCl_2$. Protein-alginate hydrogel beads were formed in the gelation solution containing only 0.1 M $CaCl_2$. The 0.5% (w/v) chitosan solution was prepared by first dissolving 5.0 g of chitosan in 500

mL 1% HCl solution. The pH of the solution was then adjusted to 5.5 with 1 M NaOH and diluted to give a final volume of 1 L. At 25 ℃, the protein–alginate solution was extruded into the gelation solutions through a 26 gauge needle from a syringe connected to compressed air. Under gentle stirring, the resulting beads were allowed to harden in the gelation solution for 60 minutes. The beads were then washed with distilled water and kept in a storage solution containing 0.9 % (w/v) NaCl and 0.05 M $CaCl_2$ at 4 ℃.

After 7 days of storage, the hydrogel beads were rinsed twice with distilled water to remove excess salt before drying. The beads were flash frozen with liquid nitrogen prior to lyophilisation using a vacuum freeze-dryer (7670520, Labconco Co., Kansas City, USA) at -45 ℃ and 12 Pa for 10 h. The dried beads were stored at room temperature away from moisture and light for further analysis.

Table 3.1 presents the different samples tested. The total concentration of polymers (protein and alginate) was maintained at 4.5% (w/v). The effects of protein sources, protein to alginate (P:A) ratios and PEC with chitosan on physicochemical properties of the resulting gels were investigated.

Table 3.1. Composition of the hydrogels

Sample	Protein source	Protein %(w/v)	Alginate %(w/v)	Protein:Alginate ratio	Gelation solution
S1	Pea protein	3.00	1.50	2:1	0.1 M Ca^{2+}
S2		3.00	1.50	2:1	0.5% Chitosan + 0.1 M Ca^{2+}
S3		3.60	0.90	4:1	0.1 M Ca^{2+}
S4		3.60	0.90	4:1	0.5% Chitosan + 0.1 M Ca^{2+}
S5		3.86	0.64	6:1	0.1 M Ca^{2+}
S6		3.86	0.64	6:1	0.5% Chitosan + 0.1 M Ca^{2+}
S7		4.00	0.50	8:1	0.1 M Ca^{2+}
S8		4.00	0.50	8:1	0.5% Chitosan + 0.1 M Ca^{2+}
S9	Canola protein	3.00	1.50	2:1	0.1 M Ca^{2+}
S10		3.00	1.50	2:1	0.5% Chitosan + 0.1 M Ca^{2+}
S11		3.60	0.90	4:1	0.1 M Ca^{2+}
S12		3.60	0.90	4:1	0.5% Chitosan + 0.1 M Ca^{2+}
S13		3.86	0.64	6:1	0.1 M Ca^{2+}
S14		3.86	0.64	6:1	0.5% Chitosan + 0.1 M Ca^{2+}
S15		4.00	0.50	8:1	0.1 M Ca^{2+}
S16		4.00	0.50	8:1	0.5% Chitosan + 0.1 M Ca^{2+}

3.3.3 Fourier transform infrared spectroscopy (FTIR)

IR-spectra of alginate, proteins, chitosan and hydrogel beads were obtained using a Nicolet iS5 FTIR spectrometer (Thermo Scientific, Madison, WI, USA). The samples were freeze-dried and ground to fine powder before test. The spectra were recorded at 32 scans and 4 cm^{-1} resolution in the 500 to 4000 cm^{-1} range. The spectra were analyzed using the OMNIC software package (version 8.2, Thermo Nicolet Corp).

3.3.4 Differential scanning calorimetry (DSC)

The thermal behavior of the hydrogel beads was investigated by DSC (Q100, TA Instruments, Inc., New Castle, DE, USA). Dried hydrogel beads were ground into powder. Alginate, chitosan, PPI and CPI were freeze-dried before the assay. Ten mg of sample was sealed in a hermetic aluminum pan. Samples were heated from 20 to 350 ℃ at a constant heating rate of 10 ℃/min under nitrogen gas (50 mL/min). The samples were run in duplicate.

3.3.5 Thermogravimetric analysis (TGA)

The thermal stability of the hydrogel beads was determined by TGA (Q500, TA Instrument, Inc., New Castle, DE). Five mg of sample was loaded into a platinum pan and heated from room temperature to 600 ℃ at a constant rate of 20 ℃/min. The analyses were carried out under a stream of nitrogen gas at a constant flow rate of 60 mL/min. Each sample was tested in duplicate.

3.3.6 Scanning electron microscopy (SEM)

The morphological properties of the freeze-dried hydrogels were examined by SEM (TM3000, Hitachi High-Technologies Co., Tokyo, Japan) at accelerated voltages of 5 and 15 kV. The hydrogel beads were cut to expose the cross-sections.

3.3.7 Equilibrium swelling test

The swelling behavior of hydrogel beads in distilled water, SGF and SIF was investigated. The swelling tests were conducted in SGF and SIF solutions without enzyme, which were prepared as outlined by Wang et al. [201]. SGF (pH 1.2) was prepared by dissolving 2.0 g of NaCl in 7 mL of concentrated HCl and adjusting the final volume to 1 L. SIF (pH 6.8) was prepared by dissolving 6.8 g of KH_2PO_4 in 250

mL of distilled water, adding 77 mL of 0.2 N NaOH solution, and diluting to a final volume of 1 L. The pH of SGF and SIF solutions were adjusted to 1.2 and 6.8 respectively. In a typical experiment, empty tea bags were immersed in the swelling medium for 24 h and hung to dry for 15 min. Thereafter the empty bags were blotted with paper towel to remove excess water and weighed (W_1). Around 0.2 g of sample was weighed (W_2) and placed in the tea bag. The loaded tea bags were closed and immersed in different swelling media for 6 hours, after which the loaded tea bags were taken out and blotted dry, and their weights were measured (W_3). The equilibrium swelling (ES) of the hydrogel beads was calculated using the following equation:

$$\mathrm{ES}(g/g) = \frac{W_3 - W_2 - W_1}{W_2} \qquad \text{(Equation 1)}$$

3.3.8 Degradation assay

To further understand the effect of chitosan on the stability of the resulting hydrogels in simulated gastrointestinal conditions, hydrogel samples with (S2 and S10) and without (S1 and S9) chitosan were selected and submitted to the degradation tests. The method used was adapted from Chen and Subirade [178]. The digestive media (SGF and SIF) are prepared in the same fashion as described in the swelling test except that enzymes were added. Pepsin (0.1% w/v) was added to the SGF and pancreatin (1.0% w/v) was added to the SIF. To conduct the test, 40 mg of lyophilized hydrogels beads was suspended in 30 ml of digestive medium and incubated at 37 ℃ with agitation at 150 rpm. At one hour intervals, 1 ml of sample was withdrawn from the liquid phase (undissolved beads were avoided). The samples were diluted and the absorbance was measured at 280 nm wavelength. Standard curves were made using known amounts of proteins (100%, 80%, 60%, 40% and 20%) contained in 40 mg of hydrogel in the digestive media. After 6 hours of incubation, these standards were diluted and the absorbance was measured at the same wavelength. The tests were conducted on three independent replicates and each sample was measured in duplicate.

3.3.9Statistical analysis

Analysis-of-variance (ANOVA) and Levene's test for homogeneity of variance were performed by using SAS® (statistical analysis system, Version 9.2, SAS Institute Inc., Cary, NC). Post-hoc multiple comparisons tests were performed to examine the significant difference by either the Tukey's test or Games-Howell test depending on whether the variance was homogenous or heterogeneous, respectively. All the tests were performed at a significant level of α=0.05. The error bars represent the standard deviation of the data.

3.4 Results and discussion

3.4.1 Synthesis of PPI and CPI hydrogel beads

Protein-alginate hydrogels were synthesized by ionotropic gelation of protein-alginate gel solutions. The protein-alginate hydrogels were crosslinked only with Ca2+ ions. Hydrogel beads with additional crosslinking were formed in the gelation solution with the addition of chitosan. These beads formed in the gelation solution with chitosan will be further referred as PEC beads. The hydrogel beads were lyophilized and they closely maintained their size and shape (Fig. 3.1).

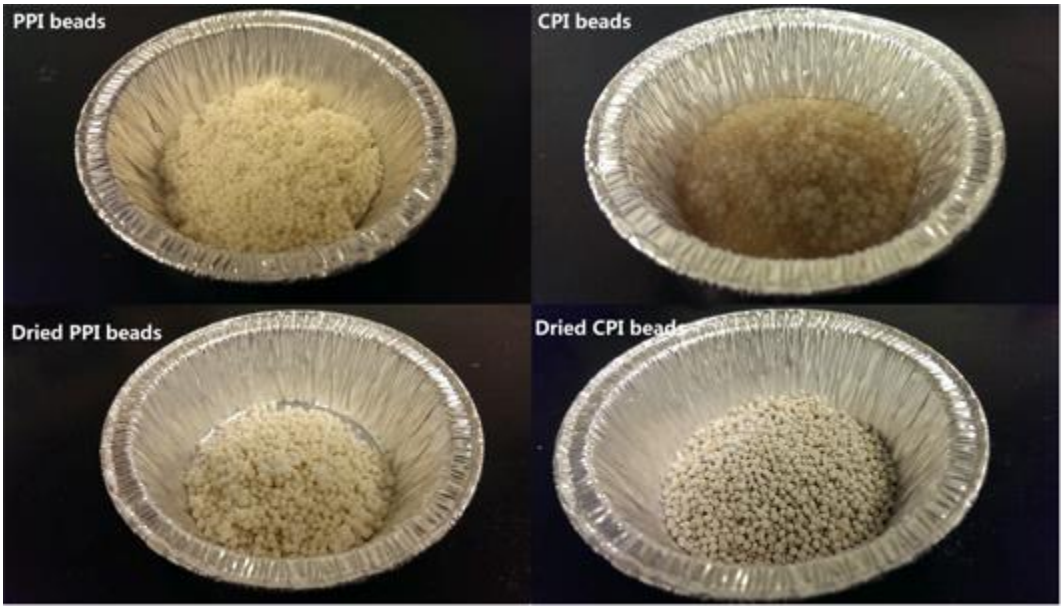

Fig. 3.1. PPI and CPI hydrogel beads

3.4.2 Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of alginate and chitosan are shown in Fig. 3.2a. Symmetric COO^- stretching vibration and asymmetric COO^- stretching vibration of alginate carboxyl groups were associated with bands at 1602 and 1405 cm^{-1}, respectively. The chitosan spectrum shows bands at 1651 and 1591 cm^{-1} which were assigned to C=O stretching of amide I and N-H bending from amide II. These observations agreed with previous observations [202]. Fig. 3.2b and 3.2c present the FTIR spectra of proteins, protein-alginate hydrogels and PEC hydrogels. The carboxyl stretch vibration from the carboxyl groups of alginate located at 1417 cm^{-1} was found in the spectra of hydrogel beads. A common peak in the 1643-1614 cm^{-1} region was assigned to the C=O stretching from the amide groups of proteins presented in these three samples. Amide II bands were located at 1519 and 1538 cm^{-1} in protein spectra which agreed with the literature [203]. These amide II bands were weaker in both PPI and CPI hydrogel beads (without chitosan) indicating a change in conformation due to the unfolding of proteins which occurred during denaturation. However, strong amide II bands at 1538 cm^{-1} were observed again in hydrogels with chitosan. The presence of these bands in PEC hydrogel beads can be explained by the occurrence of protonated $–NH_3^+$ groups of

chitosan at a pH of 5.5 [202]. This indicated the presence of chitosan in the PEC hydrogel beads.

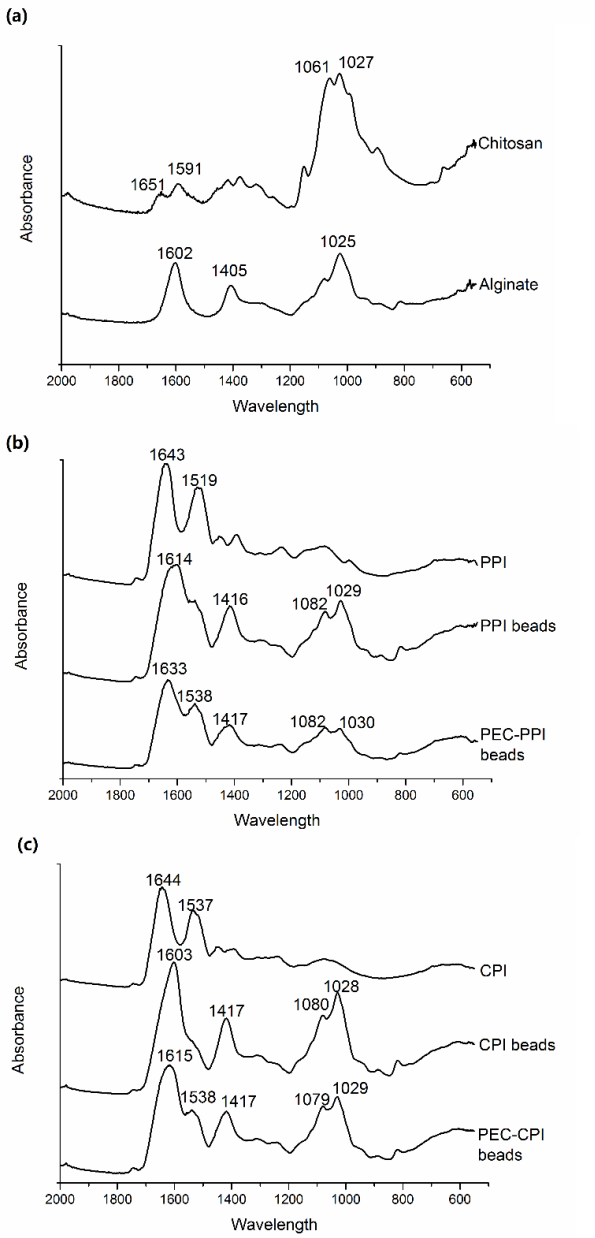

Fig. 3.2. FTIR spectra of (a) alginate and chitosan. (b) PPI, PPI beads (S3) and PEC-PPI beads (S4). (c) CPI, CPI beads (S11) and PEC-CPI beads (S12).

3.4.3 Differential scanning calorimetry (DSC)

The initial endothermic peaks corresponding to the thermal denaturation of PPI

and CPI were observed at 136.47 ℃ and 100.98 ℃, respectively (Fig. 3.3b). The later endothermic peaks at 224.50 ℃, 277.39 ℃ and 319.92 ℃ for PPI, and at 217.99 ℃ and 284.04 ℃ for CPI are related to the cleavage of peptide bonds, indicating the thermal degradation of the polypeptide chains [204]. The exothermic peaks at 245.95 ℃ for alginate and at 306.9 ℃ for chitosan are assigned to the degradation of the polysaccharide [205]. These results were confirmed by a rapid weight loss observed during the TGA tests (Section 3.4). Differing from alginate and chitosan, no exothermic peak was observed for the resulting hydrogels. This indicated that the interactions between the proteins and the polysaccharides increased the thermal stability of the hydrogels. There was no endothermic peak above 200 ℃ were observed in hydrogels with low protein content (S2, S4, S10, S12 and S14). However, one was observed for hydrogel samples with high protein content (S6, S8 and S16). A possible explanation could be that a portion of the proteins did not undergo crosslinking reaction. Therefore, the thermal properties of these loose proteins were similar to the native proteins.

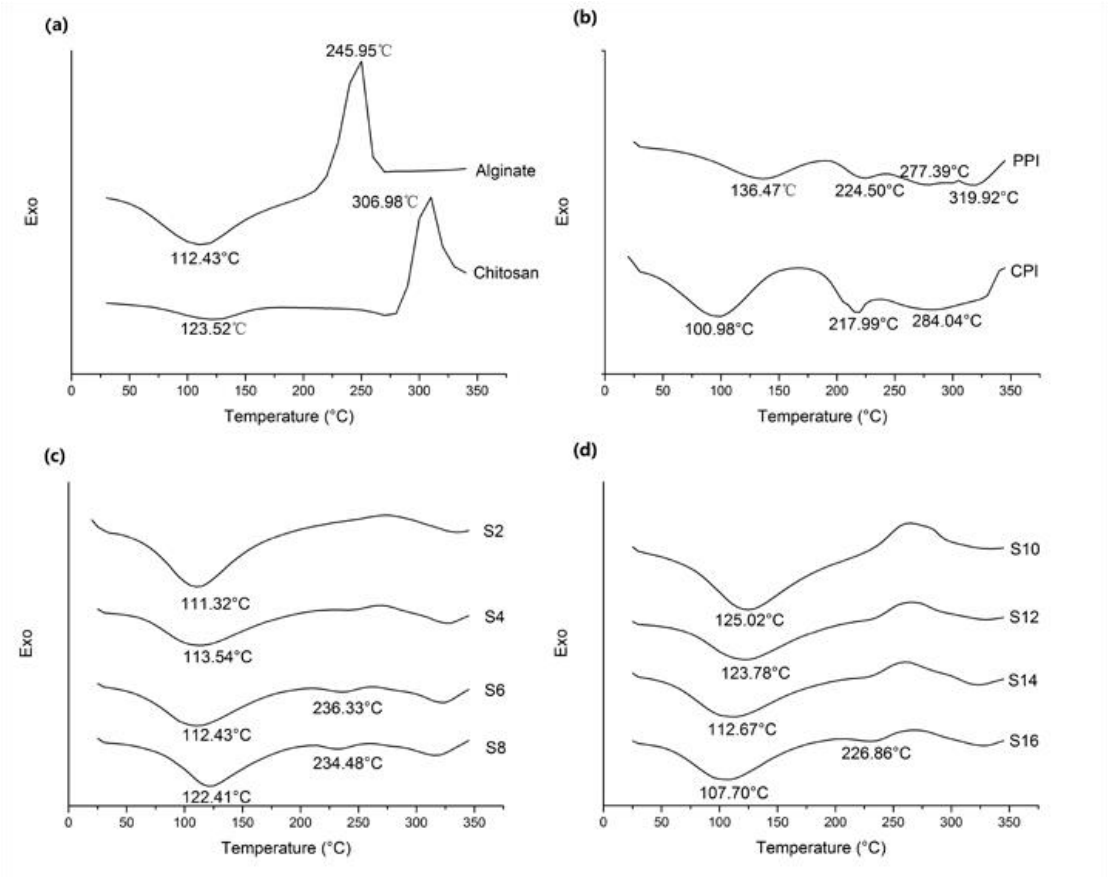

Fig. 3.3. Thermograms of (a) alginate (AG) and chitosan (CTS). (b) PPI and CPI. (c) PEC-PPI beads with ratio of PPI:AG of 2:1 (S2), 4:1(S4), 6:1(S6), 8:1(S8). (d) PEC-CPI beads with ratio of CPI:AG of 2:1 (S10), 4:1(S12), 6:1(S14), 8:1(S16)

3.4.4 Thermogravimetric analysis (TGA)

The thermal stability of hydrogels and starting materials were further characterized by TGA. A rapid weight loss (1.62 %/℃) of alginate occurred at 246.30 ℃ which corresponds to the temperature at which an exothermic peak was found in the DSC thermogram. The major weight loss of PPI and CPI occurred from 180 ℃ to 560 ℃. Fig. 3.4 shows that below 340 ℃, the hydrogel beads had a higher weight loss than the proteins. This is due to the denaturation step which resulted in the loss of the tertiary structure of proteins and therefore reduced their thermal stability.

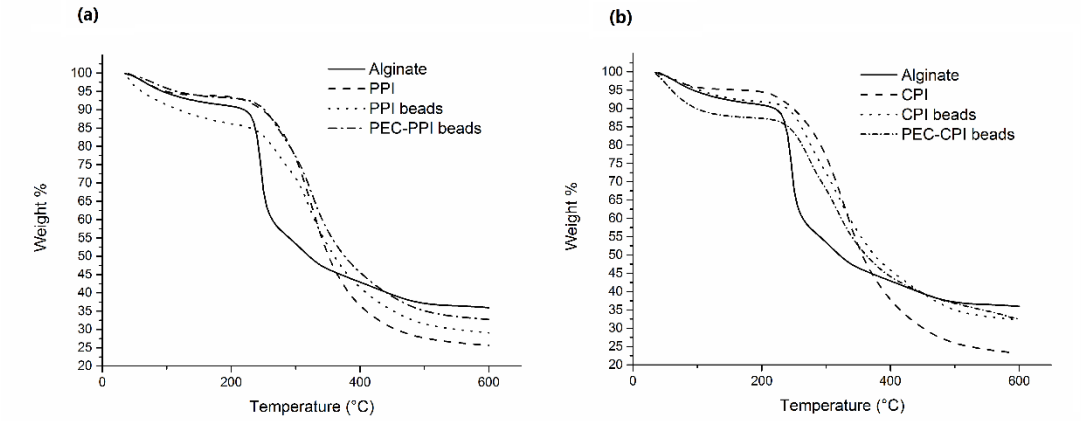

Fig. 3.4. TGA thermograms of (a) alginate, PPI and PPI beads; (b) alginate, CPI and CPI beads

3.4.5 Scanning electron microscopy (SEM)

The SEM was used to examine surface morphology and the internal structures of the hydrogel beads. A freeze-dried hydrogel bead is shown in Fig. 3.5a. The PEC beads displayed a random honeycomb network structure (Fig. 3.5b), which was not observed on the surface of protein-alginate beads (Fig. 3.5c). The cross-section of PEC beads is shown in Fig. 3.5d. The sublimation of ice reduced shrinkage of the hydrogel during

freeze-drying and thus maintained the highly porous internal structure. A bilayer 'wall' structure was observed in PEC beads (Fig. 3.5e), which is different from the single layer structure observed in protein-alginate beads (Fig. 3.5f). The bilayer 'wall' structure could have been formed by the aggregation of chitosan-alginate PEC on the surface of the beads.

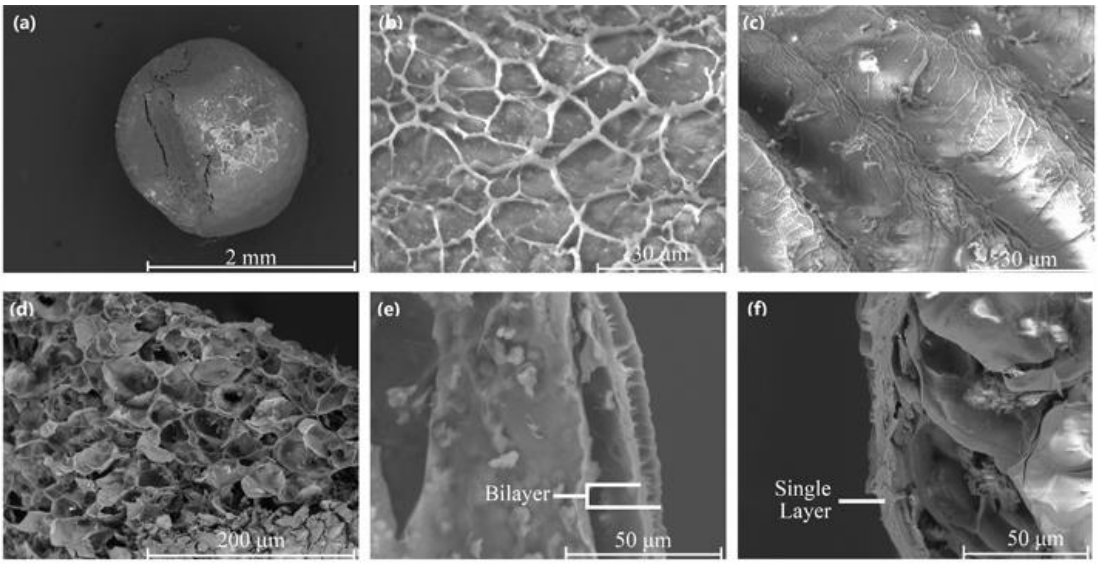

Fig. 3.5. SEM images of (a) PEC bead 50X (b) surface of PEC bead 2000X (c) surface of PPI bead 2000X (d) cross-section of PEC bead 500X (e) bilayer ‘wall’ structure of PEC bead 1500X (f) single layer ‘wall’ structure of PPI bead 1200X

3.4.6Equilibrium swelling test

The effects of the swelling media, PEC, protein sources and protein:alginate (P:A) ratio on the swelling of hydrogels were studied. The swelling tests were performed in three swelling media (distilled water, SGF and SIF) for all hydrogel samples. The equilibrium swelling was determined using Equation 1. The results are presented in Fig. 3.6. Interestingly, the swelling test showed that CPI beads have a higher swelling capacity than PPI beads for all three media ($P<0.01$) (Fig. 3.6). A possible explanation could be that CPI hydrogel has a lower crosslinking density than PPI hydrogel due to a higher content of glutamic acid and aspartic acid of PPI (Glu: 19% and Asp: 12%) [18]

than CPI (Glu: 17% and Asp: 8.32%) [14]. These two amino acids possess a carboxylate group which is negatively charged at the pH of gelation. Crosslinking of negatively charged carboxyl groups via Ca^{2+} has been suggested for the gelation of denatured proteins similar to the Ca^{2+} induced gelation of alginate [206]. Thus, the CPI hydrogels have a more flexible network which is able to expend while swelling. Moreover, the CPI used in this study has higher protein content (95 % protein) as compared to the PPI (81.73 % protein, 10.3 % sugar). The sugars in PPI are mainly sucrose and galactoside [207]. They are relatively small molecules as compared to polypeptides in proteins and thus were not likely to participate in the formation of the hydrogel network as a polymeric backbone.

After 6 hours of swelling, the average absorption capacity of PPI and CPI beads (P:A=2:1) in SIF reached 25.3 and 33.9 g/g, respectively. The maximum swelling reached were 29.17 g/g for PPI beads (P:A=2:1) and 42.3 g/g for CPI beads (P:A=2:1) (Fig. 3.6c). Compared to SIF, both PPI and CPI beads (P:A=2:1) showed relatively low water absorption in distilled water (8.9 and 10.4 g/g) and SGF (6.9 and 10.5 g/g). It should be noted that after swelling, all samples kept a shape and a size similar to their original state. After 6 hours of immersion in distilled water and SGF, the PPI and CPI beads closely maintained their original shape and size. However, during the swelling tests in SIF solution, the volume of both PPI and CPI beads increased and a gradual shape loss was observed. The swelling behavior of hydrogel beads in SIF could be explained by the diffusion of Ca^{2+} ions from the hydrogel network into the swelling medium due to the formation of calcium phosphate salts. Calcium ions are the crosslinkers of the hydrogel network and the loss of these ions would result in a decrease in crosslinking density. The hydrogel networks became more flexible, while the chain relaxation led to the exposure of hydrophilic carboxyl groups. As a result, the relaxation of the hydrogel backbone and the exposure of carboxyl groups enhanced the gel swelling [208].

Moreover, PEC had a significant effect on swelling in SIF solution ($P<0.01$). The maximum swelling capacity was 33.0 g/g for PPI beads, and 25.2 g/g for PEC-PPI beads; while it was 50.8 g/g for CPI beads and 33.8 g/g for PEC-CPI beads (Fig. 3.6d). The chitosan within the PEC beads formed extra crosslinks via interaction with alginate. Although the formation of calcium phosphate salts still occurred, the PEC beads were less affected by the loss of Ca^{2+} ions. Therefore, chain relaxation in PEC beads was less pronounced than for the other matrices and which resulted in a lower swelling degree. These findings agreed with results reported in the literature [209].

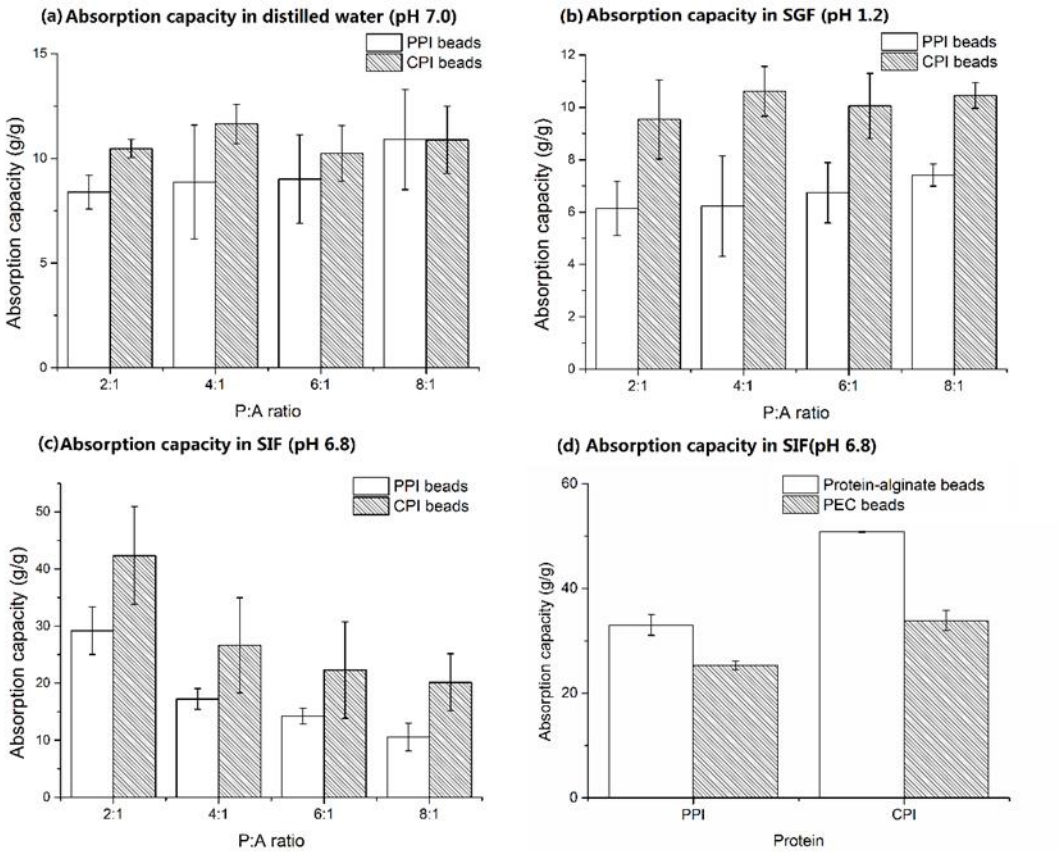

Fig. 3.6. Effects of P:A ratio on the absorption capacity of hydrogel beads after 6 hours of swelling in (a) distilled water; (b) SGF; and (c) SIF (d) The swelling capacity of protein-alginate and PEC hydrogel beads in SIF

3.4.7 Hydrogel degradation in simulated gastrointestinal fluid with enzymes

Further degradation tests were performed to investigate the degradation profile of the hydrogels in digestive media with enzymes (Fig. 3.7). Most of the beads retained their shape after 6 hours of incubation in SGF. The slight decrease in the size of the beads might have resulted from both the erosion and the shrinkage of the beads. At pH 1.2, the carboxyl groups become protonated which reduces electrostatic repulsion, thereby inducing shrinkage. Furthermore, the results did not show significant difference between the protein-alginate beads and the PEC beads. After 6 hours of incubation, around 40% of proteins within both PPI and CPI hydrogel beads were digested and diffused into the SGF.

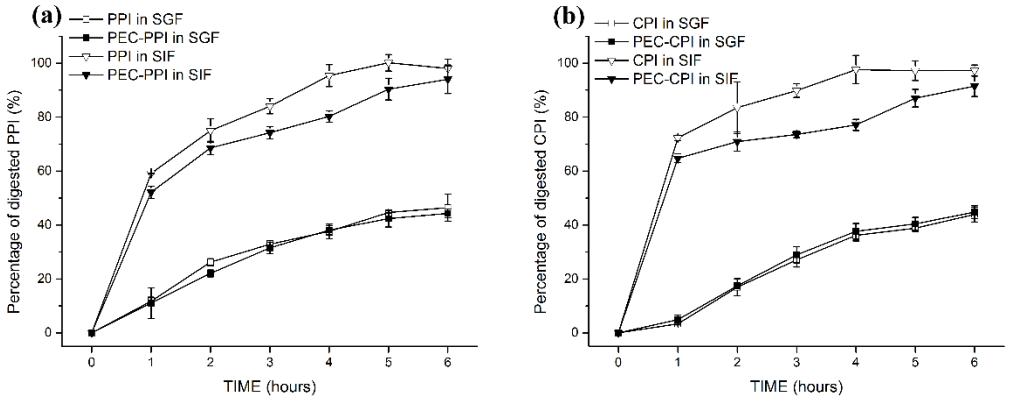

Fig. 3.7. Degradation of (a) PPI beads (S1), PEC-PPI beads (S2) (b) CPI (S9), PEC-CPI beads (S10) in digestive media.

A rapid degradation was observed in SIF media for all hydrogels regardless of the presence of chitosan. Around 59% of pea proteins and 64% of canola proteins were digested within the first hour. After 2 hours, most of the beads without chitosan had lost their shape. The rapid degradation could be explained by the direct contact of enzymes with the proteins on the bead surface. Moreover, the rapid loss of calcium due to the formation of calcium phosphate salts also reduced the crosslinking density and led to the erosion of the polymers. The erosion further led to the exposure of proteins

embedded within the hydrogel beads to the enzymes.

Interestingly, PEC with chitosan retarded the degradation of hydrogel beads from the second hour. The biggest difference between protein-alginate beads and PEC beads was observed at 4 hours for both PPI and CPI (Fig. 3.7). Hydrogel beads without chitosan showed degradation ratios of 95.37% ± 4.06 (PPI) and 97.60% ± 5.25 (CPI) respectively. These results agree with the degradation of whey protein-alginate hydrogels (around 95% at 4 hours) reported by Chen and Subirade [178]. However, the PEC hydrogel beads synthesized in this study showed significantly lower degradation ratios (80.24% ± 2.10 for PEC-PPI beads and 77.09% ± 2.06 for PEC-CPI beads). This difference can be attributed to the enhanced crosslinking density of hydrogels that contained chitosan. For hydrogel beads without chitosan, the relaxation of the hydrogel network was fast because of the loss of Ca^{2+}. The enzymes can easily penetrate the relaxed network and digest the proteins inside of the structure. For PEC beads, the chitosan provided additional crosslinks formed by PEC with alginate which delayed the erosion of the alginate. These results agreed with the results from the swelling tests presented in Section 3.6 where the PEC beads showed lower swelling in SIF media than protein-alginate beads. The PEC beads had less chain relaxation which reduced the diffusion of media through the matrix.

3.5 Conclusion

Pea protein and canola protein-based hydrogels tested in this study showed similar properties except that CPI beads had higher swelling capacities in all the media. Polyelectrolyte complexes of protein-alginate-chitosan were synthesized. Interactions between the protein and polysaccharide backbones were verified by FTIR. From the DSC and TGA results, it was observed that the hydrogels were less thermally stable than the native proteins. The surface morphology and the internal structure of the resulting gels were studied by SEM. The freeze-dried beads still closely maintained

their original shape and a porous internal structure. The swelling test showed that both PPI and CPI hydrogels had a higher absorption capacity in SIF due to the calcium-chelating effect of phosphate ions which reduced the crosslinking density. Moreover, PEC with chitosan reduced the impact of Ca^{2+} chelation on the hydrogel network. The swelling tests showed less diffusion of SIF media through PEC beads. The PEC beads are also more stable against the enzymatic digestion in SIF. But PEC did not show significant influence on the stability of beads in SGF. These findings suggest that protein-alginate-chitosan complexes could serve as prolonged release matrices.

Connecting statement

Chapter 3 showed the potential of pea and canola proteins-based hydrogel capsules as delivery matrices for oral administration of nutraceutical products. The mild gelation condition, and controllable degradation in simulated gastrointestinal fluid suggested that protein-based hydrogels could be excellent delivery matrices. Especially pea protein isolates, which have been used as food additive to increase the protein content of food, is a brilliant protein source for the synthesis of food-grade hydrogels. Chapter 4 describes the encapsulation of *Lactobacillus. Casei* ATCC 393 as a model probiotic by pea protein-based hydrogel capsules. The encapsulation efficiency and protective effect of hydrogels against gastrointestinal conditions, through freeze-drying and subsequent storage have been studied.

Chapter 4 has been accepted to be published in the Journal of Food Engineering, and it is currently in press.

CHAPTER 4

Encapsulation of *Lactobacillus casei* ATCC 393 and evaluation of their survival after freeze-drying, storage and under gastrointestinal conditions

4.1 Abstract

The aim of this work was to investigate the encapsulation of *Lactobacillus casei* ATCC 393 cells with a pea protein isolate-alginate hydrogel matrix and to study the protective effects of such matrix on the bacteria during freeze-drying, storage and under harsh gastrointestinal conditions. The encapsulation of *L. casei* achieved a high yield of 85.69% ± 4.82 which indicated that the matrix and the encapsulation technique are compatible with the probiotic strain. During the freeze-drying process, the matrix did not show any protective effect as compared to the non-encapsulated cells. The dried capsules have been taken into subsequent storage tests at three temperatures (+22, +4 and -15 ℃). After 84 days of storage, the encapsulated *L. casei* stored at -15 ℃ showed the highest survival rate among all samples (59.9% ± 17.4). After 84 days of storage, the capsules stored at -15 ℃ were submitted to further survival and release tests in simulated gastrointestinal fluids. These dried and stored capsules displayed a weaker buffering effect against acidic gastric conditions as compared to the fresh capsules which were tested right after the encapsulation. However, both stored and fresh capsules showed similar release profiles of *L. casei* in simulated intestinal fluid.

4.2 Introduction

Probiotics are microorganisms which, when consumed in adequate amounts, confer a health benefit on the host [194]. Due to the rising interest of consumers in food products containing probiotics, the volume of research related to the subject has been expanding rapidly since the year of 2000 [210]. The most important probiotic microorganisms in the food industry are lactic acid bacteria (LAB) [194]. They have been widely used for the production of fermented dairy products such as cheese, yogurt and ice cream. Dry products such as capsules, cereal products and beverage powders containing probiotics have also been developed [166]. Recent studies on LAB confirmed their health benefits on the human gut and immune system [211]. Among LAB, *Lactobacillus casei* (*L. casei*) species has been widely used in the production of fermented food [212]. It has been reported that *L. casei* can reduce the cholesterol level [213] and can be used against cancer cell proliferation [214]. However, their probiotic benefits strongly depend on the viability of these microorganisms. It has been suggested that probiotic-based products should contain at least 7 log cfu/g of viable cells at the time of consumption to provide probiotic benefits [215]. However, it has been reported that the survival rate of probiotics is relatively low in traditional dairy products [216]. The poor survival rate of probiotics during processing and storage is attributed to environmental stressors such as the variations in pH and the toxicity of oxygen and UV light. After ingestion, adequate amount of probiotic cells also have to survive through the upper digestive tract and reach the intestine of the host [22]. The harsh gastric environment combined with a variety of digestive enzymes can lead to the loss of viable probiotic cells. The detrimental effects of simulated gastric environment on several strains of *L. casei* have been reported [217].

The entrapment of probiotic cells by microencapsulation provides a physical barrier against environmental stressors [194] and therefore reduce the unavoidable loss of viability of probiotic cells during processing, storage and digestion [25]. The

selection of a wall material for encapsulation is always a challenge. The material should be food-grade and capable of entrapping and protecting probiotics. Moreover, the material and the encapsulation process should be compatible with the probiotics [216].

Food-grade natural polymers are excellent materials for microencapsulation due to their non-toxicity and good biocompatibility as well as their ability to form gels. Legume proteins have been used as substrate in the production of fermented product with *L. casei* ATCC 393 [218]. Moreover, microspheres based on legume protein isolate and alginate have been reported [164]. These microspheres have been used to encapsulate *Bifidobacterium adolescentis*, an acid sensitive probiotic. These protein-polysaccharide based microspheres showed an excellent protective effect against simulated gastric conditions. The dense gel structure formed by legume proteins and alginate served as a barrier between the probiotics and the environment.

Among legume proteins, pea proteins are increasingly attracting interest due to their high nutritional value, digestibility, bioavailability and long term health benefits [17]. Pea protein isolate (PPI) is a food-grade material that has been used as additive to enrich the protein content in food industry [18]. To the best of our knowledge, no research has investigated the encapsulation of *L. casei* with pea protein isolate-alginate (PPI-alginate) based hydrogels and the survival of *L. casei* in freeze-dried PPI-alginate hydrogels during long term storage.

The overall objective of this work was to encapsulate *L.casei* ATCC 393 with PPI-alginate hydrogel microspheres beads via extrusion. The effects of freeze-drying and subsequent storage at different temperatures on the viability of *L.casei* ATCC 393 were studied. Moreover, the microspheres were tested in simulated gastrointestinal conditions just after their preparation and after a period of 12 weeks of storage. The protective effect and the release profile of the microspheres before and after storage were compared to investigate their stability.

4.3 Materials and Method

4.3.1 Materials

Lactobacillus casei ATCC® 393™ was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Pea protein isolate (PPI) (Propulse N™, 81.73% protein, < 10.3% sugars, < 0.7% starch, 3.40% moisture, < 0.5% fat and < 4.0% ash) was obtained from Nutri-Pea Ltd. (Portage la Prairie, MB, Canada). Alginic acid sodium salt (alginate) with low viscosity (1% aq. solution: < 300 cps) was purchased from MP Biomedicals, LLC (Solon, OH). Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), sodium chloride (NaCl) and hydrochloric acid (HCl) were purchased from Fisher Scientific (Fair Lawn, NJ). Sodium hydroxide (NaOH) was purchased from EMD (Damstadt, Germany). Agar and deMan, Rogosa Sharpe (MRS) broth, ammonium sulphate ($(NH_4)_2SO_4$), potassium phosphate monobasic (KH_2PO_4) , potassium phosphate dibasic trihydrate ($K_2HPO_4 \cdot 3H_2O$), trisodium citrate ($HOC(COONa)(CH_2COONa)_2 \cdot 2H_2O$), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), pepsin from porcine gastric mucosa (powder, ≥250 units/mg solid) and pancreatin from porcine pancreas (4 ×USP specifications) were purchased from Sigma-Aldrich (St. Louis, MO).

4.3.2Methodology

4.3.2.1 Preparation of the bacteria for encapsulation

L. casei ATCC 393 dry pellet was rehydrated with 5 mL of MRS broth and incubated for 24 h (37 ℃, 200 ×rpm) in an incubator (INFORS AG CH 4103 Bottingen, Switzerland) to revive the bacteria. The liquid culture was then used to inoculate MRS agar plates via t-streak. Single colonies of L. casei were obtained after 48 h of incubation at 37 ℃ under an anaerobic environment created with a jar and anaerobic

atmosphere generation bags (Sigma-Aldrich, Oakville, Canada). MRS broth (100mL) was inoculated with one single colony and incubated for 24 h at 37 ℃ under constant agitation (200 ×rpm). Thereafter, this liquid culture was mixed with equal amount of sterilized 50% glycerol solution and stored at -80 ℃ to be used as stock culture.

Prior to encapsulation, single colonies obtained from streaking stock cultures on MRS plates, were grown in 100 mL of MRS broth for 24h at 37 ℃ under constant agitation (200 ×rpm). Cells were harvested at early stationary stage by centrifugation at 8000 rpm for 10 min at 20 ℃ (Sigma centrifuge, 3–16PK, Germany). The cells with a final concentration of 9 log cfu/mL were resuspended in 20 mL of sterile modified phosphate buffer ((NH4)2SO4 0.2%, K2HPO4•3H2O 1.83%, KH2PO4 0.6%, HOC(COONa)(CH2COONa)2•2H2O 0.1% and MgSO4•7H2O 0.02%).

4.3.2.2 Encapsulation

The L. casei loaded PPI-alginate capsules were prepared via extrusion technology [164]. Sterilized distilled water and glassware were used for the encapsulation process. The 3.6% (w/v) protein solution was prepared by dissolving PPI powder in 0.05 M NaOH solution. The solution was heated to 80 ℃ to denature and dissolve the proteins. After 30 min, the solution was cooled to room temperature in a cold water bath and neutralized to pH 7 with 1 M HCl. The solution was reheated to 80 ℃ and the alginate powder was added to produce a final concentration of 0.9% (w/v). Complete dissolution of the alginate powder was achieved at 80 ℃ under magnetic stirring for 30 min. Thereafter, the solution was cooled to room temperature. The bacterial suspension was subsequently added to the PPI-alginate solution at a bacteria-to-polymer ratio of 1:10 (v/v). The capsules loaded with bacteria were formed via extrusion of the bacteria-polymer solution through a 26G needle into a 0.05 M CaCl2 solution. The resulting capsules were allowed to harden in the CaCl2 solution for 30 min. Thereafter, the capsules were collected and rinsed with distilled water. The capsules were separated into two portions. One portion was immediately used for the survival and release tests

in simulated gastrointestinal conditions. These capsules will be referred as 'fresh capsules' throughout the text. The remaining capsules were freeze-dried for the storage test. The encapsulation and freeze-drying steps were quadruplicated.

4.3.2.3 Encapsulation Yield

In order to investigate the survival of bacteria after encapsulation, 1 g of fresh capsules was immersed in 9 g of modified phosphate buffer. The capsule suspension was incubated at room temperature under constant agitation (250 × rpm) for one hour to completely dissolve the capsules. Preliminary tests confirmed that no significant change occurred on the numbers of *L. casei* viable cells after one hour of incubation in the modified phosphate buffer at room temperature. The enumeration of viable cells was conducted by spread plating serial 10-fold dilutions of dissolved capsules and free *L. casei* cell suspension onto MRS agar plates. All the plating was done in triplicate. The plates were incubated at 37 ℃ for 48 hours in an anaerobic environment before colony counting. The encapsulation yield was calculated by applying equation (1):

$$\text{Encapsulation yield} = \frac{\text{total viable cells after encapsulation}}{\text{total viable cells before encapsulation}} = \frac{N_E \times M_E}{N_0 \times V} \quad (1)$$

Where $N_0(\text{cfu/mL})$ is the number of viable cells in cell suspension, $V(\text{mL})$ is the volume of cell suspension used for encapsulation, $N_E(\text{cfu/g})$ is the number of viable cells within 1 g of capsules and M_E (g) is the mass of capsules obtained from encapsulation.

4.3.2.4 Survival of *L.casei* after freeze-drying

The capsules were flash frozen with liquid nitrogen and then lyophilized in a vacuum freeze-dryer (7670520, Labconco Co., Kansas City, USA) for 48 h. Thereafter, the freeze-dried capsules were weighed. In order to investigate the survival of *L. casei* after freeze-drying, the freeze-dried capsules (0.1 g) were suspended in 9.9 g of

modified phosphate buffer to dissolve the capsules. After 1 hour of incubation under constant agitation (250 × rpm), the viable cells were enumerated via spread plating serial 10-fold dilutions of dissolved capsules. For the free *L. casei* cells, 100 µL of cell suspension was transferred into 1.5 mL Eppendorf centrifuge tubes. The tubes were centrifuged (8000 × rpm, 10 min, 20 ℃) to harvest the cells. The liquid phase was removed and the tubes were freeze-dried for 48 h. To enumerate the viable cells after freeze-drying, 9.9 mL of modified phosphate buffer was added into each tube to rehydrate the cell pellets. The tubes were vortexed to resuspend the cell. The tubes were also incubated for 1 h at room temperature under constant agitation (250 × rpm) before spread plating. The results were presented as the total viable cells before and after the freeze-drying process. The total viable cells after freeze-drying were calculated by applying equation (2):

$$\text{Total viable cells after freeze} - \text{drying} = N_{FD} \times M_{FD} \qquad (2)$$

Where N_{FD} (cfu/g) is the number of viable cells in 1 g of freeze-dried capsules and M_{FD} (g) is the dry weight of freeze-dried capsules.

4.3.2.5 Scanning electron microscopy (SEM)

The morphological properties of the freeze-dried capsules were examined by SEM (TM3000, Hitachi High-Technologies Co., Tokyo, Japan) at accelerate voltage of 5 kV. The capsules were sliced with a scalpel to expose the cross section. The free *L. casei* cells were lyophilized as positive control. After 24 h of incubation, free *L. casei* cells were harvest via centrifugation (8000 × rpm, 10 min, 20 ℃). The cell pellets were suspended in a modified phosphate buffer and centrifuged again. The liquid phase was removed. The cell pellets were frozen at -80 ℃ for 8 h and then freeze-dried for 48 h before microscopy.

4.3.2.6 Storage of *L.casei* at different temperatures

To investigate the shelf life of encapsulated bacteria under various storage temperatures, the freeze-dried capsules were transferred into 50 mL conical centrifuge tubes covered with an aluminum foil. The tubes were stored at room temperature (+22 ℃), in a fridge (+4 ℃) and in a freezer (-15 ℃). The capsules (0.1 g) were withdrawn from the tubes every 7 days for the first 35 days of storage and after 84 days of storage. The samples were dissolved, diluted and plated on MRS agar plates to enumerate the viable cells after storage. For the free L. casei cells, the freeze-dried cell pellets were stored separately in 1.5 mL Eppendorf centrifuge tubes under the same conditions as the capsules. At predetermined time points (7, 14, 21, 28 and 35 days), the tubes were taken out and 1 mL of modified phosphate buffer was added into each tube. The tubes were gently vortexed and then incubated at room temperature under constant agitation (250 ×rpm) for 1 h to rehydrate the cells before spread plating. The survival rate of L. casei during storage was calculated by applying equation (3):

$$\text{Survival rate} = \frac{N_t}{N_{FD}} \times 100\% \qquad (3)$$

Where $N_{FD}(\text{cfu/g})$ is the number of viable cells after freeze-drying and $N_t(\text{cfu/g})$ is the number of viable cells within the capsules after storage.

4.3.2.7 Survival and release of *L. casei* in simulated gastrointestinal fluids

In order to assess the protective effect of encapsulation on *L. casei* in simulated gastric condition and the release of *L. casei* in simulated intestinal condition, fresh capsules and the capsules which were stored for a period of 84 days have been tested. The free *L. casei* cells have been used as control. The capsules stored for a period of 84 days at -15 ℃ have been used for these tests due to their highest viability among all three temperatures. They will be referred as ‘preserved capsules’ to distinguish them

from fresh capsules.

Survival of *L. casei* in simulated gastric fluid

The simulated gastric fluid (SGF) was prepared by dissolving 2.0 g of NaCl and 6.0 g of pepsin in 7 mL of concentrated HCl. The solution was diluted with distilled water to a final volume of 1 L. The SGF was adjusted to pH 2.0 with 1 M NaOH by using a Symphony SB70P pH meter (VWR, Wayne, NJ, US). The prepared SGF was pre-warmed to 37 ℃ in an incubator before the survival test. The free *L. casei* cell suspension (1 mL) and the fresh capsules (1 g) were separately added to test tubes containing 9 g of SGF. The tubes were gently shaken and then incubated at 20 ℃ under a constant agitation (200 × rpm). The preserved capsules were tested in the same way except that 0.1 g of sample was suspended in 9.9 g of SGF. For both fresh and preserved capsules, three centrifuge tubes were prepared and used for the enumeration of viable cells at time intervals of 30, 60 and 120 min. At each time point, the capsules were harvested, rinsed twice with distilled water and used for the enumeration of viable cells. The survival of *L. casei* was presented as numbers of viable cells (log cfu/g).

Release of *L.casei* in simulated intestinal fluid

The simulated intestinal fluid (SIF) was prepared by dissolving 6.8 g of KH_2PO_4 in 250 mL of sterilized water followed by the addition of 77 mL of 0.2 N NaOH solution. The final volume was adjusted to 1 L with sterilized water. Pancreatin powder was added to the SIF to produce a concentration of 1 % (w/v) and the pH of SIF was adjusted to 6.8. The survival of *L. casei* and the release profile of *L. casei* from PPI-alginate capsules were investigated. Free *L. casei* cells, fresh capsules and preserved capsules were incubated in SIF in a similar fashion as the SGF test. The free *L. casei* cells and the capsules were added into the tubes containing pre-warmed SIF and were incubated at 37 ℃ under constant agitation (200 × rpm). At predetermined time points (20, 40, 60, 120 min), 100 μL of the liquid phase was withdrawn from the tubes and immediately

used for the enumeration of viable cells.

4.3.2.8 Statistical analysis

Analysis-of-variance (ANOVA) and Levene's test for homogeneity of variance were performed by using SAS® (statistical analysis system, Version 9.2, SAS Institute Inc., Cary, NC). Post-hoc multiple comparisons tests were performed to examine the significant difference by either the Tukey's test or Games-Howell test depending on whether the variance was homogenous or heterogeneous, respectively. All the tests were performed at a significant level of α=0.05. The error bars represent the standard deviation of the data.

4.4 Results and discussion

4.4.1 The survival of L. casei after encapsulation and freeze-drying

Lactobacillus casei ATCC 393 was encapsulated within the PPI-alginate capsules via extrusion technique. After the extrusion step, the capsules were collected from the $CaCl_2$ solution. A part of the capsules were immediately tested in simulated gastrointestinal conditions after preparation (fresh capsules) (Fig. 4.1a) while the remaining was freeze-dried for storage (Fig 4.1b). It has been reported that the formation of extracellular ice crystals may damage the cell membranes which reduce the viability of probiotic cells [219]. Moreover, the freezing rate can influence the nucleation of ice crystals. Fast freezing can create smaller ice crystals which have less detrimental effects on cells [220]. Thus, liquid nitrogen was used in this study to flash freeze the capsules. The preparation and processing of *L. casei* loaded capsules is summarized in Fig. 4.2.

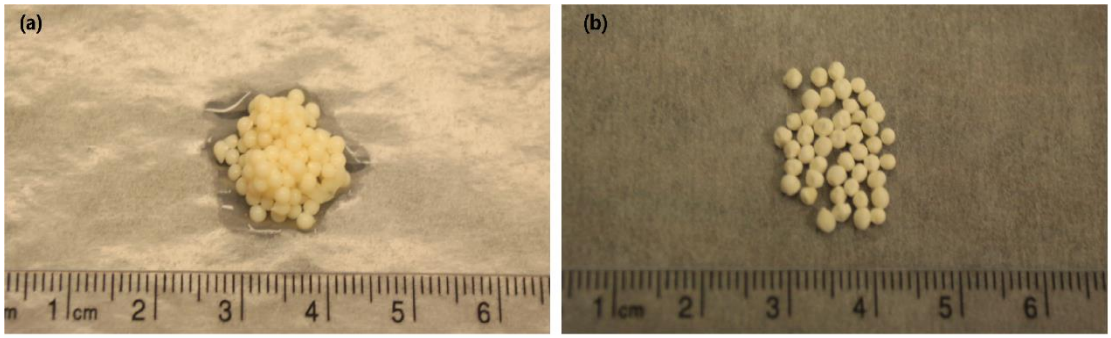

Fig. 4.1. (a) Fresh capsules after preparation and (b) freeze-dried capsules

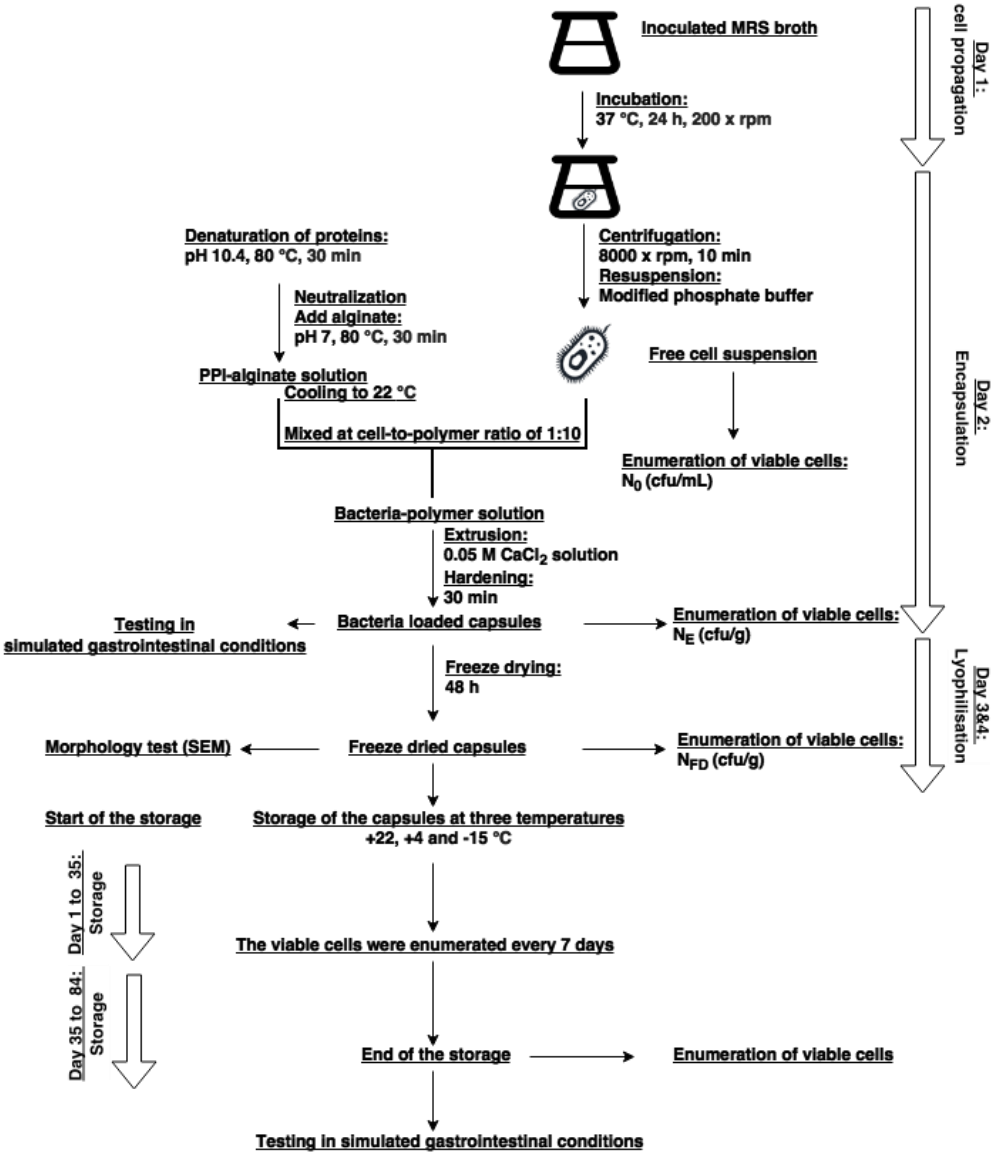

Fig. 4.2. Flowchart of the preparation of *L. casei* loaded capsules, freeze-drying, storage and tests in simulated gastrointestinal conditions.

Overall, the encapsulation process was gentle (Table 4.1). A loss of 0.09 log cfu of total viable cells (survival rate of 85.69% ± 4.82) was observed after encapsulation. This number is considered robust as compared to *L. casei* encapsulated within alginate-pectin capsules as the encapsulation yield of this system was reported to range from 54% to 79% [221]. This high encapsulation yield supports the idea that PPI is a compatible material for the encapsulation of *L. casei*.

The viable cells were enumerated for freeze-dried capsules just after the freeze-dying step. Although there is no significant difference between the reduction in total viable cells for encapsulated and free *L. casei*, the reduction of viable cells within the freeze-dried capsules (1.42 log cfu) was higher than the non-encapsulated cells (0.97 log cfu) (Table 4.1). This could be attributed to the formation of ice crystals during the freeze-drying step which were likely to induce cell damages.

Table 4.1

Survival of free *L. casei*, *L. casei* encapsulated in fresh and in freeze-dried capsules

	Cell suspension	After encapsulation		After 48 h of freeze-drying	
	Total viable cells (log cfu)	Total viable cells (log cfu)	Reduction (log cfu)	Total viable cells (log cfu)	Reduction (log cfu)
Encapsulated *L. casei*	9.72 ±0.31	9.63 ±0.32	0.09	8.21 ±0.36	1.42
Free *L. casei* control	9.62 ±0.07			8.65 ±0.06	0.97

4.4.2 Microstructure of capsules

After 48 h of freeze-drying, the capsules were pale yellow with a diameter of about 1.8 mm (Fig. 4.3a). Compared to the *L. casei* loaded capsules (Fig. 4.3b, 4.3c), the blank control (Fig. 4.3e, 4.3f) has a ‘clean’ internal structure and slightly larger pore sizes. *L.*

casei were freeze-dried to be used as a positive control (Fig. 4.3d). *Lactobacillus* are non-spore-forming bacteria which explains why they maintained their rod shape after freeze-drying [222]. Similar rod shapes have been observed in the pores of capsules (× 3000) which are very likely to be the bacteria (indicated with black arrows in Fig. 4.3f).

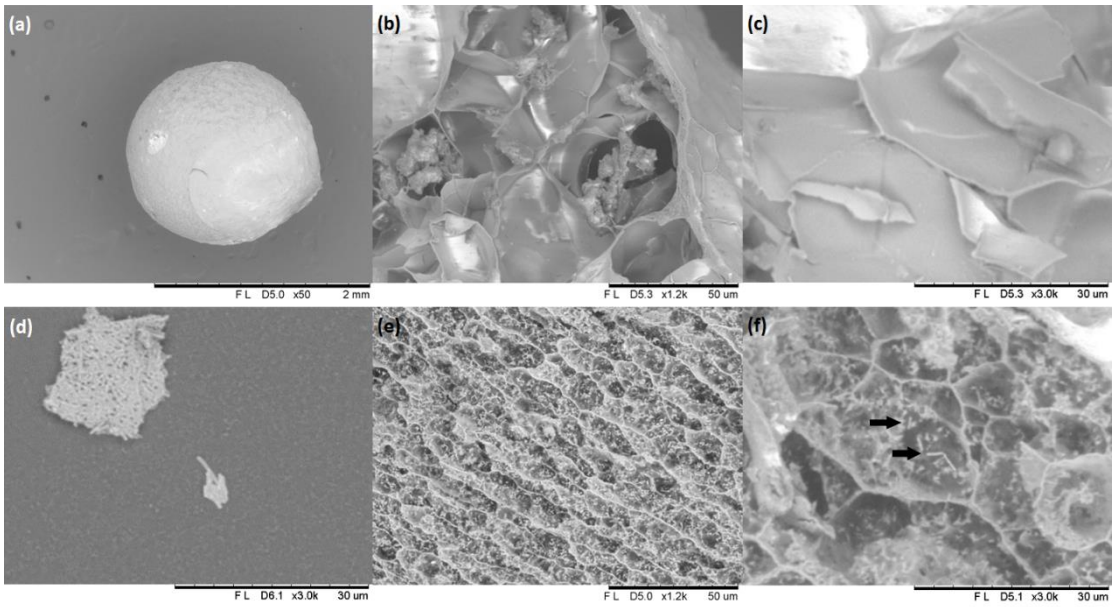

Fig. 4.3. Scanning electron microscopy images of (a) a freeze-dried capsules (×50), (b, c) the internal structure of a capsule without bacteria (×1200, ×3000), (d) freeze-dried *L. casei* ATCC 393 (×3000), (e, f) the internal structures of a *L. casei* loaded capsule （×1200, ×3000).

4.4.3 Influence of temperature on the storage of *L. casei*

The freeze-dried free *L. casei* cells and capsules were stored at +22, +4 and -15 ℃ which simulated three common storage conditions in real life (shelf, fridge and freezer). Free *L. casei* cells and capsules were tested every 7 days for 35 days (Fig. 4.4a) and the storage test of the capsules was extended to 84 days (Fig. 4.4b). In general, after 35 days, the numbers of viable cells for both capsules and free *L. casei* cells stored at +4

and -15 ℃ were above 7 log cfu/g. The capsules stored at -15 ℃ achieved the highest survival rate (66.9% ± 16.8) among all samples. From Fig. 4.4a, it can be seen that the survival rate of free *L. casei* was higher at +4 ℃. This result agrees with a previous report describing that low temperatures close to above 0 ℃ reduce the rate of detrimental chemical reactions which could lead to cell damages [166]. However, when the temperature dropped to -15 ℃, non-encapsulated bacteria showed a dramatically lower survival rate. A possible reason is that the ice crystals formed at that temperature caused cell damages. However, for encapsulated *L. casei*, the highest survival rate was obtained at -15 ℃. The high survival rate can be attributed to the protection of the capsule which provided a physical barrier between the bacteria and the ice crystals formed in the test tube. After 84 days of storage, the protective effect of PPI-alginate capsules on bacteria at -15 ℃ was obvious. The encapsulated *L. casei* displayed a survival rate of 59.9% ± 17.4 which was the highest among all three temperatures.

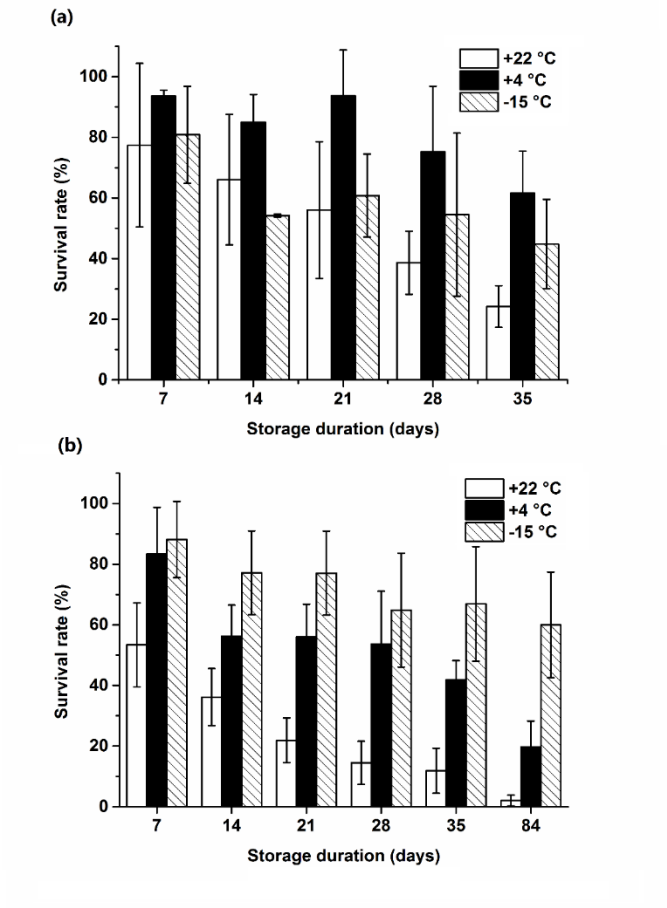

Fig. 4.4. Survival of *L. casei* at different temperatures in forms of (a) free *L. casei* cells (35 days of storage) (b) encapsulated in PPI-alginate capsules (84 days of storage) (n=4, error bars represent standard deviation between replications).

At temperatures of +22 and +4 ℃ the encapsulated *L. casei* showed relatively low survival rates (Fig. 4.4). Such results could be explained by the reason that, during the storage test, the capsules were stored in 50 mL conical centrifuge tubes. Every time the samples were withdrawn from the tube for testing, the moisture from the atmosphere could enter the tube and thus elevate the water activity in the sample. It has been reported that an increase in water activity can cause detrimental effects on probiotics during the storage [166]. However, the results showed that the encapsulated *L. casei*

stored at -15 ℃ have not been influenced by the elevated water activity and showed a relatively high survival rate. This result could be explained by the low temperature which attenuated the negative effect of the elevated water activity on the survival of the bacteria. This agrees with the literature since it has been reported that humidity has less deteriorative effect on probiotics at lower temperature [223, 224]. For free *L. casei* cells, since they were stored separately in 1.5 mL Eppendorf centrifuge tubes, the humidity level was not influenced by the action of sampling.

4.4.4 Survival of encapsulated *L. casei* in simulated gastric fluid (SGF)

Free *L. casei* cells (free cells), *L. casei* loaded capsules after preparation (fresh capsules) and dry capsules after 84 days of storage at -15 ℃ (preserved capsules) were submitted to the survival test in SGF (Fig. 4.5). The free cells showed a significant reduction (5.22 log cfu/g) in the number of viable cells after 2 h of incubation in SGF. This result is in agreement with the literatures stating that *L. casei* ATCC 393 is an acid sensitive strain [225, 226]. The number of viable cells of *L. casei* in fresh capsules showed no significant reduction after 2 h of incubation in SGF. Only a slight decrease (0.41 log cfu/g) was observed. For the *L. casei* in preserved capsules, a significant reduction (2.24 log cfu/g) in viable cells was observed in the first 60 min and then stabilized as no significant decrease (0.03 log cfu/g) was detected during the period from 60 to 120 min. It indicated that the *L. casei* in preserved capsules were more sensitive to the SGF than those in fresh capsules. The freeze-drying process created a highly porous structure which favored the diffusion of H^+ ions into the matrix. Thus, the freeze-dried capsules were expected to have a weaker buffering effect than the fresh capsules. Moreover, Santivarangkna *et al.* [227] studied the effects of freezing, dehydration and storage on the functionality of cell membranes. It is known that the

basic function of cell membranes is to protect the cells from the surrounding environment. It has been reported that cell membranes are the major site of damage occurring during the dehydration and the rehydration process of cells. The rupture of the cell membranes due to the dehydration process increased the sensitivity of the cells to ions. In addition, the removal of moisture in cells during the drying process combined to membrane lipid oxidation during the storage can both influence the fluidity and the properties of cell membranes [228, 229]. This could also render the cells more sensitive to the acidic environment. These reasons explained the rapid reduction (2.24 log cfu/g) in viable cells in preserved capsules after 60 min of incubation in SGF. The bacteria which survived after 60 min might have less or no damage from the freeze-drying and storage steps.

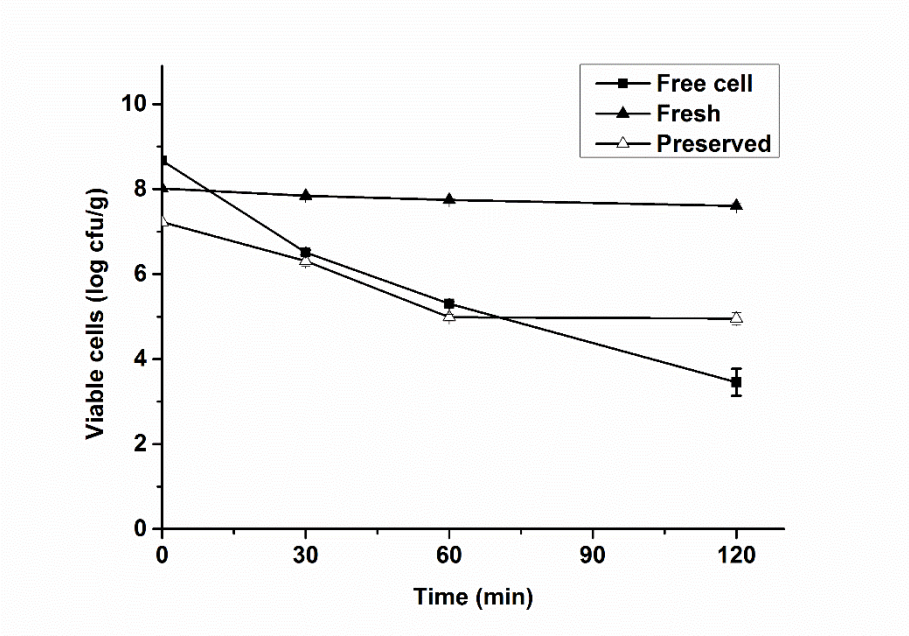

Fig. 4.5. Survival of free *L. casei* (free cells), *L. casei* in capsules which were stored for 84 days (preserved) and *L. casei* in fresh capsules (fresh) under simulated gastric condition (pH 2.0) (n=3, error bars represent standard deviation between replications).

4.4.5 Release of encapsulated *L. casei* in simulated intestinal fluid (SIF)

The survival of free *L. casei* in SIF with and without pancreatin has been

investigated. The results showed that their survival is not dependent on the presence of pancreatin. Regardless of the pancreatin, *L. casei* showed a reduction of 0.7 log cfu/ml of viable cells in the first 30 min prior to stabilize (Fig. 4.6a). Before the release test, the fresh capsules and preserved capsules were dissolved and the viable cells were enumerated. This initial number of bacteria was used as the theoretical number for a complete release of the bacteria. The results of the release test are presented in Fig. 4.6b. The fresh and preserved capsules showed similar release profiles. A rapid release was observed in the first 20 min. This could be explained by the decrease of crosslinking density of the hydrogel matrix induced by the loss of calcium ions and the hydrolysis of pea proteins. Due to the existence of phosphate ions in SIF, the calcium ions, which are the crosslinkers of the hydrogel network, formed calcium phosphate salts. Thus, less calcium ions participated in the crosslinking of the hydrogel network which reduced the crosslinking density [209]. The reduced crosslinking density of the hydrogel network led to the erosion of alginate which accelerate the release of *L. casei*. Moreover, in the presence of intestinal enzymes, the proteins were quickly digested which resulted in the collapse of the hydrogel network [172]. The degradation of the hydrogel network led to the release of *L. casei* cells into the media. The difference between the fresh and the preserved capsules was that the preserved capsules stopped releasing *L. casei* after a period of 60 min while the fresh capsules constantly released the bacteria until reaching the theoretical complete release number of 8.51 log cfu/g at 120 min. The final number of viable cells released from the preserved capsule was 0.2 log cfu/g lower than the theoretical number (7.54 log cfu/g). This result indicated that *L. casei* in preserved capsules were more sensitive to the salt which agrees with the results from the survival test in simulated gastric condition.

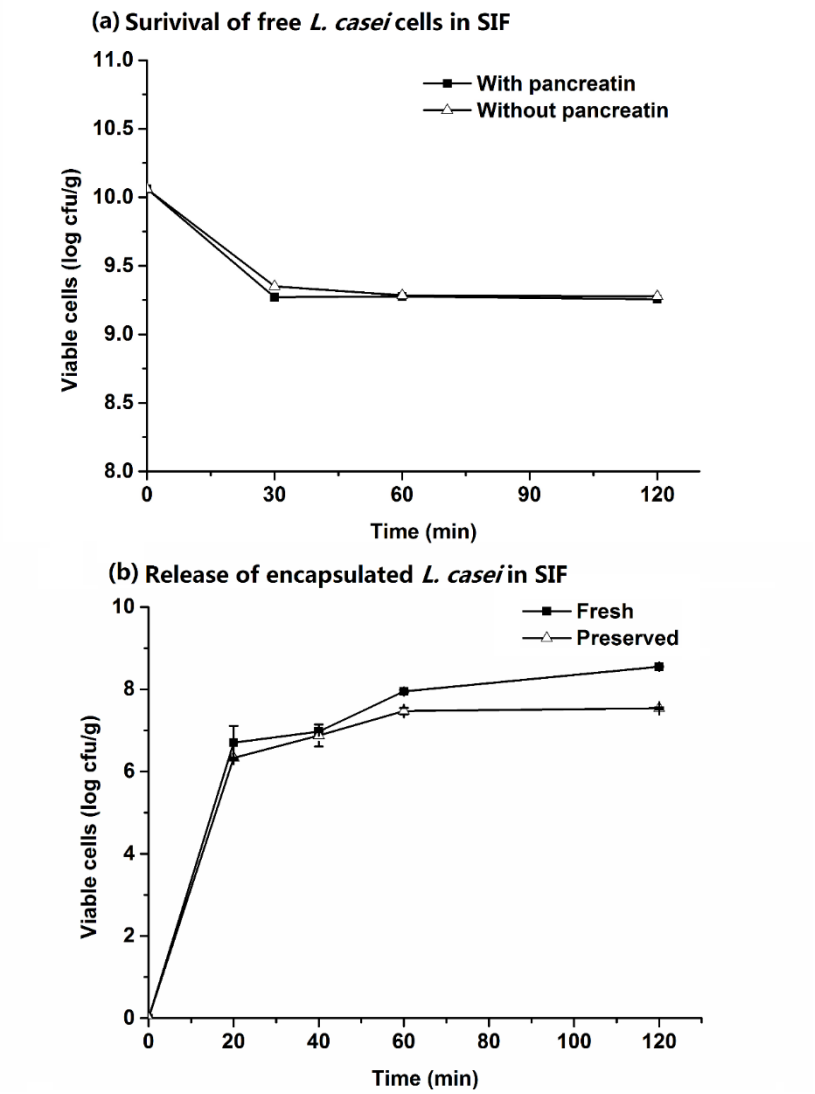

Fig. 4.6. (a) Survival of free *L. casei* cells in SIF with and without pancreatin, (b) release of *L. casei* from freeze-dried capsules after 84 days of storage at -15 ℃ (preserved) and fresh capsules (fresh) under simulated intestinal conditions (n=3, error bars represent standard deviation between replications).

4.5 Conclusion

This study presented the encapsulation of an acidic sensitive probiotic strain *L. casei* ATCC 393 in PPI-alginate hydrogel capsules via extrusion technology. The matrix and the encapsulation process were considered compatible with the probiotic strain and

showed an encapsulation yield of 85.69% ± 4.82. However, during the subsequent freeze-drying step, the capsules showed a lack of protection on the bacteria. The effects of freezing and dehydration on the viable cells in PPI-alginate hydrogels could be of interest in future works. Incorporation of extra freeze-drying protectant within the capsule could also be a target. During the storage test, the dried capsules displayed an excellent protection on *L. casei* at a storage temperature of -15 ℃. After freeze-drying and after 84 days of storage at -15 ℃, the capsules showed a weaker protective effect against acidic gastric conditions than fresh capsules but showed a similar release profile in SIF to fresh capsules. These results indicate that PPI-alginate hydrogel capsules may serve as carriers for the long term preservation of *L. casei* cells.

CHAPTER 5

GENERAL CONCLUSION AND RECOMMENDATION

5.1 General conclusion

The objectives of this thesis were to 1. Synthesize and characterize protein-polysaccharide-based hydrogels where the protein sources used were canola and pea protein isolates and 2. To encapsulate *Lactobacillus casei ATCC 393* using pea protein isolated-alginate hydrogels and determine the survival rate of the bacteria in simulated gastrointestinal fluids, after a freeze-drying operation and prolonged storage time. Detailed conclusions related to both objectives are listed as follows:

1. Hydrogels based on protein-alginate-chitosan polyelectrolyte complexation were prepared. The DSC, TGA and FTIR assays showed that hydrogels based on pea protein isolates and canola protein isolates have similar properties. Generally, canola protein isolate-based hydrogels showed a higher swelling capacity. Further degradation tests confirmed that the polyelectrolyte complexation with chitosan attenuated the effect of alginate erosion on the stability of hydrogels. The addition of chitosan in the hydrogels' formulation delayed their degradation in simulated intestinal fluid.

2. Pea protein isolate-alginate hydrogels have been used to encapsulate an acid sensitive probiotic strain. The encapsulation yield and protective effects of the matrix on probiotic cells during freeze-drying, storage and in simulated gastrointestinal conditions were investigated. The matrix and the encapsulation process were considered compatible with the probiotic strain as the encapsulation yield was around 85%. The matrix showed an excellent protective effect on *L. casei* during the storage at -15 ℃. A survival rate of 59.9 % ± 17.4

was achieved after 84 days of storage. The matrix also provided significant protection on probiotic cells against the simulated gastric fluid. The reduction of viable cells of encapsulated *L. casei* was 0.41 log cfu/g (fresh capsules) and 2.27 log cfu/g (dry capsules) compared to 5.22 log cfu/g for free *L. casei* cell after 2 hours incubation in simulated gastric fluid. The results showed that pea protein isolate-alginate hydrogels are an excellent matrix for the encapsulation and the long term storage of *L. casei* ATCC 393.

5.2 Recommendations and future studies

The main weakness of pea protein isolate-alginate hydrogels is their lack of protection on *L. casei* during freeze-drying. The effects of freezing and dehydration on the survival of probiotic cells have not been fully investigated and could be performed in future work. Moreover, the addition of extra freeze-drying protectants (e.g. trehalose, sucrose and glycerin) into the matrix should enhance the protective effect of the matrix during freeze-drying. The selection of proper freeze-drying protectants can be of interest for future studies.

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