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Biomedical Technology and Cell Therapy Research Laboratory
Department of Biomedical Engineering
Faculty of Medicine

McGill University

Microcapsules combining Alginate, Chitosan, Poly-l-lysine and Polyethyelene Glycol for Liver Cell Transplant and Cell Therapy Applications

A thesis submitted to McGill Univeristy in partial fulfillment of the requirements of the degree of Master's of Engineering (Biomedical)

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May, 2005

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ISBN: 0-494-12610-8
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ISBN: 0-494-12610-8

Abstract

Liver diseases are the eighth leading cause of death in North America. Currently, liver transplant is the available treatment for patients with liver failure. However, the shortage of donors and the requirement of immunosuppressant remain a disadvantage. Microencapsulation of living cells is an emerging technology which may serve as an alternative therapy for patients requiring organ transplants. One of the limiting factors in the progress of such therapy is attaining a biocompatible and mechanically stable polymer. In the following thesis, a novel microcapsules combining alginate, poly-l-lysine, chitosan and polyethylene glycol (ACPPA) was designed and evaluated for its use in the treatment of liver failure. In vitro studies were also conducted to compare the novel membrane, with other microcapsules, including the widely studied APA microcapsules as well as alginate coated with chitosan (AC), APA with PEG (APPA) and AC with PEG (ACP). Results show that the novel membrane can support liver cell proliferation and function and is capable of providing cell immuno-protection. The study reveals that chitosan and PEG containing microcapsules can be an alternate material for cell microencapsulation to be used for live cell delivery and other biomedical applications. Further in-vivo studies are recommended to evaluate the full potentials.

Résumé

Les Maladies du Foie sont la huitième plus importante cause de mort en Amérique du Nord. Présentement la transplante rénale est le seul traitement pour les patients qui ont des maladie du foie. Par contre le manque de dons et la nécessité des immunosuppresseurs sont un désavantage à cette méthode. L'encapsulation des cellule est une nouvelle technologie qui pourrait servir de thérapie alternative pour les patients qui ont recours aux transplantes rénales. Un des facteurs qui limite cette technologie est d'atteindre un matériel polymère qui est biocompatible et mécaniquement stable. Dans cette thèse, de nouvelles microcapsules composées d'alginate, poly-l-lysine, chitosan et PEG (ACPPA) ont été formulées et évaluées dans l'usage en tant que traitement pour un échec du foie. Des études in vitro ont aussi été faites pour comparer les nouvelles micro capsules avec d'autres micro capsules telles que l'APA bien connue, et aussi alginate enrobe de chitosan (AC), l'APA avec PEG (APPA) et l'AC avec PEG (ACP). Les résultats indiquent que la nouvelle micro capsule peut aider à la prolifération et la bonne fonction des cellules du foie. L'étude montre que les microcapsules contenant la chitosan et la PEG pourraient être un matériel alternatif pour la micro encapsulation de la cellule qui serait livrée vivante et pour d'autres usages médicaux. Des études in vivo additionnelles sont recommandées pour évaluer les possibilités au complet.

Acknowledgments

I would like to acknowledge the co-authors, Hongmei Chen, Wei Ouyang, Christopher Martoni, Bisi lawuyi and Alexandra Malgorazata Urbanska for their assistance in conducting experiments and contributions for the original papers entitled: "In vitro study of alginate-chitosan microcapsules: An alternative to transplant for the treatment of liver failure" (accepted in Biotechnology Letters), "Design of a novel microcapsule membrane combining alginate, chitosan, polyethylene glycol and poly-l-lysine for cell transplantation"(submitted to International Journal of Artifical Organs, and "Superior cell delivery features of polyethylene glycol incorporated alginate, chitosan and poly-l-lysine microcapsules" (published in Mol. Pharm., **2** (1), 29 -36, 2005).

I would also like to thank my Biomedical Technology and Cell Therapy Research Laboratory colleagues. Specifically I would like to acknowledge Terrence Metz, Mariam Mirzai, Trisnawati Halim and Fatmeh Afkhami for their continuous assistance.

I would like to recognize the advice, direction and assistance provided by my supervisor Dr. Satya Prakash. I would also like to acknowledge CIHR and NSERC research operating grants awarded to Dr. Prakash for conducting the research presented in this thesis.

In addition, I extend my gratitude to Dr. Maryam Tabrizian's laboratory for their permission in utilizing their equipment as well as providing training for their facilities.

I gratefully acknowledge financial support from the Natural Sciences and Engineering Research Council (NSERC) of Canada, PGSA scholarship.

Finally, I would like to thank my family for their continuous support throughout this research.

Preface

In accordance with the McGill University thesis preparation and submission guidelines, as stated in section I-C, I have taken the option of writing the experimental section of this thesis as a compilation of original papers suitable for publications. The papers are presented in chapters 3, 4 and 5 and are subdivided into sections including abstract, introduction, materials and methods, results, discussion and conclusion. A common abstract, general introduction, literature review, summary of results, overall conclusions and references are included in the thesis as required by the guidelines.

List of Abbreviations

AC	Alginate- chitosan microcapsules
ACP	Alginate-chitosan-PEG microcapsules
ACPPA	Alginate-Chitosan-PEG-PLL-Alginate microcapsules
APA	Alginate-PLL-Alginate microcapsules
APPA	Alginate-PLL-PEG-Alginate microcapsules
BSA	Bovine Serum Albumin
DMSO	Dimethyl Sulfoxide
FBS	Fetal Bovine Serum
FTIR	Fourier Transform Infrared Spectroscopy
MEM	Minimum Essential Eagle Medium
MTT	Tetrazolium salt (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide)
PBS	Phosphate Buffer Solution
PEG	Polyethylene glycol
PLL	Poly-l-lysine
RPM	Rotation per minute
SEM	Scanning Electron Microscopy

Units:

cm	centimeter
kg	kilograms
mL	milliliters
mm	millimeters
nm	nanometers
μm	micrometers

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1.0 General Introduction

The liver is the largest organ in the human body and it is responsible for carrying out a diverse range of metabolic functions[2]. Some of the processes controlled and performed by the liver include, the removal and neutralization of toxins and bacteria from the blood, production of immune agents to control infection, absorption of fats and fat soluble vitamins and synthesis of enzymes, plasma proteins and bile.

Due to the vast role the liver plays in the human body, any damage or disease altering the liver function can lead to many severe adverse affects[3-5]. The major diseases affecting the liver include viral hepatitis and liver cirrhosis which occurs when healthy liver tissues are replaced by scarred tissues as a result of chronic liver diseases, severe physical damage to liver tissues or excess consumption of alcohol. Liver cirrhosis ranks as the eighth leading cause of death and it is estimated that the mortality rate for hepatic failure ranges between 80-90% [6]. The liver is also a target of many inborn errors of metabolisms. For example, Wilsons disease in which excess copper accumulates in the body due to the livers inability to eliminate it, α-1-antitrypsin deficiency where the liver is incapable of producing sufficient AAT proteins which causes severe lung damage and potentially cirrhosis, and Type I glycogen storage disease which results in an enlarged liver and abnormal growth due to a deficiency of the enzyme glucose-6-phosphatase.

Although some of the diseases inflicting the liver such as Wilsons disease may be treated using various drugs, most liver diseases, in particular liver cirrhosis, presently have no marketed treatment [6,7]. Currently, liver transplant is considered to be the only effective therapeutic solution for many hepatic disorders including acute liver failure and chronic and inborn errors of metabolism.[6,7]. However, the scarcity of donors continues to be a barrier in the treatment of such disorders. It is estimated that 20-30% of patients on the waiting list for an organ die before an available organ is found [3,5,8]. Many liver support systems have developed to supply liver functions as a bridge to transplantation. For example, hemoperfusion with adsorbents such as resins or activated charcoal remains an option for patients with hepatic failure. The adsorbents purify the patient's blood by removing toxins that cause hepatic coma [9,10]. Although hemoperfusion is practiced

clinically, this non biological hapatic support system is incomplete because it does not supply the proteins and hormones produced by a functioning liver.

The diverse function of the liver, therefore, makes it difficult to construct an effective hepatic support system. Researchers thus are trying to construct a biological artificial liver that is stable, capable of removing toxins and has the ability to supply proteins to patients[8].

One of the most appealing possibilities to treat several liver related diseases transplanting isolated hepatocytes as opposed to whole liver transplants [3,5,11,12]. Investigations of hepatocyte transplantation have been a subject of research for the past 20 years and may potentially to be used clinically to treat liver diseases. Hepatocytes transplanted in the liver or the spleen can support liver function in times of hepatic insufficiency. In cases of metabolic diseases, the hepatocytes are capable of expressing and providing the enzyme and proteins lacking in the host liver. Although hepatocyte transplantation in theory is a sound alternative to whole organ transplant, in practice it is not used in most clinics. One of the reasons for the slow progression of the therapy is that hepatocyte cells are not always readily available. It is necessary to attain new sources of hepatocytes either via replication of cell lines, xenogenic hepatocytes or hepatocytes derived from stem cells [7]. Additional drawbacks of direct hepatocyte transplantation is that the cells do not always engraft successfully and host immuno-suppression is required which may not be optimal for patients receiving long term cellular transplants[3,6,12,13].

Immuno-isolation technologies, such as artificial cell microencapsulaton, for the long term transplantation of biologically active materials have been a focus of research for the treatment of several diseases including liver failure. Microencapsulation of allogenic or xenogenic cells within a semipermeable membrane is an emerging technology which may overcome the problem of immunogenic reactions during transplantation. The membrane allows the diffusion of small particles such as nutrients, oxygen, wastes and therapeutic products however impedes the passage of high molecular weight components such as immune cells, antibodies, cytokines, immunoglobulins and complement factors. This provides an immunological barrier between the entrapped cells and its surrounding environment which can protect the cells from the host immune system when used in

transplantation. Microencapsulation also protects immobilized cells from damage during handling [6,14-19].

The entrapment of cells within alginate-calcium spheres is a well established technique which can be used for mammalian cell encapsulation. In the 1960's Chang first reported the use of artificial cells to encapsulate biologically active materials. He successfully entrapped cells within a cross-linked protein membrane and proposed the potential of microencapsulation for hepatocytes and islet cells [17]. Lim and Sun reported the first successful transplantation of islet cells in alginate-poly-L-lysine (APA) capsules approximately 23 years ago [20,21]. Since this finding, many researchers continue to investigate the potential of polymer microencapsulation of live cells as an alternative and improvement to transplantation.

Several factors need to be considered for the clinical implementation of liver cell microencapsulation. The primary characteristic for microcapsules used in cell immobilization is that the membrane material needs to be completely biocompatible and support cell growth without interfering with its differential function. The material must not trigger any immune responses from the host and the membrane must exhibit sufficient mechanical strength and stability for long term cell transplantation [13,22,23]. Alginate is the most suitable membrane biopolymer for cell encapsulation with regards to biocompatibility. To increase mechanical strength, alginate microcapsules are commonly coated with an additional layer of poly-l-lysine and dilute alginate to form alginate-poly-l-lysine-alginate microcapsules (APA). Although this membrane is widely studied for the encapsulation of biologically active materials, previous research has shown the membrane to induce necrosis of encapsulated cells and fibrotic tissue growth around the membrane surface is observed when transplanted directly in rat models [6,21,24]. As a result, improvements to this membrane as well as the design of new microcapsule membranes continue to be studied in order to instigate the technology of microencapsulation. This is the main research goal of the presented thesis.

1.1: Research Objectives:

The current study investigates the potential of a novel microcapsule membrane combining alginate, chitosan, poly-ethylene glycol (PEG) and poly-l-lysine (PLL) for liver cell encapsulation and cell therapy applications. The research objectives are:

1) To test the use of chitosan for liver cell encapsulation and study its cytotoxicity.
2) To design a novel microcapsule composed of alginate, coated with chitosan, PEG and PLL and test its mechanical stability.
3) To encapsulate live HepG2 cells in the novel microcapsule and perform in-vitro studies on cell proliferation, protein production, immuno-protection and cryopreservation.
4) To compare the novel microcapsules with the widely studied alginate-poly-l-lysine- alginate (APA), alginate-chitosan (AC) and PEG incorporated APA as well as PEG incorporated AC microcapsules.

2.0 Literature Review

2.1 Liver Function and Diseases:

The liver is the largest organ in the human body and it is responsible for carrying out a diverse range of metabolic functions[2]. Some of the processes controlled and performed by the liver include, the removal and neutralization of toxins and bacteria from the blood, production of immune agents to control infection, absorption of fats and fat soluble vitamins and synthesis of enzymes, plasma proteins and bile. The liver is conjugated to two circulatory systems that include the hepatic artery which brings oxygenated blood from the heart and the portal vein through which nutrients from the intestines are passed through the liver. Approximately 70% of liver cells are hepatocytes and the liver has the unique capacity to regenerate itself following various injuries [2,25].

Due to the vast role the liver plays in the human body, any damage or disease altering the liver function can lead to many severe adverse affects to the body [3-5]. The major diseases inflicting the liver include viral hepatitis and liver cirrhosis which occur when healthy liver tissues are replaced by scarred tissues as a result of chronic liver diseases, severe physical damage to liver tissues or excess consumption of alcohol. Liver cirrhosis ranks as the eighth leading cause of death and it is estimated that the mortality rate for hepatic failure ranges between 80-90% [6]. The liver is also a target of many inborn errors of metabolisms. These include Wilsons disease in which excess copper accumulates in the body due to the livers inability to eliminate it, Alpha-1-antitrypsin deficiency where the liver is incapable of producing sufficient AAT proteins which causes severe lung damage and potentially cirrhosis, and type I glycogen storage disease which results in an enlarged liver and abnormal growth due to a deficiency of the enzyme glucose-6-phosphatase.

Although some diseases inflicting the liver such as Wilsons disease may be treated using various drugs, most liver diseases, in particular liver cirrhosis, presently have no marketed treatment. The prime option for patients inflicted with liver diseases is to undergo a liver transplant.

2.2 Current Treatments for Liver Failure:

Currently, orthotopic liver transplantation is considered to be the only effective therapeutic solution for many hepatic disorders including both acute liver failure and chronic and inborn errors of metabolism [2,26]. However, the scarcity of donors continues to be a barrier in the treatment of such disorders. It is estimated that 20-30% of patients on the waiting list for an organ die before an available organ is found [3,5,8,26]. Many liver support systems have developed as a bridge to transplantation. For example, hemoperfusion with adsorbents such as resins or activated charcoal remains an option for patients with hepatic failure. The adsorbents purify the patient's blood by removing toxins that cause hepatic coma. Although hemoperfusion is practiced clinically, this non biological hapatic support system is incomplete because it does not supply the proteins and hormones produced by a functioning liver [27,28].

The diverse function of the liver makes it difficult to construct an effective hepatic support system. Therefore, researchers are thus trying to construct a biological artificial liver that is stable, capable of removing toxins and has the ability to supply proteins[8].

Gene therapy using in vivo and ex vivo liver gene transfer is an option being investigated for the treatment of genetic and acquired hepatic diseases [2,25,26]. Although the application of gene delivery is immense, ethical issues remain a concern. A problem observed in studies is that the required transgene expression level to attain therapeutic effectiveness in gene transfer to the liver may trigger an immune response[2].

One of the most appealing options for the treatment of several liver related diseases is the transplantation of isolated hepatocytes as opposed to whole liver [3,5,11,12]. Investigations of hepatocyte transplantation have been a subject of research for the past 20 years and have the potential to be used clinically to treat liver diseases. Hepatocytes transplanted in the liver or the spleen can support liver function in times of hepatic insufficiency. In cases of metabolic diseases the hepatocytes are capable of expressing and providing the enzyme and proteins lacking in the host liver. Although hepatocyte transplantation in theory is a sound alternative to whole organ transplant, in practice it is not used in most clinics. One of the reasons for the slow progression of the therapy is that hepatocyte cells are not always readily available. It is necessary to attain hepatocytes

either via replication of cell lines, xenogenic hepatocytes or hepatocytes derived from stem cells [7]. Studies have revealed that less than 30% of the hepotocytes engraft and integrate with the surroundings after implantation. Transplanted hepatocytes have a lower survival advantage in comparison to the host cells [26]. An additional drawback of direct hepatocyte transplantation is that immuno-suppression of the host is required which may not be optimal for patients receiving long term cellular transplants [3,6,12,13].

2.3 Microencapsulation as an Alternative Treatment:

Microencapsulation technologies for the short and long term transplantation of biologically active materials has been a focus of research for the treatment of several diseases including inborn errors of metabolism, enzyme deficiencies, cancer, CNS diseases as well as liver failure[20,23,24,29]. Microencapsulation is an emerging technology which may overcome the problem of immunogenic reactions as well as graft rejection and non integration during transplantation. Hepatocytes can be encapsulated within a semi-permeable membrane which allows the diffusion of small particles such as nutrients, oxygen, waste and therapeutic products however impedes the passage of high molecular weight components such as immune cells, antibodies, cytokines, immunoglobulin and complement factors. This provides an immunological barrier between the entrapped cells and its surrounding environment which can protect the cells from the host immune system when used in transplantation. Microencapsulation also protects immobilized cells from damage during handling [6,14-19]. The technology of microencapsulation can be applied to treat liver failure by direct transplantation in the peritoneal cavity. The peritoneal cavity is an attractive site for transplantation because of its location and ease of access[26]. Microcapsules can also be used in cell based bioreactors for application in bioartificial assist devices [9,10,30].

The following sections summarize the technology of microencapsulation, its properties and limitations.

2.4 Microencapsulation Technology:

The theory of microencapsulation was first proposed and termed "artificial cells" by Chang in the 1960's [22,24,31]. Chang demonstrated that enzymes, cells, microorganisms and various adsorbents can continue to perform their respective functions on their surrounding environment while being enclosed within a protective membrane [22,31-33]. The semi-permeable membrane is capable of supporting cell proliferation, metabolism and differentiation [18,32,34]. Microencapsulation therefore has the potential to be used for various biotechnological applications. Currently, microcapsules containing adsorbents are being used clinically in hemoperfusion to remove toxins from the bloodstream of patients with liver failure or drug poisoning. Live cells and tissues such as islet cells and endocrine tissues can be encapsulated to treat diabetes as well as various endocrine diseases[1,32,35]. Microencapsulated genetically engineered microorganisms have been investigated as a means of urea and ammonia removal during kidney failure[29,36,37]. Artificial cells containing hemoglobin can act as blood substitutes by being used to transport oxygen and microcapsules enclosing certain enzymes can be used to convert or remove substrates and metabolites[22,31]. Microcapsules have been studied for either direct transplantation, oral delivery or for usage in extracorporeal devices and bioreactors [1,32]. Table 2.1 summarizes various uses of artificial cells and modes of application.

Microcapsules generally range between 0.3-1.5 mm in size. The small size of microcapsules provides a large surface area to volume ratio which is advantageous for mass transfer [18]. The ideal approach for the application of cell microencapsulation would be to directly implant encapsulated cells into the body for long term function. The use of cells as opposed to immobilized peptides to supply therapeutic proteins offers the advantage of 'de novo' produced proteins and it also enables chemical stability of the product [38]. Since the membrane is capable of immuno-isolating the enclosed cells, microencapsulation can be applied for both allogenic or xenogenic transplantation [24,31,38]. However, to implement the technology of microencapsulation it is necessary to attain a proper membrane material and delivery procedure that conform to the quality and biosafety standards applied to other drug delivery systems [38].

Disease	Enclosed material	Mode of Application	Reference
Diabetes	Islet cells to secrete insulin	Transplantation	1,35
Renal Failure	Genetically engineered Ecoli DH5, *lactobacillus*	Oral delivery	1,29,37,39
Liver Failure	Hepatocytes	Transplantation	14,15,31,40
Phenylketonuria (PKU)	Phenylalanine	Oral delivery	31
IBD and Crohn's Disease	*lactobacillus* and *bifidobacterium*	Oral delivery	41,42
Blood loss	Hemoglobin and Enzymes	Injection	31,32
Parkinson's	Genetically engineered kidney fibroblasts, Chromaffin cells, Dopamine from PC12 cells	Implantation (gene therapy)	1,43,44
Hemophilia	Genetically engineered kidney cells	Implantation (gene therapy)	24,45
Neurodegenerative diseases	Genetically engineered cells producing glucuronidase	Injection	1,46

Table 2.1: Applications of microencapsulation as a mode of therapy for various diseases

2.5 Essential Characteristics of Microcapsule Membrane:

For optimum function of the microcapsules, several factors need to be considered. These include membrane permeability, strength and biocompatibility and immuno-protection.

2.5.1 Permeability:

The survival of the enclosed cells is dependant primarily on the permeability of the capsule membrane which determines the supply of essential nutrients as well as the elimination of toxic metabolites [18,47]. Adequate amount of oxygen must be provided to the enclosed cells at a sufficient rate to permit proliferation and metabolic activity [47]. The space available for cell growth within the capsule is often limited due to substrate and oxygen transfer[48]. The permeability characteristics of a capsule membrane consist of two factors including the molecular size cut-off for the entry and exit of desired and undesired

molecules and the diffusion rate of permeating molecules. The rate of diffusion is directly dependent on the type and size of solute being transported, its interaction with the membrane and the membrane thickness [24,38]. The water content within the hydrogel used for encapsulating also determines the absorption as well as diffusion of solutes across the membrane [20]. It is suspected that the molecular transport through the capsule membrane is through pores within the microcapsules; however, research and information about the actual pore size of the capsules are limited [18,20]. Pores may be formed during synthesis by phase separation or they may be present as smaller pores within the hydrogel network[20].

2.5.2 Mechanical Strength:

The mechanical property of the microcapsule is also an essential characteristic determining the feasibility of cell microencapsulation. The mechanical stability of the microcapsule indicates the membrane integrity during application as well as the durability of the capsules during preparation, production and handling [18]. Depending on the membrane strength, microcapsules containing cells can potentially be preserved at low temperatures for long term cell storage [49]. The possibility of capsules rupturing when exposed to stress is dependant on membrane strength, thickness as well as the properties of the microcapsule core. Microcapsules can be improved in strength by modifying the capsule design by varying the polymer used for the capsule, integrating different polymeric additives and altering the coating time as well as the extent of membrane surface modification[18].

2.5.3 Biocompatibility and Immunogenicity:

In order to implement the technology of cell microencapsulation it is necessary for the microcapsule material to be completely biocompatible and not elicit any immune response when exposed to in vivo physiological conditions. The biocompatibility of microcapsules is determined by the capsule's ability to support interior cell proliferation and prevent any apparent foreign body reaction when implanted. The capsule material must not interfere with the function and viability of the encapsulated tissues or cells [18]. A lack of biocompatibility can result in fibrotic overgrowth around the microcapsule surface post surgical transplantation. This restricts the transport of oxygen and nutrients

to the enclosed cells which, in turn, results in a decreased cellular function and eventual cell necrosis and failure of the microcapsules [18,24,31,33]. It is reported that over 40% of initially implanted islet grafts are damaged as a result of overgrowth of fibroblasts and macrophages [50].

An important factor to consider for the application of microcapsules in tissue transplantation is the assessment of the immuno-protection ability of the capsule. The diffusive permeability of the membrane affects the extent of immunogenic barrier and the requirements for immuno-protection vary depending on whether the capsules will be used for allogenic or xenogenic transplantation. There are several pathways that are involved in the rejection of immuno-isolated cells. In allografts, immune response is triggered by a direct pathway involving T-lymphocyte sub populations such as CD8+ which act on the donor cells. This interaction can be prevented if the microcapsule's membrane provides sufficient immunological barrier by preventing any cell to cell contact between the enclosed cells and the surrounding host cells. If these criteria are satisfied, the implant can be accepted by the host [24]. For xenogenic transplantations, the immuno-protection potential of the microcapsules is primarily determined by the molecular weight cut-off (MWCO) of the membrane. It is imperative that the microcapsule prevents the diffusion of antibodies reacting to foreign substances as well as complement components which are cytotoxic to xenogeneic tissues [18,24,32,50]. Figure 2.1 represents the different molecular weight limitations of different polymers.

Biocompatibility and the immuno-protection characteristics of microcapsules can be improved by the use of highly purified and biocompatible polymers during synthesis. The uniformity, spherical shape and size of the microcapsules affects the biocompatibility of microcapsules [24,38]. Irregularities, such as tails in the microcapsules can affect the integrity of the capsule, which may trigger the growth of fibroblasts and macrophages after implantation [50]. A larger capsule volume generally results in more frequent adverse side effects in recipients. The biological acceptance of encapsulated cells can also be influenced by the surgical procedure, site of implantation, type of cell used and the cellular products being secreted. Integrating chemical agents such as vitamins D3 and E or other cells that can counter antibodies, may be used to enhance the immuno-barrier capacity of microcapsules [24].

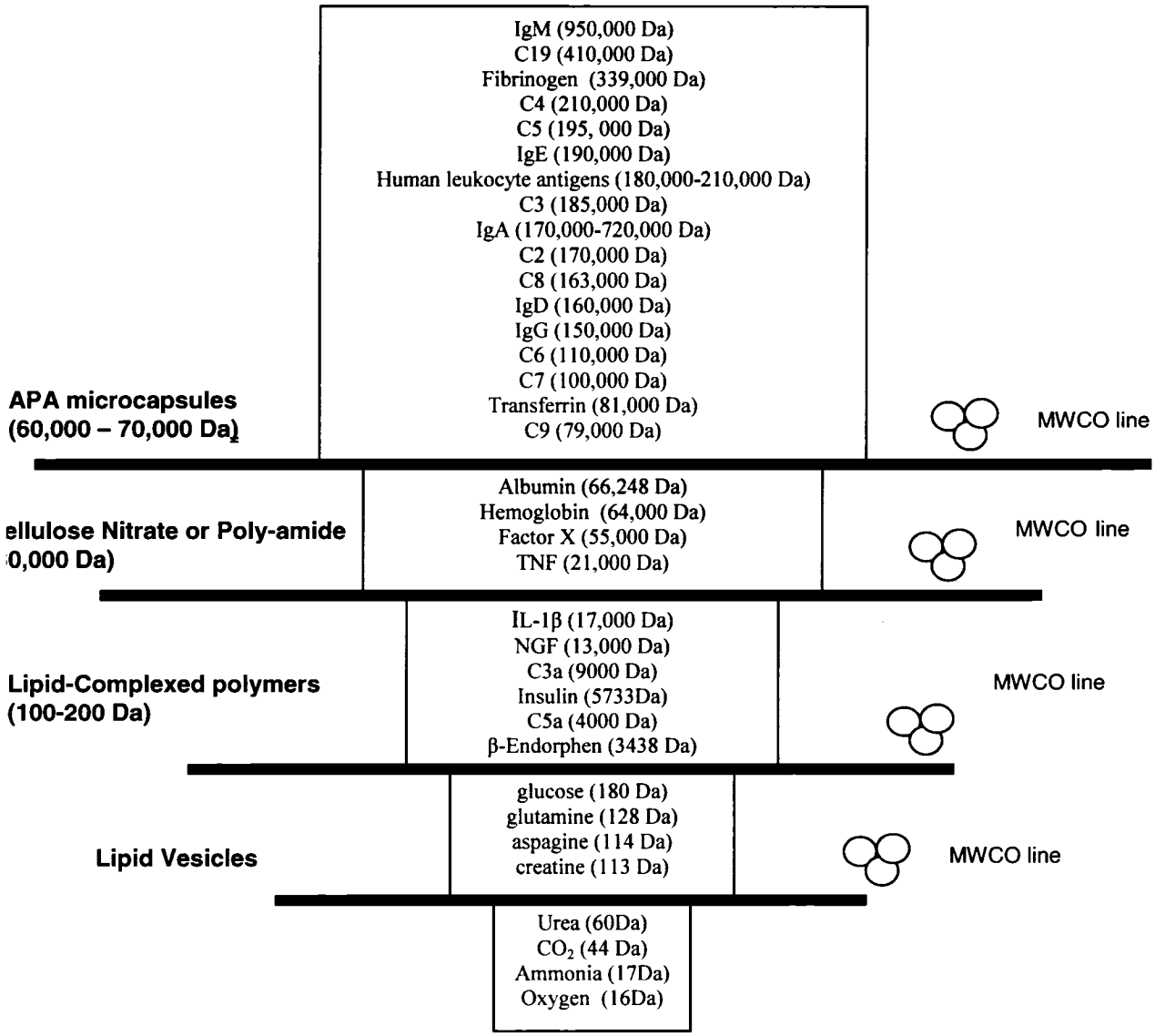

Figure 2.1: Molecular weight of various molecules and molecular weight cut-off for different microcapsules (MWCO). Microcapsules are permeable to elements below the MWCO line and impermeable to elements above the MWCO line. The image is adapted from Chang and Prakash. [1]

2.6 Materials used in Microencapsulation:

Over the past two decades, many types of microcapsules composed of different hydrogels including synthetic and naturally occurring polymers have been developed.

The biomaterial used for cell encapsulation must be impermeable to high-molecular weight species such as antibodies to avoid immune rejection, be permeable to small molecules to allow transport of metabolites and the microcapsule material must be able to support cell growth and not interfere with their differentiated function [34]. The porosity and thereby permeability of microcapsules can also be controlled by varying the membrane composition. This permits the diffusion of anabolic compounds such as oxygen and glucose, as well as cellular products such as hormones and proteins, while excluding immunoglobulin. Microcapsules are designed primarily using hydrogels because the flexibility of the gel reduces irritation to the surrounding and enclosed tissues. Hydrogels offer minimal protein and cell adhesion to surfaces due to their hydrophilic nature and permeability to low molecular mass substances such as nutrients and metabolites [18,34].

The encapsulation of tissue cells within a protective microcapsule has been researched as a means to eliminate the problems associated with immune rejection during transplantation. Depending on the type of polymer used, their characteristics in terms of MWCO varies, making them suitable for separate applications. Table 2.2 summarizes some of the membranes studied for live cell encapsulation.

Microcapsule membrane material	Characteristics	References
Alginate	*Advantages:* Biocompatible, mild preparation process. *Limitations:* Batch to bath differences affects purity and function. Possibility of Ca loss, cell leakage.	24,51,52
APA	*Advantages*: Excellent biocompatibility and biodegradability. Moderate mechanical strength, well established for encapsulation of many cell types. *Limitations:* Inflammatory response.	18,21,24
HEMA/MMA based microcapsules	*Advantages:* High stability, durability and good mass transfer properties. *Limitations:* Requires matrix for anchorage dependent cells, encapsulation process may not be suitable for all cell types, further studies on immunogenicity required.	18,23,24,53,54
Chitosan	*Advantages*: Increases biocompatibility in comparison to APA. Non-toxic and compatible with soft tissue. *Limitations:* Requires acidic condition, further studies on immunogenicity and cell encapsulation still under investigation.	50,55-57
Agarose	*Advantages:* Increased mechanical stability in comparison to APA, biocompatible *Limitations:* No distinct MWCO and possibility of becoming engrafted with host tissue	58
Barium-alginate	*Advantages:* Improved mechanical and chemical stability in comparison to Ca.. *Limitations*: Ba cation may not be suitable for all cell types since it is an inhibitor of K+ channels. Questionable adequacy of immuno-protection	18,24
Cellulose sulfate	*Advantages:* 1 step method preparation, high mechanical stability, individual parameters can be adjusted to suit application. *Limitations:* Sulfuric acid and n-propanol required as an agent. Mechanical strength and permeability still under investigation.	24,52,59

Table 2.2: Summary of advantages and limitations of various microcapsule membranes used for cell encapsulation

2.6.1 Synthetic Polymers/HEMA-MMA:

Synthetic polymers have been investigated for the use in mammalian cell encapsulation for various tissue engineering and therapeutic applications [54,60,61]. A widely studied polymer is known as hydroxyethyl methacrylate-methyl methacrylate (HEMA-MMA) and it is formed using a method called interfacial precipitation. These microcapsules offer an adequate balance between mechanical strength and permeability[18,34]. Chinese Hamster Ovary fibroblasts (CHO's) were successfully immobilized in HEMA-MMA microcapsules. The cells remained viable and a molecular weight cut-off of approximately 100 kDa was observed [34]. The encapsulation technique was further investigated for the encapsulation of both human and rat hepatoma cells, islets cells as well as transfected cells including mouse myoblasts and embryonal kidney cells[62]. Uludag reports an in vitro study of protein release from encapsulated hepatocytes in HEMA-MMA polymers. It is concluded that by using an attachment substrate such as matrigel with HEMA-MMA microcapsules, the polymer permits the proliferation of hepatocytes and does not interfere with the cells differentiated functions [34].

Several modifications have been studied on the HEMA-MMA capsule including the addition of methacrylic acid sodium salt and using a two step encapsulation process to form capsules with four layers [13,54,63]. The four step design was investigated as an improvement to the mechanical strength for immobilizing rat hepatocytes [63]. Yin et al. reports the use of HEMA-MMA-MAA microcapsules for the application in bioartificial liver assist devices [13,64].

HEMA-MMA microcapsules have the advantage of improved mass transfer, stability and durability [24]. However, despite the varying applications of synthetic microcapsules using HEMA-MMA polymers, there exist several limitations to the system. The inherent disadvantage of synthetic polymers is the preparation process requires exposure to organic solvents, toxic monomers and possible irradiation which are undesirable for the use in some cell culturing [53,61]. In vivo studies have also demonstrated the risk of capsules clumping after implantation [62].

2.6.2 Agarose Microcapsules:

Agarose, a nontoxic polysaccharide derived from seaweed, has also been studied for the entrapment of mammalian cells including islet cells, hybridoma cells, fibroblasts and myoblasts [65-67]. Agarose is composed of alternating units of 3,6-anhydro-α-L-galactopyranosyl and β-D-galactopyranosyl and it gels at temperatures below 25°C [18,58]. Kobayashi et al. reports the use of agarose microcapsules for the transplantation of allogenic islet cells in mice models [58]. The study concluded that agarose can supply sufficient immuno-protection to cells [58]. However, other studies performed on the transplantation of agarose microencapsulated islet cells transplanted into rats have revealed that some of the implanted microcapsules triggered an inflammatory response [67]. One of the drawbacks of using agarose microcapsules for transplantation is that agarose does not have a distinct MWCO. Cytokines can potentially permeate through the agarose membrane and attack the enclosed cells. Implanted agarose in the peritoneal cavity has also been found to become embedded with the host tissues. This makes it difficult to remove the graft once transplanted [58].

2.6.3 Alginate Microcapsules:

Ionotropic gelation using calcium alginate polymers is the most extensively studied material for live cell and tissue microencapsulation [53]. In regards to biocompatibility, alginate is the most suitable membrane biopolymer for cell encapsulation [24]. Studies have shown the feasibility of alginate-based microcapsules for transplantation of mammalian cell lines, recombinant mammalian cells, microbial cells, allogenic and xenogenic tissues as well as the application of drug and protein delivery [48,55,68-72].

Alginate is one of the most abundant naturally occurring polymers. It constitutes a family of unbranched anionic polysaccharides mainly extracted from brown algae. Alginates are composed of binary copolymers 1-4 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G). The monomers are arranged in a pattern of blocks along the chain with regions of alternating M and G blocks. The composition and sequence varies depending on the type of alginate which in turn affects the polymers functionality [11,18,70,71]. Gelation is induced by cross linking the anionic alginate with oppositely charged divalent ions such as Ca^{2+} and Ba^{2+}. Ca^{2+} binds preferentially to the G block to

form a spherical shape. Figure 2.2 represents an image of alginate molecules interacting with Ca^{2+} ions to form a spherical structure.

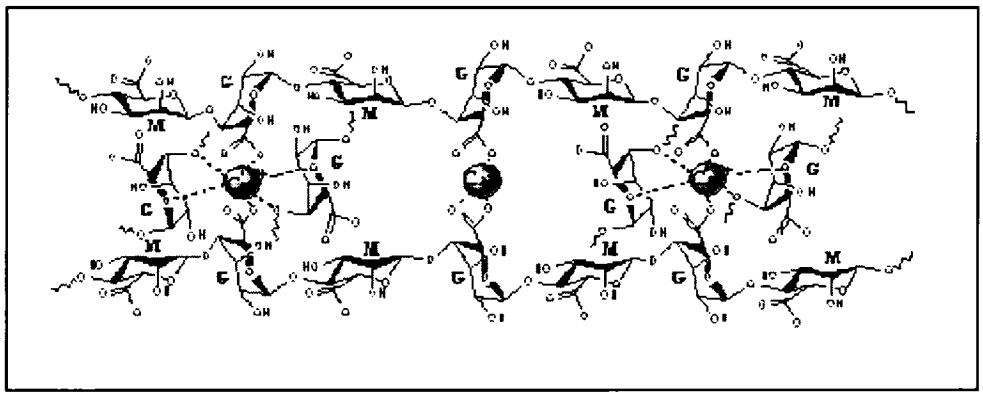

Figure 2.2: Molecular structure of alginate interacting with Ca^{2+} ions

It is reported that beads made of alginate with a G content greater than 70% provide high mechanical strength[21,69]. Ba^{2+} cations can bind with both G and M blocks of alginate. Although the long term integrity of capsules is greatly improved when Ba^{2+}, replaces Ca^{2+} , it is an inhibitor of K^+ channels which makes it a difficult alternative for cell encapsulation [18,71].

Alginate immobilization is viewed as a mild process involving non-toxic components with a pH, osmolarity and temperature suitable for preserving mammalian cell viability. Alginate can form stable gels at temperatures ranging from 0 to 100°C. Previous studies show that alginate encapsulated hepatocytes can maintain function both in vitro and in vivo. Encapsulated rat hepatocytes in calcium alginate membranes can survive and function for over 1 week in vitro and is capable of synthesizing urea and albumin which indicates the ability of the encapsulated hepatocytes to express liver specific functions. However, studies on the long term entrapment within alginate capsules in both in vivo and in vitro applications exhibit reduced functionality of the cells as well as protrusion of cells through the membrane [51]. Although alginate capsules are generally inert since they are composed of up to 95% water, positively charged proteins can be attracted to the carboxylic acid sites on the alginate matrix and compete with the Ca^{2+} ions. This may reduce the integrity and functionality of the microcapsule [69]. Studies on the use of

alginate microcapsules for cell culture in a bioreactor show similar results to those investigated in transplantation. Cell protrusion and leakage through the alginate membrane is observed resulting in the presence of both immobilized and free suspended cells within the surrounding solution [51]. Modification of the alginate however can create a stable gel core which can form a capsule strong enough to keep proliferating cells within the capsule over extended time periods [21].

2.7 Modifications and Alternatives to Alginate Microcapsules for Cell Encapsulation:

The biocompatible and mild preparation process makes alginate an ideal biomaterial for cell encapsulation applications. Its properties can be enhanced by surface coating or incorporating other materials to its structure. The most commonly investigated alginate capsules for the application of transplantation is the use of a 1.5% alginate solution cross-linked in calcium chloride and coated with poly-l-lysine (PLL) followed by another layer of alginate. The PLL layer improves biocompatibility by modifying the molecular weight cutoff of the microcapsule membrane [71]. Other potential additions to the alginate microcapsules include coating with chitosan as well as incorporating polyethylene glycol[18,55,61].

2.7.1 Poly-l-lysine Based Microcapsules:

Microcapsules with an alginate core and polyanion-polycation membrane to increase capsule strength have been widely investigated for various applications of cell encapsulation. The most commonly used polycation for capsule formation is poly-l-lysine (PLL) which forms APA microcapsules [55]. PLL binds to alginate via electrostatic interaction between the positively charged PLL and negatively charged alginate. This results in the interfacial adsorption of the PLL which forms a thin polymeric layer on the surface of the alginate microcapsules [18]. The molecular structure of PLL is shown in figure 2.3.

Coating alginate capsules with a polycation such as poly-l-lysine (PLL) can enhance capsule stability and immunogenicity. Alginate coated with PLL microcapsules are usually re-coated with a dilute alginate layer to remove any excess charge remaining on

the membrane surface which may attract protein adhesion. APA microcapsules have been studied extensively for enzyme, protein, bacteria, mammalian cell and drug encapsulation [39,73]. Many researchers investigated the use of APA microcapsules for the encapsulation of hepatocytes as well as other living cells. Chang studied the potential of encapsulating rat hepatocytes in APA microcapsules that were treated with calcium chelating agents such as phosphate, lactate or citrate to dissolve the calcium alginate gel core. It was found that intraperitoneal implanted encapsulated hepatocytes prolonged survival time of rats with hepatic failure and can lower bilirubin levels in Gunn rats [14].

Hepatocyte function within a membrane depends on various parameters. It is reported that alginate coated with high molecular weight PLL results in membranes with a greater molecular weight cutoff and a lower alginate content. Alginate coated poly-l-lysine capsules can be permeable to molecules up to 120 000 daltons which is the cutoff size for immunoglobulin and most complement molecules [19]. Cell stability and liver-specific function is also enhanced when hepatocytes are grown in a three dimensional spheroid formation [17].

Although APA microcapsules support cellular growth and increases capsule stability, recent research reveals some major shortcomings of the membrane which include the potential for an inflammatory response to capsule fragments upon breakage during transplantation and the possible cytotoxic characteristics of PLL. Islet cells encapsulated in APA membranes have been transplanted in rats and survival was observed up to 3 months. However, the xenograft failed as a result of overgrowth of fibroblast and macrophage like cells on the membrane surface [60].

Research has shown that in the short term between 7-12 days, and in some cases up to 30 days, intraperitoneally transplanted APA microcapsules are capable of supporting metabolic liver functions and increasing the survival of animals with hepatic failure [59]. Long term studies however indicate that beyond 4-6 weeks considerable loss of function and viability is observed. Capsule integrity is also reduced and physical breakdown of the capsules are apparent. It is suspected that the degeneration of the encapsulated hepatocytes causes ionic and pH changes within the microcapsule. The pH changes, in turn, weaken the polyelectrolyte membrane which disrupts the membrane stability. The same study noted no change in capsule integrity in the control where blank capsules were

transplanted into the peritoneal cavity of rats. Microencapsulated cell therapy would require repeated transplantation for long term treatment. To improve its potential as a more effective method of treatment, a more suitable membrane would be required [15].

One study of injecting alginate coated with PLL capsules in the peritoneal cavity of rats found no infiltration of inflammatory cells or lymphocytes in the capsules up to 4 days after implantation. The same study noted that the capsules induced peritoneal inflammation and were surrounded by fibroblasts and newly developed blood vessels. Therefore, it was concluded that in a clinical setting, the transplantation of alginate-PLL capsules can cause an aggravated fibrotic response which could pose a risk to the patient. For the safe and effective treatment of acute liver failure in patients, an extracorporeal system consisting of microencapsulated hepatocytes certainly has the potential of development in the near future. However, studies have shown that alginate-PLL-alginate capsules are too fragile to use in a bioreactor environment. In addition, the observation of necrosis of hepatocytes in the capsules suggests that alternative materials for the capsule layer needs to be investigated [19].

Rokstad et al performed a study on optimizing microcapsules in terms of mechanical stability, cell growth and protein secretion [21]. The study compared alginate microcapsules with and without a PLL coating. It was found that stability of alginate capsules are greatly enhanced by the addition of a PLL coating. As noted in previous studies, penetration of cells from uncoated alginate capsules was observed. The study also demonstrated that the functional time of microcapsules containing proliferating cells is limited due to reduced access to oxygen and nutrients from the accumulation of waste products. The use of cell encapsulation for therapeutic protein release requires further investigation because a method of controlling and hindering cellular growth would be needed. Although alginate microcapsules support cellular growth, the structural stability of these capsules needs to be improved for proliferating cells. It is suggested that the stability can be improved by increasing the PLL layer. However, this may not be advantageous for cell transplantation purposes [21].

All of these studies indicate that the APA microcapsules can support tissue proliferation and protect implanted tissues against immunoglobulins. However, fibrotic

tissue growth around the capsule remains a barrier for this polymer to be used in cell transplantation as the tissue growth inhibits the function of the encapsulated cells.

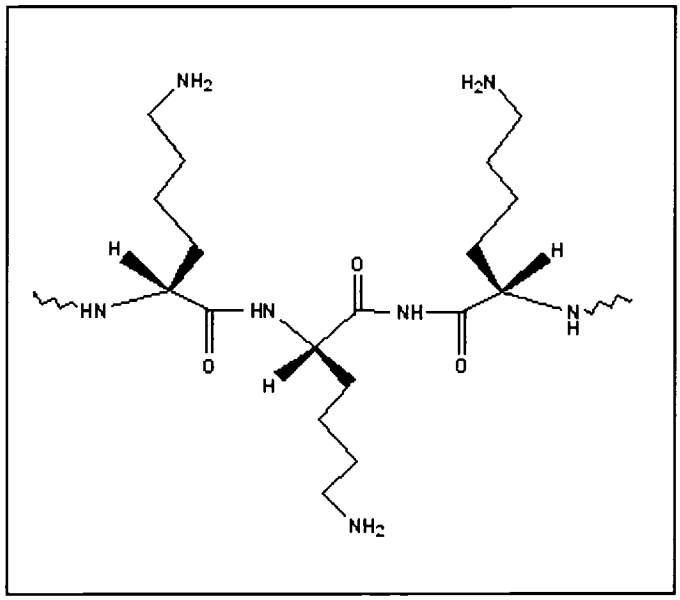

Figure 2.3: Molecular structure of poly-l-lysine

2.7.2 Chitosan Based Microcapsules:

Chitosan is recommended as a potential replacement of PLL. Chitosan is a cationic polysaccharide comprised of 1-4 linked N-acterylglucosamin residues. It is a biocompatible and biodegradable material which is normally insoluble in aqueous solutions with a pH above 7. However, when exposed to dilute acids (pH<6), the free amino groups become protonated allowing the molecule to dissolve. The protonated amino groups have a high charge density in solution which permits chitosan to form ionic complexes with polyanionic substances [12,57,74]. At lower pH, chitosan can interact with negatively charged ions to form crosslinked hydrogels [75]. It has been shown that chitosan is bioadhesive, non toxic and compatible with soft tissue [57,74].

Chitosan can cross-link with anionic alginate by displacing calcium ions, which results in the formation of a surrounding membrane [74-78]. The protonated amino groups have a high charge density in solution which permit chitosan to form ionic complexes with the carboxyl group of the alginate molecule[12]. Chitosan can form a stronger complex with alginate than PLL because the distance between the charges is approximately the same for the two molecules. The electrostatic interaction of the carboxyl group of alginate with the amine groups of chitosan results in the entanglement of the polymer membrane which can act as an enclosure for active substances. The molecular structure of chitosan is shown in figure 2.4. Studies have shown that alginates containing a high G block content bind more rapidly with chitosan than alginate with a high M block content. The presence of calcium ions also facilitate and improve binding [55]. The interaction between alginate and chitosan is almost irreversible and is optimum when the porosity of the alginate capsule is high and the molecular weight of the chitosan is low. This allows higher diffusion of the chitosan into the alginate matrix [55,56]. Studies on the effect of chitosan's molecular weight on microcapsules reveal that low molecular weight chitosan results in microcapsules having a thick and strong membrane that is less prone to swelling[79]. It is reported that chitosan membranes can range in thickness from 30-50 μm in comparison to 3-13 μm for PLL coatings [74]. Although there are more positive charged amine groups in higher molecular weight chitosan, which results in more binding sites available for alginate interaction, diffusion is hindered due to the large size [79].

Chitosan had been widely investigated for microencapusulation of drugs and proteins as well as some animal cells [57,68,77-80]. One study observed the potential of directly encapsulating two types of fibroblasts in a chitosan matrix and comparing it to APA capsules. The study concluded that chitosan can be used as a cell immobilizing matrix that is capable of supporting anchorage-dependent cells. In comparison to PLL coated alginate capsules, it was found that chitosan microcapsules resulted in cells forming discrete aggregates in the dense core of the gel. For one of the cell line studied (R208N.8), it was observed that viable cells were located primarily between the alginate and poly-l-lysine layer. The observation supports earlier studies which indicate that anchorage dependent cells prefer cationic surfaces. The author concludes that alginate alone may therefore be inadequate for supporting all cell types [75].

A study on the stability and permeability of chitosan capsules reveals that chitosan-alginate forms a high stability complex which provides the membrane the potential to be used for artificial organs. It is reported that capsule strength is directly correlated with the amount of chitosan that is bound to the interior of the alginate matrix[56,80]. A similar study on the release of immobilized dextran in chitosan coated alginate microcapsules has shown that a higher porosity and diffusion rate is obtained for capsules containing calcium ions [55,80]. However, other studies have shown that diffusion is not affected by the concentration of $CaCl_2$ used during the encapsulation process [74]. DeGroot et al. performed a study on the use of alginate coated with chitosan and alginate coated with PLL microcapsules for the encapsulation of urease as a model enzyme [74]. Results from the study revealed that upon exposure to chymotrypsin, enzymatic activity in microcapsules coated with intermediate molecular weight chitosan was least affected in comparison to PLL coatings. The activity retention is attributed to the fact that a thicker membrane may result in reduced permeability[74].

There have not been many reported studies on the use of microencapsulated hepatocytes in chitosan. One of the limiting factors of using chitosan bound microcapsules is the long binding time required between it and alginate as well as the requirement of a slightly acidic environment for solubility. It is reported that the time required for chitosan binding to produce stable beads is at least 30 minutes[56]. Research has been conducted on the use of chitosan scaffolds to support proliferating liver

cells[12,16,59]. Chitosan has a structure similar to glycosaminoglycans which are components found in the liver extracellular matrix. Since all vertebrae cells are known to possess unevenly distributed negative charges, chitosan having a high positive charge permits hepatocytes to bind tightly via electrostatic interactions. One research group observed the functionality and viability of rat hepatocytes seeded on chitosan-alginate scaffolds by monitoring albumin and urea synthesis. It was observed that the ionic complex formed between chitosan and alginate is beneficial to cell attachment and permits hepatocyte proliferation as well as maintenance of metabolic activity [12].

Another study on the permeability of alginate-chitosan matrix revealed that multi-layer of chitosan and alginate may be required to ensure that the capsule is impermeable to IgG and thereby not elicit an immune reaction if used in transplantation. For the application of chitosan-alginate microcapsules it is imperative that the release of polycation be as low as possible since both chitosan and PLL in solution may initiate an immune reaction [56].

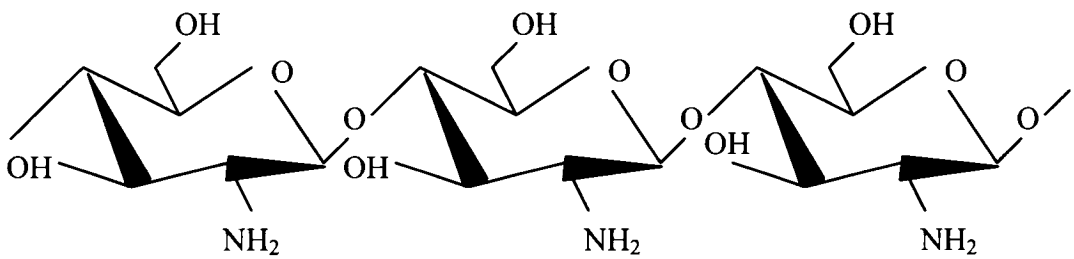

Figure 2.4: Molecular structure of chitosan

2.7.3 PEG Based Microcapsules:

Polyethyelene glycol (PEG) is a biocompatible, hydrophilic and non ionic polymer that is protein resistant, biodegradable and has both low toxicity and immunogenicity [81]. Despite its non aqueous nature, PEG does not exhibit cytotoxicity to live cells and tissues and remains relatively inert in body fluids [62,82]. It has been observed that PEG can eliminate the immunogenicity of proteins while preserving their biological properties. The addition of PEG to polymers reduces the adsorption and adhesion of cells and proteins. Therefore, PEG can potentially improve the biocompatibility of polymers[53,81,83]. PEG incorporated microcapsules has been investigated for the use in drug delivery, blood cells, proteins as well as mammalian cell encapsulation for tissue engineering applications [48,82-86].

Both alginate-PLL and alginate-chitosan microcapsules contain a high density of positive and negative charges which can be exposed in the case of any disturbances of the membrane. The exposed charges in turn attract proteins via hydrophobic and coulombic interaction with the charged surface. Studies have shown that PEG-poly-l-lysine copolymer coated capsules can eliminate the fibrous tissue growth around the membrane surface. This was observed when encapsulated islet cells formed by gelation of alginate-PLL capsules in the presence of PEG were implanted intraperitoneally in mice. Chen et al revealed that APA microcapsules coated with PEG amines can be used for xenogenic transplantation since such microcapsules containing rat islet cells prevented cell overgrowth on the capsule surface when implanted in mice [83]. However, to ensure long term stability, the strength of the microcapsules needs to be improved [53,60,61].

Morphological studies of alginate-PEG microcapsules indicate that with increasing PEG concentration, pore size decreases while the number of pores on the membrane increases. Therefore, the pore diameter can be optimized for growth of various cell lines by modifying the PEG concentration. This in turn would minimize cell leakage because voids created by the presence of PEG in the alginate matrix produces areas of concentrated alginate which contribute to increasing the strength of the microcapsules [51].

Chandy et al. studied the use of chitosan and PEG coatings around calcium alginate microspheres to increase capsule strength, flexibility and biocompatibility [53]. Red blood cells were encapsulated in alginate coated with chitosan microcapsules containing 0.1%

PEG and cross linked with glutaraldehyde. Reported SEM studies indicate that PEG is incorporated within the alginate gel core membrane. The addition of glutaraldehyde during gelation improves surface texture [53].

The effect of BSA (Bovine Serum Albumin) loading and release through a chitosan-PEG capsule was studied to observe the permeability influence of PEG on microcapsules. It was found that BSA release rate is accelerated by the presence of PEG. The entanglement of PEG chains with chitosan molecules hinders the packed and rigid bonding between chitosan and BSA. Therefore, relatively loose structure of chitosan containing PEG microcapsules resulted in a high rate of BSA release. The study demonstrates that PEG incorporated capsules can be used for protein delivery or therapeutic applications [12].

2.8 Research Justification:

As indicated above, although many microcapsules have been investigated for various usages including drug, protein and cell delivery, there exist several limitations to the technology. This thesis investigates the design of a novel microcapsule formulation combining alginate, PLL, chitosan and PEG, and evaluates its preclinical efficacy as a treatment for liver diseases and cell therapy in vitro.

Preface for Chapter 3, 4 and 5

The results from the current study have been presented in the following papers. Chapter 3 is an investigation of alginate coated with chitosan microcapsules to verify whether chitosan can be used for liver cell encapsulation. Chapter 4 focuses on the design and properties of the novel ACPPA microcapsule and Chapter 5 provides a comparative study of the novel membrane with other microcapsule membranes containing PEG. In accordance with the McGill University regulations, the three manuscripts are presented in their original forms as submitted.

Research Articles Presented in the Thesis Chapters 3, 4 and 5:

1) **Tasima Haque**, Hongmei Chen, Wei Ouyang, Christopher Martoni, Bisi Lawuyi, Alexandra Urbanska and Satya Prakash. In vitro study of alginate-chitosan microcapsules: An alternative to transplant for the treatment of liver failure. Biotechnology Letters, 2005. In Press.

2) **Tasima Haque**, Hongmei Chen, Wei Ouyang, Christopher Martoni, Bisi Lawuyi, Alexandra Urbanska and Satya Prakash. Design of a novel microcapsule membrane combining alginate, chitosan, polyethylene glycol and poly-l-lysine for cell transplantation. International Journal of Artifical Organs, 2004. Submitted.

3) **Tasima Haque**, Hongmei Chen, Wei Ouyang, Christopher Martoni, Bisi Lawuyi, Alexandra Urbanska and Satya Prakash. Superior cell delivery features of polyethylene glycol incorporated alginate, chitosan and poly-l-lysine microcapsules. Molecular Pharmaceutics. **2** (1), 29 -36, 2005. Published.

Contributions from the Current Research that are not Included in the Thesis:

1) **Tasima Haque**, Hongmei Chen, Wei Ouyang, Terrence Metz, Bisi Lawuyi and Satya Prakash. Effect of integrating polyethylene glycol to alginate-poly-l-lysine and alginate-

chitosan microcapsules for oral delivery of live cells and cell transplantation for therapy. Proceedings CMBEC28 Conference (Canadian Medical and Biological Engineering Conference) 2004. *Research was awarded 3rd place for Canadian Medical and Biological and Engineering Society Student (CMBES) Paper Competition*

2) **Tasima Haque**, Hongmei Chen, Wei Ouyang, Christopher Martoni, Bisi Lawuyi, Alexandra Urbanska and Satya Prakash. Investigation of a new microcapsule membrane combining alginate, chitosan polyethylene glycol and poly-l-lysine for cell transplantation applications. International Journal of Artificial Organs. 2004. Under Revision.

Abstracts:

1) **Tasima Haque**, Hongmei Chen, Wei Ouyang, Christopher Martoni, Bisi Lawuyi, Alexandra Urbanska and Satya Prakash. Design of a novel microcapsule membrane combining alginate, chitosan, polyethylene glycol and poly-l-lysine for cell transplantation. Abstract. XXXI Annual ESAO Congress, 8-11 September 2004, Warsaw-Poland.

2) **Tasima Haque**, Hongmei Chen, Wei Ouyang, Terrence Metz, Bisi Lawuyi and Satya Prakash. Effect of integrating polyethylene glycol to alginate-poly-l-lysine and alginate-chitosan microcapsules for oral delivery of live cells and cell transplantation for therapy. 28th CMBES Conference, September 9-11, 2004. Quebec City, Canada

3) **Tasima Haque**, Hongmei Chen, Wei Ouyang, Terrence Metz, Christopher Martoni and Satya Prakash. Investigation of a novel microcapsule membrane integrating polyethylene glycol to alginate, poly-l-lysine and chitosan microcapsules for the application of liver cell transplantation. XXth International Congress of the Transplantation Society September 5-10, 2004, Vienna, Austria.

Contribution of Authors:

I am the first author for all of the above articles and was responsible for the research design, procedure and data analysis. All the other authors have also contributed significantly and are reported as co-authors. The last author, Dr. Satya Prakash, is the research advisor and is also the corresponding author in all of the manuscripts, abstracts and proceedings written throughout the course of the current Masters project.

Contributions to Other Research:

As a co-author in the following papers, I have also contributed significantly in these areas:

1) Hongmei Chen, Wei Ouyang, Mitchell Jones, **Tasima Haque**, Bisi Lawuyi and Satya Prakash. *In-vitro* analysis of APA microcapsules for oral delivery of live bacterial cells" Journal of Microencapsulation, 2004. In Press.

2) Terrence Metz, Mitchell L. Jones, Hongmei Chen, Trisnawati Halim, Maryam Mirzaei, **Tasima Haque**, Devendra Amre, Satya Prakash. Targeted delivery of thalidomide using polymeric microcapsules for the treatment of Crohn's disease. Cell Biochemistry and Biophysics, 2004. In Press.

3) Hongmei Chen, Mitchell L Jones, Wei Ouyang, Terrence Metz, Christopher Martoni, **Tasima Haque**, Rebecca Cohen, Bisi Lawuyi, and Satya Prakash. Design, preparation and in-vitro characterization of genipin cross linked alginate-chitosan microcapsules for live cell encapsulation for cell therapy. Cell Biochemistry and Biophysics, 2004. In Press.

Chapter 3:

In Vitro Study of Alginate-Chitosan Microcapsules: An Alternative to Liver Cell Transplant for the Treatment of Liver Failure

Tasima Haque, Hongmei Chen, Wei Ouyang, Christopher Martoni, Bisi Lawuyi, Aleksandra Malgorzata Urbanska, and Satya Prakash*

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Biotechnology Letters (2005). In Press

Chapter 3: In Vitro Study of Alginate-Chitosan Microcapsules: An Alternative to Liver Cell Transplant for the Treatment of Liver Failure

3.1 Abstract:

The application of alginate-chitosan (AC) microcapsules to liver cell transplantation has not been investigated. In the current in-vitro study, we investigate the potential of AC microcapsules for the encapsulation of liver cells. Results show that the AC membrane supports the survival, proliferation and protein secretion by entrapped hepatocytes. The AC membrane provides cell immuno-isolation and has the potential for cell cryopreservation. The study reveals that the AC microcapsule has several advantages compared to the widely investigated alginate-poly-lysine (APA) microcapsules for the application of cell therapy.

Keywords: Alginate, Chitosan, Cell viability, Hepatocytes, Microencapsulation

3.2 Introduction:

The diverse function of the liver makes it difficult to construct an effective hepatic support system. As a result, research continues to be performed on constructing a biological artificial liver that is stable, capable of removing toxins and has the ability to supply proteins to patients [8]. An appealing possibility to treat liver failure without the requirement of immunosuppressants is the use of cell microencapsulation. Allogenic or xenogenic cells entrapped within a semi-permeable membrane are protected and immuno-isololated from its surroundings. This prevents the direct contact of the cells with immune cells, cytokines, immunoglobulins and complement factors when directly used in transplantation. Microencapsulation also protects immobilized cells from damage during handling [18,22,87].

Most research to date on encapsulating hepatocytes are based on alginate-poly-l-lysine-alginate (APA) membranes; however, previous research has shown APA microcapsules to be inadequate for long term transplantation. Although APA capsules can support hepatic function when transplanted in rats, the polymer may cause fibrotic reactions and exhibit cytotoxic effects in some cases [21,56]. Therefore, finding a

biocompatible polymer enabling the long-term entrapment and growth of hepatocytes without causing adverse host immune responses is still an undergoing area of research. Alginate-Chitosan (AC) microcaspules have been studied as an alternate microcapsule membrane, however details regarding its use in cell encapsulation have not been reported extensively [12,53,55,75]. The current research investigates the potential and properties of AC microcapsules as an alternative membrane for encapsulating liver cells and a new direction for cell encapsulation technology.

3.3 Materials and methods:

Chemicals:

Sodium Alginate (MW 80 000-120 000, catalogue # A2033), Polyethylene glycol (PEG) (MW 10000, catalogue #P6667), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide or Thiazolyl blue, catalogue #M-5655), Poly-l-lysine hydrobromide (MW 27,400, catalogue # P-7890) and Bradford Reagent (catalogue #B-6196) were purchased from Sigma Chemicals (St. Louis MO, USA). Chitosan 10 (lot# ELE2169) was obtained from Wako Chemicals, Japan.

Cell lines and Growth conditions:

Cell lines HepG2 and lymphocyte leukemia cells were purchased from ATCC and routinely sub-cultured in MEM (minimum essential eagle media) supplemented with 10% FBS and 1% penicillin-streptomycin obtained from Sigma Aldrich. The cells were grown in 75 cm^2 culture flasks and incubated at 37 ^{o}C with an air atmosphere of 5% CO_2.

Method for Cell Encapsulation:

HepG2 cells were encapsulated in alginate microcapsules using previously established procedures [14,17]. Briefly, HepG2 cells were trypsinized and then centrifuged at 1000 rpm for 10 minutes at 20 ^{o}C. The media was decanted and the cells were resuspended in 30 ± 10 mL of sterile filtered 1.5% sodium alginate solution in order to attain a final cell concentration of $1.5x10^6$ cells/mL. The solution was extruded through an INOTECH microencapsulator using a 60 mL syringe and a 300 μm nozzle. The

parameters applied to the encapsulator machine in order to form spherical capsules include: A volatage of 0.577kV, a voltage of 21V was supplied to the pump motor to maintain the syringe flow and a frequency of 710Hz. The gelation process took place in a 0.1M $CaCl_2$ solution for 10 minutes. APA microcapsules were prepared by immersing the alginate capsules in a 0.05% (w/v) poly-l-lysine solution dissolved in 0.45% (w/v) NaCl for 10 minutes and recoating with a layer of 0.1% alginate for 5 minutes. AC microcapsules were prepared by coating the alginate capsules with a 0.5% (w/v) chitosan solution dissolved in dilute acetic acid at a pH of 5.2 for 30 minutes. The pH of 5.2 was the highest pH possible that the given conentration of chitosan can form a homogenous solution without comprimising cell viability. The microcapsules were stored in complete growth media (MEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin) used for culturing free cells at 37 °C and 5% CO_2. The media was changed once every 2 days. The entire procedure was performed under sterile conditions in a Microzone Biological Containment Hood (Microzone Corporation ON, Canada) and all solutions were autoclaved with the exception of chitosan, PLL and the alginate solution which were 0.22 μm sterile filtered prior to usage.

Method of Monitoring Metabolic Activity and Viability of Encapsulated Cells:

The microcapsule membranes were tested for cytotoxicity and suitability for live cell encapsulation using an MTT based colorimetric assay. Previously established procedures as described by Uludag [23] was used with some modifications. Approximately 30±2 capsules were incubated with 100 μL of media and 25 μL of an MTT solution (1% thyiazolyl blue in PBS) for 24 hours in 96 well plates. The media and MTT solution were removed from the wells and the formazan crystals formed by the reaction of MTT with the live cells was dissolved in 100 μL of DMSO. After 30 minutes of incubation, the absorbance was measured using a μQuant multiwell spectrophotometer purchased from Bio-Tek Instruments Inc. at a wavelength of 570 nm. The cell number was obtained using a calibration curve of cell quantity versus absorbance.

Protein Secretion by Encapsulated HepG2 cells:

The Bradford Assay was used to detect the amount of protein secreted by 100±5 microcapsules containing HepG2 cells grown in serum free media for 48 hours. The quantity of protein secreted by the cells was measured by taking 15 μL of the media in which the microcapsules were stored in, and adding 0.3 mL of Bradford reagent into 96 well plates. The absorbance was measured at 595 nm after 20 minutes of incubation. The protein concentration was attained using a calibration curve of albumin concentration versus absorbance.

Method for Testing Immunogenicity of Microcapsules:

To investigate the ability of the novel membrane to provide cell required immuno-protection, approximately 200±5 APA and AC microcapsules containing HepG2 cells were grown in 1 mL of media containing an initial concentration of $9X10^4$ ± 500 cells/mL of lymphocytes in 24 well plates. Lymphocytes secrete cytokines, therefore, the viability of the encapsulated hepatocytes would depend on the immuno-protection potential of the microcapsule membrane. Samples were withdrawn every 48 hours for a period of 7 days. The viability of the HepG2 was determined using an MTT assay.

Cryopreservation Studies:

The possibility of long terms storage of encapsulated HepG2 cells in the membranes was tested. 150±10 capsules were washed with media and transferred to a 2 mL cryovial containing 1 mL of complete growth media supplemented with 0.10 mL DMSO. The vials were placed at -20 °C for 1 hour prior to being stored at -80 °C. After 30 days of storing, the microcapsules were thawed by immersing the vials in a 37 °C water bath and re-cultured in media. The MTT assay was used to obtain the number of viable cells remaining.

3.4 Results and Discussion:

APA microcapsules have been previously studied for the encapsulation of hepatocytes to be used for therapy [14,15,31]. Several studies however have revealed that improvements to the membrane in terms of mechanical strength and biocompatibility are

required for its application [14,15,21,40]. The current research demonstrated that chitosan coated microcapsules may be an alternative membrane for the encapsulation of hepatocytes.

Morphological Studies:

APA and AC microcapsules were prepared. Microscopic analysis revealed no significant difference in the images of the two types of membranes (fig. 1). The capsule diameter was found to be 450 ± 30 μm for both membranes and the encapsulated HepG2 cells appeared to grow in clumps distributed within the membrane.

Viability of Encapsulated Cells:

To test whether the AC microcapsule can support the proliferation of hepatocytes and observe how it compares with APA capsules, the cell viability was monitored for a period of 25 days using an MTT assay. Figure 2 represents the cell number for 30±2 capsules at various time intervals. Alginate coated chitosan microcapsules were able to support the proliferation of liver cells; however, according to the results, a reduction in viability is apparent in comparison to the APA membrane. After 1 week of encapsulating, the APA membranes display an increase in HepG2 activity resulting in a maximum cell number of $1.40x10^6$ $\pm 1.02x10^5$ at 9 days. After the 9th day of encapsulating, a gradual decrease in cell activity is observed for the remainder 25 day study. The AC microcapsules behaved slightly differently. AC microencapsulated cells reveal a maximum cell number of $1.21x10^6 \pm 8.80x10^4$ which is attained after the fourth day of encapsulation.

The differences observed may be due to the longer coating procedure required by the chitosan. Chitosan requires at least 30 minutes of exposure in order to complete crosslinking with the alginate molecules [55]. Despite the harsher conditions, cell proliferation and metabolic activity remained apparent throughout the 25 day study. Both the APA and AC membranes showed fluctuations in the viability. The fluctuations in cell viability may be a result of mass transfer of either nutrients or the MTT itself across the membrane to the cells. Since hepatocytes are anchorage dependent cells, the clusters of cells within the microcapsules can cause a delay or decrease in the rate of diffusion [23].

Total Protein Secretion from Encapsulated Cells:

Despite a significant difference observed in the cell viability of the APA and AC microcapsules, the total protein secreted by the membranes did not differ significantly (Fig. 3). A peak in protein secretion is apparent between the 5th and 11th day after which a decrease is observed implying reduced functionality of the HepG2 cells. A maximum protein concentration of 1.31 ± 0.030 mg/mL is attained for the APA. The maximum concentration of proteins secreted by the AC encapsulated HepG2 cells was 1.29 ± 0.044 mg/mL. The quantity of protein secretion is dependent on the diffusion and permeability properties of the membrane [18,47]. The permeability of the membrane is in turn dependant on several properties including charge distribution, porosity, hydrophilicity as well as the properties and size of the substance being transferred across the membrane. These characteristics would vary depending on the interaction of PLL with alginate microcapsules as well as the interaction of chitosan with alginate [47,69,76]. A higher surface concentration of alginate improves PLL binding [88] and the protein release rate is generally reduced by increasing the molecular weight of the chitosan [56]. Further studies on the permeability characteristics of both APA and AC microcapsules would need to be performed to assess their properties of protein secretion from hepatocytes.

Immunogenic Properties of Microcapsules:

According to the results, the AC membrane gave a lesser immunogenic response than the APA microcapsules (fig 4). Throughout the 7 day analysis the cell count for APA encapsulated HepG2 cells decreased from $9.60x10^5 \pm 1.17x10^5$ to $6.59x10^5 \pm 1.44x10^5$. Encapsulated HepG2 cells in AC microcapsules grown in the presence of lymphocytes showed no reduction in cell viability for the 7 day study. These results suggest that chitosan is better at providing immuno-protection and may be a better direction to investigate for the use in xenogenic transplantation. The hydrophobic nature of chitosan may be what causes the HepG2 cells within the microcapsules to be unaffected by the surrounding lymphocytes [53,79].

Cryopreservation Properties:

The AC and APA microcapsules were stored at -80 °C for a period of 30 days. Results show the HepG2 viability to reduce from 5.94 $x10^5 \pm$ 8.2 $x10^4$ cells prior to freezing to 5.18 $x10^5 \pm$ 5.8 $x10^4$ cells after re-growing for the AC membranes (Fig. 5). APA microcapsules had a greater reduction from $9.47x10^5 \pm 1.0 \times 10^4$ cells to $5.07x10^5 \pm 5.5 \times 10^4$ after re-growing. Morphological studies of the APA membranes revealed the membranes to lose its spherical and uniform shape. The membrane surface formed wrinkles as presented in figure 6. The viability was most likely compromised as a result of the freezing and re-growing process, implying a weaker mechanical strength. Chitosan forms stronger complexes with alginate than PLL [56,75]. This may be what contributes to the better cryopreservation properties of AC capsules. The mechanical failure of microcapsules may be attributed to osmotic swelling of the alginate core [89]. Future investigations on the mechanical strength of both membranes will need to be performed to verify the reason why chitosan microcapsules resulted in better cryopreseravation of the cells.

3.5 Conclusion:

The current study revealed that AC microcapsules may be an alternative membrane to encapsulate cells for therapy. The AC microcapsules support liver cell proliferation and function and excels the widely studied APA membrane in terms of both immunogenicity and cryopreservation properties. The AC microcapsules may thus be a forefront for an alternative membrane to be investigated for cell encapsulation. In order to determine its complete application however, further studies on its permeability, mechanical stability as well as its in-vivo behaviour will require future investigation.

3.6 Acknowledgements:

This work was supported by research grants form the Canadian Institute of Health Research (CIHR) to Dr. Prakash. We would also like to acknowledge NSERC post graduate scholarships to Haque, Chen, and Martoni. Ouyang acknowledges Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT) Post Doctoral Fellowship.

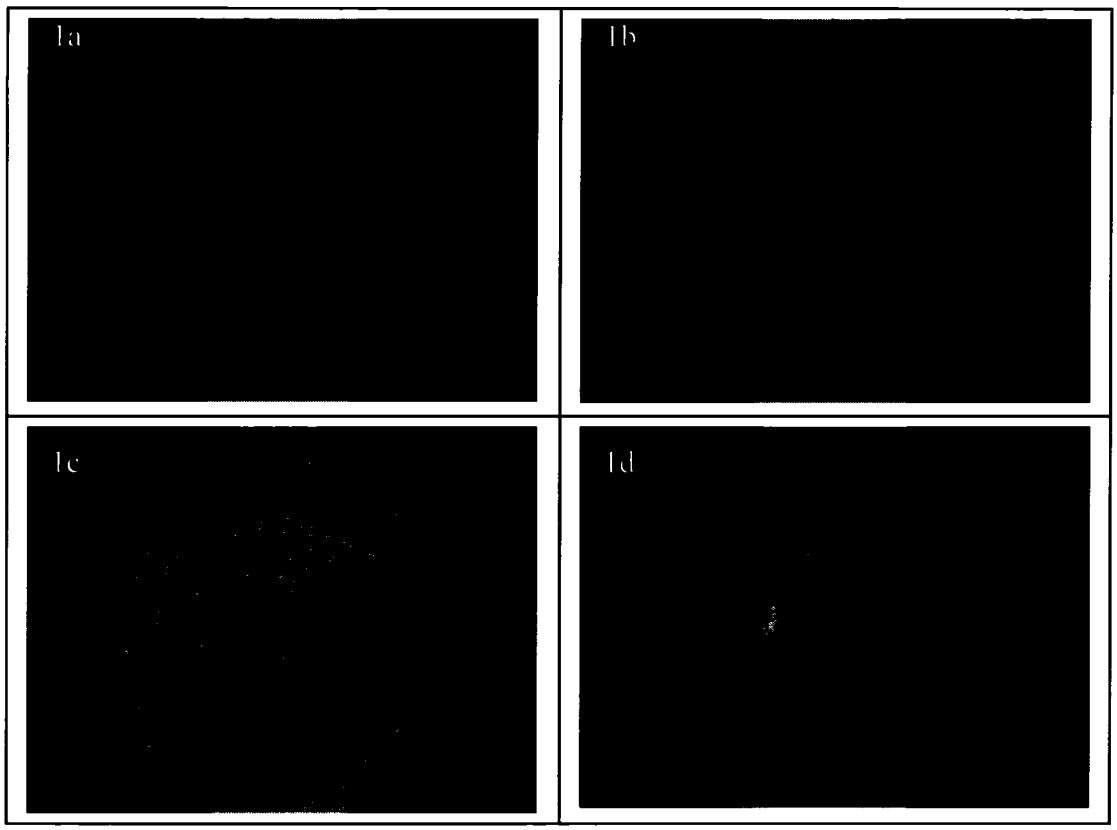

Figure 3.1: Photomicrograph of Alginate-Poly-l-lysine-Alginate (APA) and Alginate-Chitosan (AC) microcapsules containing HepG2 cells. Magnification: 10X; capsule size: 450±30 μm. *1a: APA, 5 days after encapsulation, 1b: APA, 12 days after encapsulation. 1c: AC, 5 days after encapsulation, 1d: APA, 12 days after encapsulation.*

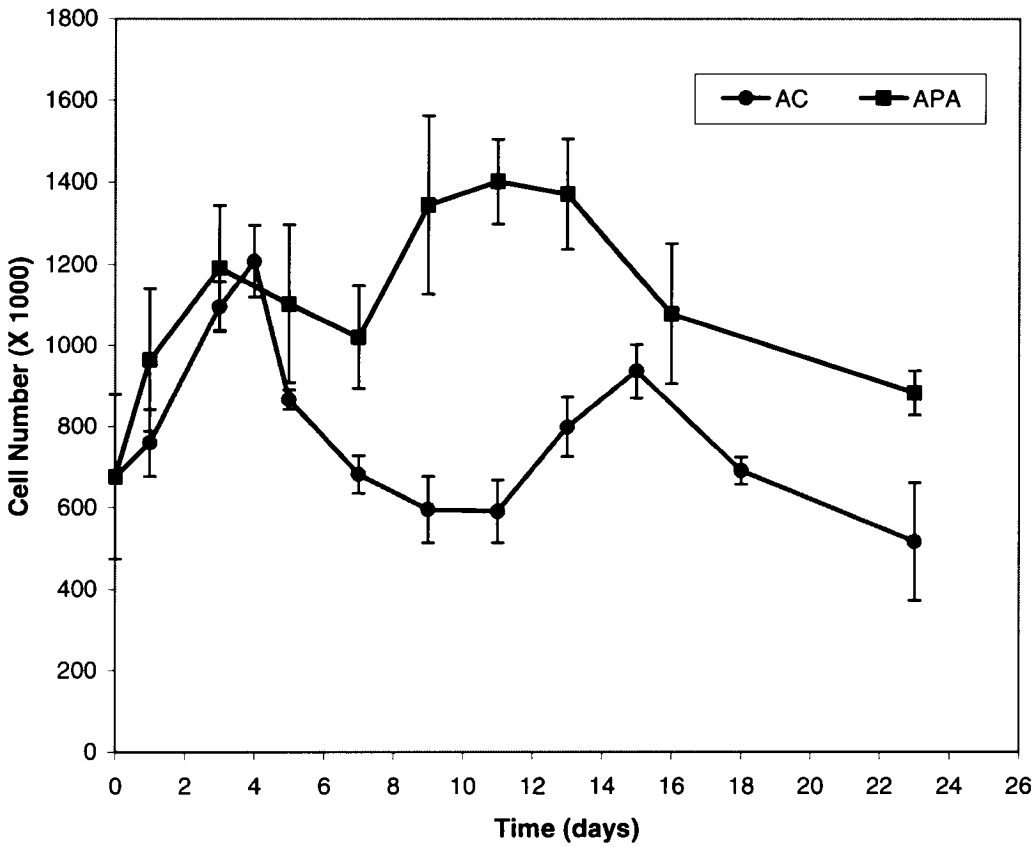

Figure 3.2: Study of cell viability for Alginate-Poly-l-lysine-Alginate (APA) and Alginate-Chitosan (AC) microcapsules containing HepG2 cells.

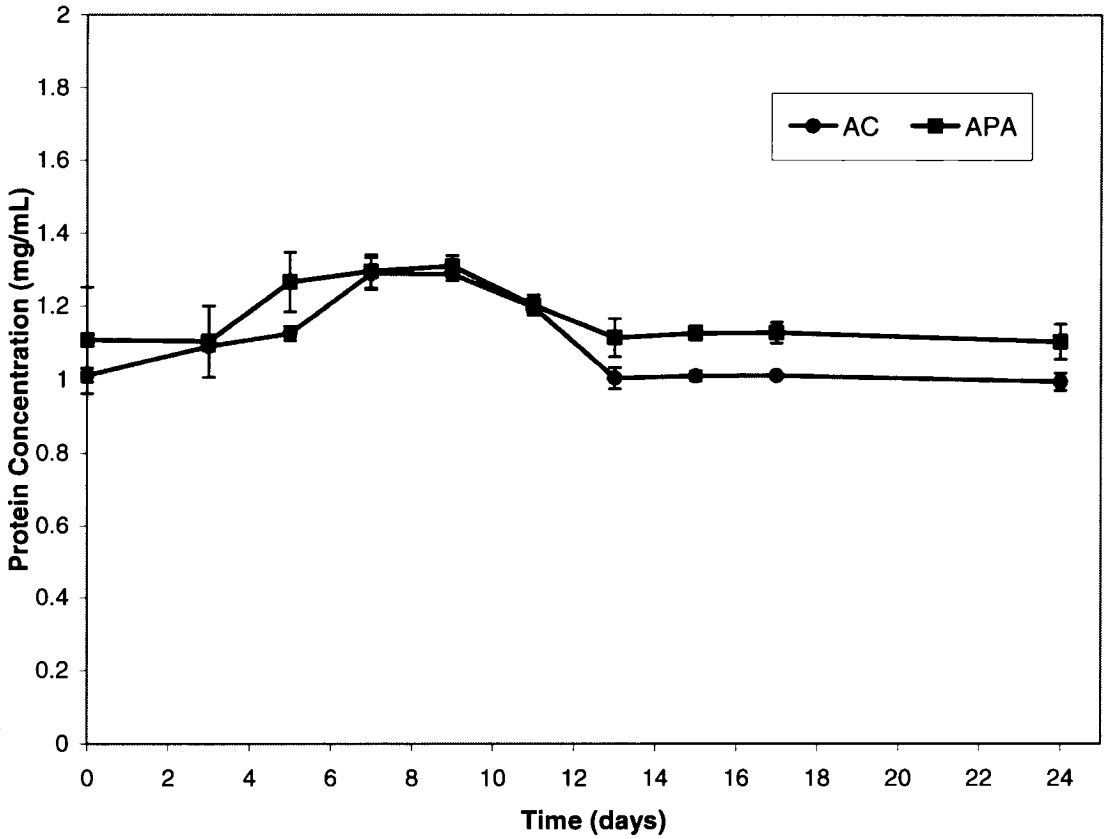

Figure 3.3: Study of protein secreted by encapsulated HepG2 cells in Alginate-Poly-l-lysine-Alginate (APA) and Alginate-Chitosan (AC) microcapsules.

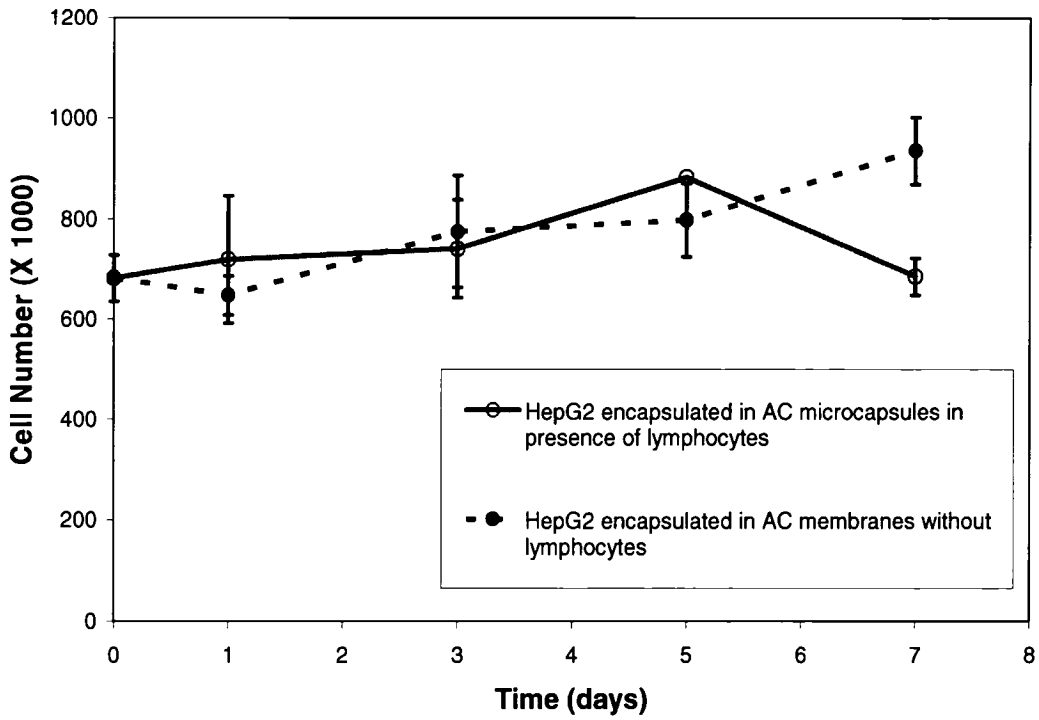

Figure 3.4a: Study of cell viability for Alginate-Chitosan (AC) microcapsules containing HepG2 cells grown in the presence of lymphocytes.

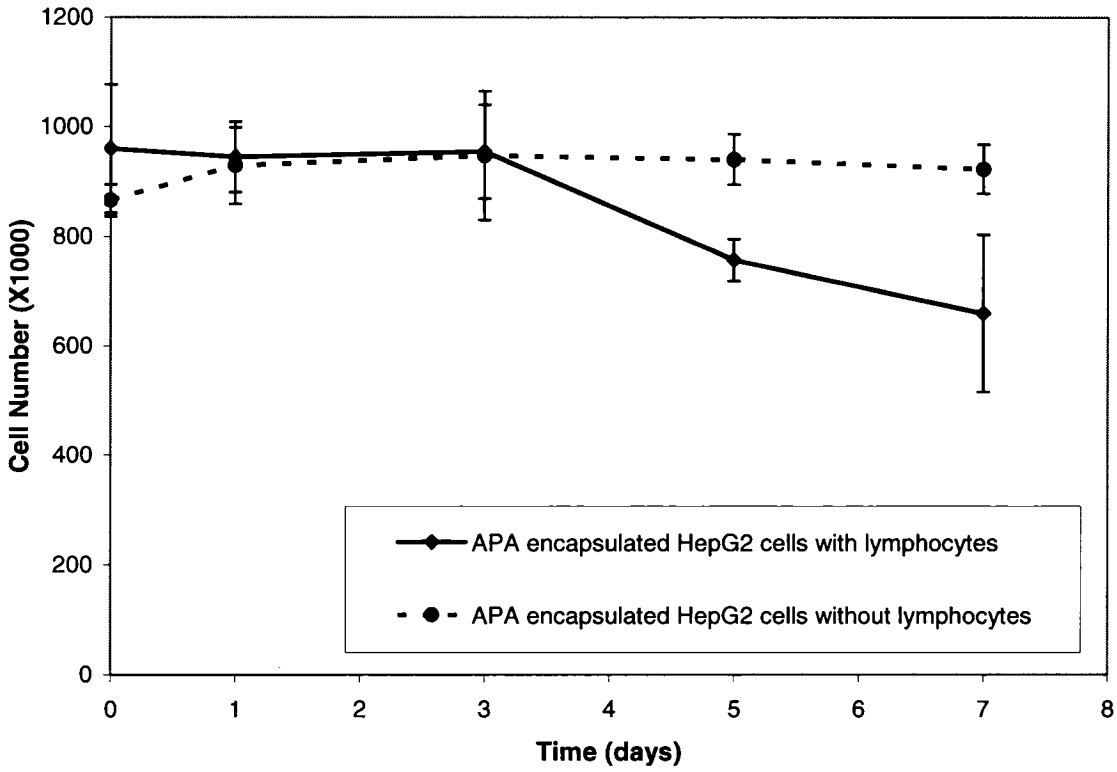

Figure 3.4b: Study of cell viability for Alginate-Poly-l-lysine-Alginate (APA) microcapsules containing HepG2 cells grown in the presence of lymphocytes.

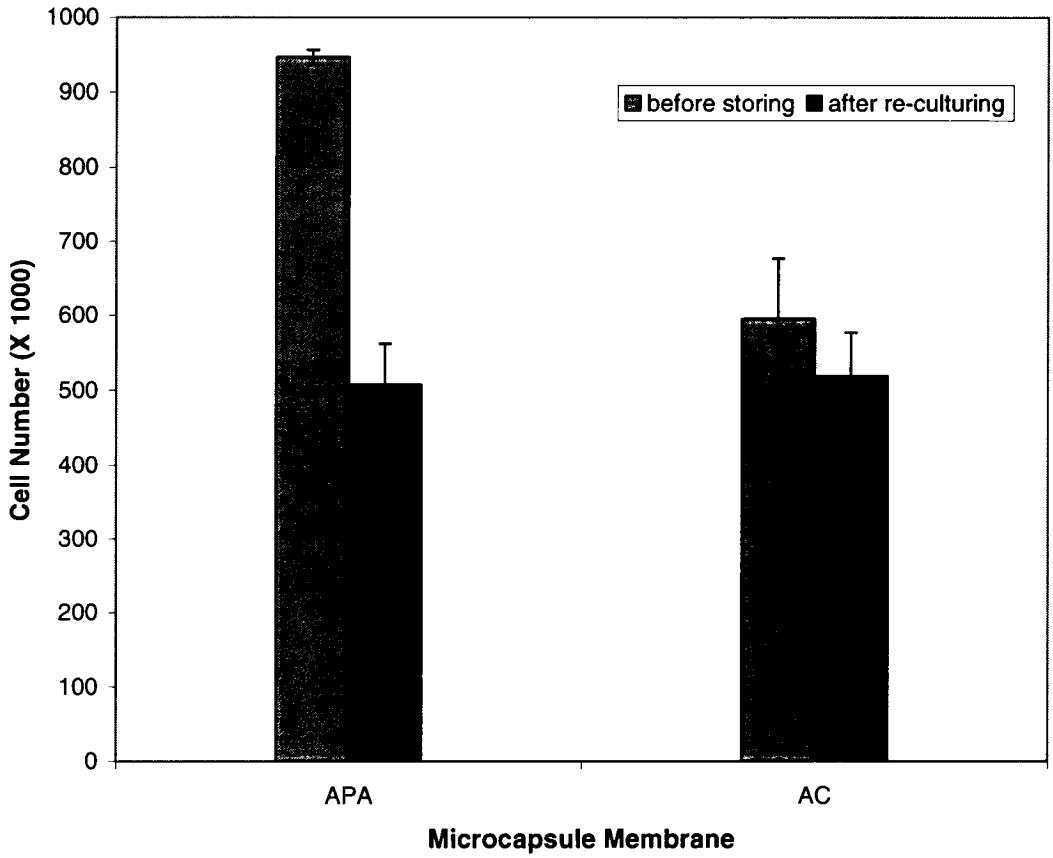

Figure 3.5: Comparative study of cell viability for Alginate-Poly-l-lysine-Alginate (APA) and Alginate-Chitosan (AC) microcapsules containing HepG2 cells after 30 days of storage at -80°C.

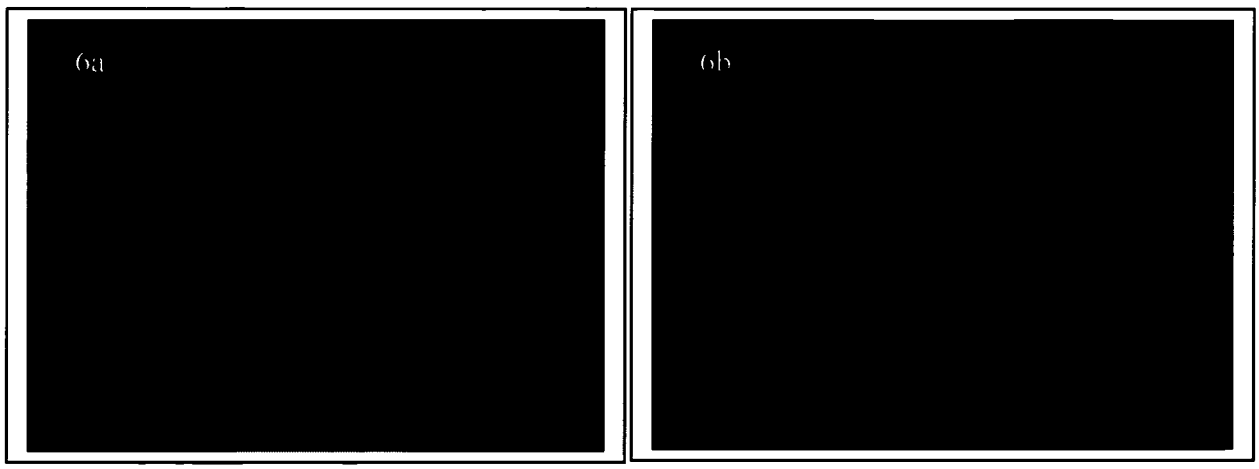

Figure 3.6: Photomicrograph of Alginate-Poly-l-lysine-Alginate (APA) and Alginate-Chitosan (AC) microcapsules containing HepG2 cells after 30 days of storage at -80 °C. Capsule size: 450 ±30 µm. *1a: APA, magnification: 10X, 1b: AC, magnification 6.5X.*

Chapter 4:

Design of a novel microcapsule membrane combining alginate, chitosan, polyethylene glycol and poly-l-lysine for cell transplantation

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Manuscript Submitted to International Journal of Artificial Organs (2004)

Chapter 4: Design of a novel microcapsule membrane combining alginate, chitosan, polyethylene glycol and poly-l-lysine for cell transplantation

4.1 Abstract:

Microencapsulation of living cells has been limited due to the need for a biocompatible polymer capable of sustaining cell viability while maintaining mechanical strength and immunoprotection. The current study investigates a novel membrane combining alginate, chitosan, polyethylene glycol (PEG) and poly-l-lysine (PLL) with the objective of proposing a membrane suitable for cell entrapment that may overcome some of the shortcomings of the widely studied alginate-poly-l-lysine-alginate (APA) microcapsules. Results show that using a 1.5% alginate solution coated with 0.5% chitosan, 0.1% PEG, 0.05% PLL and 0.1% alginate solutions, stable, uniform shaped novel membrane ACPPA microcapsules can be prepared. The mechanical stability of the ACPPA microcapsule membrane was comparable to the APA microcapsule membranes. HepG2 cells, encapsulated within the membrane, reveal the novel microcapsules to be non toxic, capable of sustaining cell proliferation, and can provide sufficient immuno-protection. This study demonstrates that the membrane has the potential to be used for cell encapsulation for developing bioartificial organs.

Keywords: Microencapsulation, alginate, poly-ethylene-glycol, chitosan, transplantation, immuno-isolation.

4.2 Introduction:

Immuno-isolation technologies for the short term and long term transplantation of biologically active materials such as tissues and cells has been a focus of research for the treatment of several diseases including inborn errors of metabolism, enzyme deficiencies, cancer, as well as CNS diseases [20,23,24,29]. A potential method of protecting living cells and tissues without causing adverse host response includes microencapsulation. The concept of microencapsulation involves enclosing a biologically active material within a polymeric semi-permeable membrane. The membrane allows the diffusion of small particles such as nutrients, oxygen, wastes and therapeutic products, however, impedes

the passage of high molecular weight species such as immune cells, antibodies and leukocytes. This prevents any direct contact between the entrapped cells and its surrounding environment, which may eliminate the requirement for immunosuppressant when used in transplantations [20,24]. Microencapsulation of cells and tissues thus represents an exciting approach for organ replacement.

Several factors need to be considered for the clinical implementation of cell microencapsulation. The primary characteristic for microcapsules used in cell encapsulation is that the membrane material needs to be completely biocompatible and support cell growth without interfering with its normal differential functions. The material must not trigger host immune responses and the membrane must exhibit sufficient mechanical strength and stability for both short term and long term cell transplantation [13,18,90]. Alginate is the most suitable membrane biopolymer for cell encapsulation with regards to biocompatibility, however it lacks suitable mechanical stability [24]. To increase mechanical strength, alginate microcapsules are commonly coated with an additional layer of poly-l-lysine and dilute alginate to form alginate-poly-l-lysine-alginate microcapsules (APA). Although this membrane is widely studied for the encapsulation of biologically active materials, previous research has shown the membrane to induce necrosis of encapsulated cells and fibrotic tissue growth around the membrane surface is observed when transplanted directly in rat models [6,21,38]. As a result, improvements to this membrane as well as the design of new microcapsule membranes continue to be studied in order to instigate the technology of microencapsulation.

This study investigates the potential of a microcapsule incorporating alginate capsules coated with chitosan, PLL and PEG to verify whether it can lead to an improved microcapsule membrane material to be used for cell encapsulation.

4.3 Material and Methods:

Chemicals:

Sodium Alginate (viscosity 2%), Polyethylene glycol (PEG) (MW 10000), MTT (Thiazolyl blue), Poly-l-lysine hydrobromide (MW 27,400) and Sodium citrate were purchased from Sigma Chemicals (St. Louis MO). Chitosan 10 was obtained from Wako Chemicals, Japan.

Microcapsule Preparation:

To prepare alginate microcapsules, 50mL of a 1.5% low viscosity sodium alginate solution in deionized H_2O was sterile filtered through a 0.22 μm filter. The solution was extruded through an INOTECH microencapsulator using a 60 mL syringe and 300 μm nozzle. The gelation process took place in a 0.1M $CaCl_2$ solution for 10 minutes. The novel microcapsule (ACPPA) was prepared by coating the alginate capsules with a 0.5% chitosan solution dissolved in dilute acetic acid at a pH of 5.2 for 30 minutes. The capsules were washed twice with physiological solution and immersed in a 0.5% solution of PEG dissolved in 0.45% NaCl for 10 minutes. The microcapsules were then washed once and transferred to a solution of 0.05% PLL solution dissolved in 0.45% NaCl for 10 minutes. They were washed twice with physiological solution and finally coated with 0.1% alginate for 5 minutes prior to being washed and stored in physiological solution.

Microcapsule Stability Tests:

The mechanical stability of the ACPPA microcapsule membrane was determined using a rotational stress test. Cell-free ACPPA microcapsules were treated with a 0.05 M sodium citrate solution for 4 minutes to dissolve the alginate core. 200±10 capsules were placed in 25 mL volumetric flasks containing 3 mL of physiological solution. The flasks were rotated in an ENVIRON shaker at a speed of 150 rpm at 37 °C. The number of damaged capsules was observed and counted under a light microscope at various time intervals.

Microcapsule Stability in Fetal Bovine Serum (FBS):

The stability of the microcapsule membrane in a transplantation setting was analyzed using fetal bovine serum as a model for the interior physiological environment. 100±5 cell-free microcapsules were immersed in FBS solution and incubated at 37 °C for 3 days. The integrity of the membranes was observed using light microscope.

Cell lines and Growth conditions:

Cell lines HepG2 and lymphocyte leukemia cells were purchased from ATCC and routinely sub-cultured in MEM (minimum essential eagle media) supplemented with 10% FBS and 1% penicillin-streptomycin obtained from Sigma Aldrich. The cells were grown in 75 cm^2 culture flasks and incubated at 37 °C with an air atmosphere of 5% CO_2 in a Sanyo MCO-18M multi-gas incubator. HepG2 were epithelial hepatocellular carcinoma tissues derived from human organisms. The cells were detached and subcultured every 10 days using Trypsin 0.53 mM/EDTA (purchased from ATCC). Lymphocyte leukemia cells were derived from *mus-musculus* host and its cellular products consist of interleukin. Fresh media was added every 2 days for cell culturing.

Method for Cell Encapsulation:

HepG2 cells were encapsulated in alginate microcapsules using previously established procedures [14,17]. Briefly, HepG2 cells were trypsinized and then centrifuged at 1000 rpm for 10 minutes at 20 °C. The media was decanted and the cells were mixed with 0.5 mL of fresh media and 30 ± 10 mL of sterile filtered 1.5% alginate solution to attain a concentration of $1.5x10^6$ cells/mL. The encapsulation process followed the same procedure as described for alginate microcapsule preparation. The alginate capsules were coated to form ACPPA microcapsules which were stored in complete growth media used for culturing free cells at 37 °C and 5% CO_2. The media was changed once every 2 days. All the procedure was performed under sterile conditions in a Microzone Biological Containment Hood (Microzone Corporation ON, Canada) and all solutions were autoclaved with the exception of chitosan, PEG, PLL and the alginate solution which were 0.22 μm sterile filtered prior to usage.

Method of Testing ACPPA Membrane Cytotoxicity and Metabolic Activity of ACPPA Encapsulated Cells:

The ACPPA membrane cytotoxicity and its suitability for live cell encapsulation was determined using an MTT colorimetric assay. The MTT was also used to detect metabolic activity of cells within the microcapsules. The test was performed following the procedure described by Uludag with some modifications [23]. The MTT assay is based on the ability of mitochondrial dehydrogenase enzymes present in viable cells to convert

MTT into insoluble, purple formazan crystals. The amount of formazan formed can be quantified and used to detect the level of cellular activity [91]. Approximately 30±2 capsules were incubated with 100 μL of media and 25 μL of an MTT solution (1% MTT in PBS) for 24 hours in 96 well plates. The media and MTT solution were removed from the wells and the microcapsules were washed once with physiological solution. The formazan crystals formed by the conversion of MTT was dissolved in 100 μL of DMSO. After 30 minutes of incubation, the absorbance was measured using a multiwell spectrophotometer at a wavelength of 570 nm.

Method for Testing Immunogenicity of Cells:

To investigate the ability of the novel membrane to provide cell required immunoprotection, approximately 200±5 ACPPA microcapsules containing HepG2 cells were grown in 1mL of media consisting of $9X10^4 \pm 500$ cells/mL of lymphocytes in 24 well plates. Samples were withdrawn at every 48 hours for a period of 1 week. The volume in each well was kept constant by adding fresh media after taking the sample. The viability of the HepG2 was determined using an MTT assay and the viability of lymphocytes was assessed using a trypan blue dye test. The microcapsule membrane was analyzed microscopically using a light microscope.

Comparative study between ACPPA and APA membranes:

The properties of the novel ACPPA membrane were compared with the conventionally studied APA microcapsules. APA membranes were prepared by immersing alginate capsules in a 0.05% poly-l-lysine solution dissolved in 0.45% NaCl for 10 minutes and then recoating with a layer of 0.1% alginate for 5 minutes after washing twice with physiological solution. HepG2 cells were immobilized within the microcapsule and cell metabolic activity, immunogenicity and membrane stability were tested using the same procedure as described above for the ACPPA capsules.

4.4 Results:

ACPPA Microcapsules:

The biomatrix used for developing microcapsules are of primary importance for addressing the complex problems associated with cell encapsulation for therapy. They should provide mild conditions for live cell encapsulation, be non toxic to the cell and host, be biocompatible, have sufficient membrane permeability to allow passage of both cell nutrients and cell products but be impermeable to antibody-size molecules. The microcapsule membrane must also have the ability to sustain the exterior environment that it will be exposed to. This study investigated the potential of a novel ACPPA microcapsule incorporating alginate capsules coated with chitosan, PLL and PEG. The membrane was designed based on the known fact that alginate can form strong complexes with poly-cations including chitosan, polypeptides such as PLL and synthetic polymers such as PEG [18,24,53,83,90]. To prepare the microcapsule membrane, alginate beads were coated with a layer of 0.5% chitosan followed by a 0.1% PEG solution and a 0.05% solution of PLL. A final layer of 0.1% alginate was added to remove any positive charge residues. Results show that using this combination of biomaterials, a novel ACPPA microcapsules of approximately 450±30μm can be prepared (Fig. 1). Light microscopic analysis reveals the membrane to retain a uniform, spherical shape with a smooth surface. The novel ACPPA membrane was tested for its stability in Fetal Bovine Serum (FBS). The FBS was used for as a model condition for the interior physiological environment that the capsules may be exposed to when used for cell transplantation. Results show that microcapsules remained stable in FBS for over 48 hours and exposure to FBS did not lead to any changes in the capsule integrity or shape.

Rotational stress test:

The membrane strength of the ACPPA microcapsules was tested. This was performed by treating ACPPA microcapsules with citrate and then subjecting the citrate treated microcapsules to mechanical rotational stress in a shaker flask at 37 ^{o}C at a rotational speed of 150 rpm. Upon citrate treatment, the ACPPA microcapsules remained intact and sustained its uniform, spherical shape and size. When subjected to the mechanical stress of rotation, however, 19.8 ±2% of the ACPPA membranes were found to have ruptured within 2 hours. After 10 hours of rotational test exposure, a total of 39 ±1% ACPPA microcapsules had ruptured (Fig. 2).

Encapsulated HepG2 viability:

To assess whether the ACPPA membrane is suitable for cell encapsulation and whether it can sustain cellular growth, HepG2 cells were encapsulated in ACPPA microcapsules as shown in figure 3. Metabolic activity of the cells within the membrane was tested by an MTT assay using 30±2 microcapsules containing live HepG2 cells following the procedure described by Uludag [23] The absorbance was correlated with a calibration plot of cell number verses formazan absorbance to attain a viability measurement of the cells within the capsules. Results are shown in figure 4. The results reveal that cells remained viable indicating that the membrane does not exert any toxicity to the cells and can support proliferation. During the entire study period of 48 hours, it is apparent from the graph that the cell viability within the capsules increases with time. The cell number increased from $5.86x10^5 \pm 6.5x10^4$ after 5 hours of incubation to $1.05x10^6 \pm 1.71\ x10^5$ after 2 days.

Encapsulated HepG2 cells with lymphocytes:

In order to test if the membrane can provide immunogenic protection, HepG2 were encapsulated (Fig 5) and grown in media containing lymphocytes that produce interleukin [92]. To perform the test, 30±2 microcapsules were incubated in the presence and absence of lymphocytes in 100 μL of media and 25 μL of MTT solution. Results (Fig 6) show similar viability between encapsulated HepG2 cells grown in the presence and absence of lymphocytes for the first 3 day period. The study revealed that free HepG2 cells grown in the presence of lymphocytes resulted in complete loss of viability of the lymphocyte cells. ACPPA membrane encapsulated HepG2 cells shows a reduction in metabolic activity only after the third day of incubation compared to free cells. Throughout the 7 day analysis, HepG2 cell number in ACPPA membranes reduced from $9.28x10^5 \pm 7.1\ x10^4$ to $6.73\ x10^5 \pm 5.4\ x10^4$.

Experiments were also designed to evaluate the viability of lymphocytes in the presence of encapsulated HepG2 cells. Results are presented in figure 7, which compares the loss of viability with respect to time for free lymphocyte cells and for lymphocytes grown with encapsulated HepG2 cells. The results present the percent of non-viable

lymphocyte cells after 1,3,5 and 7 days of incubation. The fraction of dead lymphocyte cells grown with ACPPA encapsulated HepG2 cells increased from 4±3% after the first day of incubation to 15±1.8% and 22±1.3% for the third and fifth day respectively. After the seventh day of incubation, a total loss of viability of 24 ±0.02% was found due to the presence of encapsulated HepG2 cells in the growth media.

Comparison between ACPPA with APA microcapsules:

The potentials and advantages of the novel ACPPA membrane was analyzed by comparing its performance with the widely used APA microcapsules. APA microcapsules having a diameter of 430±30 μm were prepared. The mechanical stability of ACPPA microcapsules compared to APA microcapsules was tested using the rotational stress test by challenging these capsules to a 150 rpm rotational test in an environ shaker at 37 ^{o}C. Results, presented in figure 8, show both membranes to have similar mechanical stability trends. After 1 hour, 4.4±7.6 % ACPPA compared to 3.3±5.7% of APA had ruptured. Within 6 hours, 36±2.9% of ACPPA and 31±2.9% of APA were found to have broken. Within the 10 hour study, a total of 39 ±1% ACPPA microcapsules in comparison to 34 ±5% of APA microcapsules were found ruptured from the mechanical impact of shaking.

To compare the biocompatibility of the membranes, HepG2 cells were encapsulated in both ACPPA and in APA microcapsule membranes. The MTT assay was performed to compare the cell viability efficacy of these two membranes. Results are presented in figure 9. As apparent from the graph, both microcapsules membranes support cell proliferation. After 48 hours of incubation, the HepG2 cell count for the APA microcapsule membrane was $1.19X10^{6} \pm 1.53\ x10^{5}$ compared to $1.05x10^{6} \pm 1.71\ x10^{5}$ for the ACPPA microcapsules.

The immunogenicity of the membranes was tested by growing immobilized HepG2 cells in media containing lymphocytes. Figure 10 displays results for the viability of APA encapsulated HepG2 cells. Similar to the ACPPA membrane, a reduction in metabolic activity of the HepG2 cells is apparent after the third day of incubation. During the 7 day analysis, the quantity of HepG2 cells in APA membranes reduced from $9.6x10^{5} \pm 1.17\ x10^{5}$ to $6.59\ x10^{5} \pm 1.44\ x10^{5}$, while for ACPPA membranes it reduced from 9.29

$x10^5$ ±7.1 $x10^4$ to 6.73 $x10^5$±5.4 $x10^4$. The percent of non viable lymphocyte cells grown with encapsulated HepG2 cells in the APA membrane increased from 14.5±4.8 after one day of incubation to 29±1.8% on the third day (fig 11). At the same time interval, ACPPA membranes had a percent cell death of 3.5±3.0% which increased to 14.5±1.8% after the 3 day incubation. Results, presented in figure 11, show that the lymphocyte viability of APA encapsulated cells resulted in the greatest loss of viability at 34 ±4% in comparison to the ACPPA membrane which had a total loss of 24 ±2%.

4.5 Discussion:

The APA microcapsules are the most widely studied membrane for the entrapment of living cells in the application of transplantation [90]. Although the membranes exhibit ideal properties for cell encapsulation including mechanical stability and mild preparation procedures, in vivo studies have revealed the membrane to have insufficient biocompatibility. APA membranes can potentially cause an inflammatory response and studies in transplanting APA microcapsules in rodents have revealed fibrotic overgrowth leading to cell necrosis [12,15,21,83]. As a result, the development of a biocompatible and immunosuppressive material for the encapsulation of mammalian cells to be applied in either xenogenic or allogenic transplantation remains to be developed. Many authors have investigated potential alternatives and improvements to the membrane which include the use of chitosan membranes and the incorporation of PEG to alginate.

Chitosan is a biocompatible, biodegradable and non-toxic polymer that has been shown to be compatible with soft tissues [16,75]. Although it is normally insoluble in aqueous solutions having a pH above 7, when exposed to dilute acids (pH<6), the free amino groups become protonated allowing the molecule to dissolve. The protonated amino groups have a high charged density in solution which permit chitosan to form ionic complexes with the carboxyl group of the alginate molecule [12]. It is predicted that chitosan can form a stronger complex with alginate than PLL because the distance between the charges is approximately the same for the two molecules [55,75]. Chitosan has been explored for the microencapsulation of drugs, however, very few studies on the use of chitosan for mammalian cell entrapment have been reported [53].

It is essential for the microcapsule surface to be free of charges in order to prevent foreign body reactions in the case of transplantation [24]. PLL binds to alginate via electrostatic interaction between the positively charged PLL and negatively charged alginate. This results in the interfacial adsorption of the PLL which forms a thin polymeric layer on the surface of the alginate microcapsules [18]. Incomplete coating or the use of low quality alginate may result in cationic charges remaining on the surface of the APA microcapsules. The addition of a PEG coating has been studied as an attempt to reduce surface charges [83]. PEG is a hydrophilic, non-ionic compound that is relatively inert in body fluids [82]. Previous studies have revealed PEG to improve biocompatibility by reducing surface protein adhesion and exhibiting higher resistance to fibrous overgrowth in vivo [53,61,90]. The exact mechanism of how PEG interacts with chitosan and PLL remains to be studied. It is hypothesized that the PEG polymer may form an interpenetrating network near the microcapsule membrane surface. Other studies reveal that PEG incorporates itself directly into the alginate matrix [51,53]. Although PEG displays many advantages, preparing a PEG incorporated membrane that preserves the immunogenic quality, mechanical strength and mild processing procedures as the APA remains a challenge [83]

Results of the present study demonstrate the potential application of a novel membrane combining PLL with chitosan, a naturally-derived compound which can form strong bonds with alginate, and PEG, a compound previously shown to improve biocompatibility by reducing surface protein attraction and thrombogenicity of material surfaces [53,83]. Based on the properties of these compounds, the new membrane (ACPPA) may provide mechanically stable microcapsules with greater immunoprotection and biocompatibility with reduced surface charges.

Results revealed mechanical strength of the new membrane to be almost identical to APA membranes as presented in figure 2. When the capsules were subjected to sodium citrate, which dissolves the alginate core, ACPPA membrane remained stable and intact implying a stable interaction between the chitosan, PEG and PLL layer. Figure 11 shows a picture of liquefied ACPPA capsules. Further studies however need to be conducted in order to determine the exact chemical interaction between chitosan, PEG and PLL.

The metabolic activity of encapsulated HepG2 cells in both APA and APPA membranes were monitored for a period of 48 hours to ensure the membrane does not exert any toxicity to the cells. The ACPPA membrane revealed slightly reduced viability in comparison to APA. This may be a result of the longer coating procedures needed for the chitosan layer. Chitosan requires at least 30 minutes of exposure in order to complete crosslinking with the alginate molecules [55]. Despite the harsher conditions required for coating with chitosan, results still express the ability of the membrane to sustain cell proliferation and viability. No cell leakage or capsule breakage was apparent for both membranes.

The most important characteristic of microcapsules to be used for both allogenic and xenogenic transplantation is host immunoprotection and biocompatibility. The microcapsule membrane must be able to sustain cell viability without causing any adverse host immune responses which may not only limit microcapsule cell function but also hinder normal cell activity in the host [24]. ACPPA membranes containing HepG2 cells, grown in the presence of lymphocyte leukemia cells derived from mice, revealed positive cell viability. As apparent from the graph on figure 6, the metabolic activity of HepG2 cells in the ACPPA membranes were less affected by the presence of lymphocytes than HepG2 cells encapsulated in the APA membranes (figure 10). The viability of both lymphocytes as well as the encapsulated liver cells was analyzed. Lymphocytes grown in the presence of free HepG2 cells showed no signs of viability after 1 day of culturing. In comparison to APA, the ACPPA membrane was found to reveal a higher viability of lymphocyte cells than APA membranes as shown on figure 11. These results emphasize the possibility of the novel membrane to provide better immunoprotection than the widely studied APA membranes. However, the observed reduction in lymphocyte cell number may be due to competition for nutrients in the media between the two types of cells. Further investigations would be needed to determine this.

4.6 Conclusion:

This study has demonstrated that alginate-chitosan-PEG-PLL-alginate (ACPPA) microcapsules may be used for the entrapment of live cells for the development of

bioartificial organs. The ACPPA membranes provide mechanically stable and smooth microcapsules that are capable of supporting cell growth and may result in reduced host immune responses when compared to APA membranes. This microcapsule design provides a new direction for live cell encapsulation which can be applied to either xenogenic or allogenic transplantation to be used for human therapy. In order to test its full potentials however, further detailed studies on the biocompatibility of the membrane in-vivo remain to be tested.

4.7 Acknowledgements:

This work was supported by research grants (to Prakash) form Natural Sciences and Engineering Research Council (NSERC) of Canada and Canadian Institute of Health Research (CIHR). NSERC post graduate scholarship to Haque, Chen, and Martoni is acknowledged. Ouyang acknowledges Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT) Post Doctoral Fellowship.

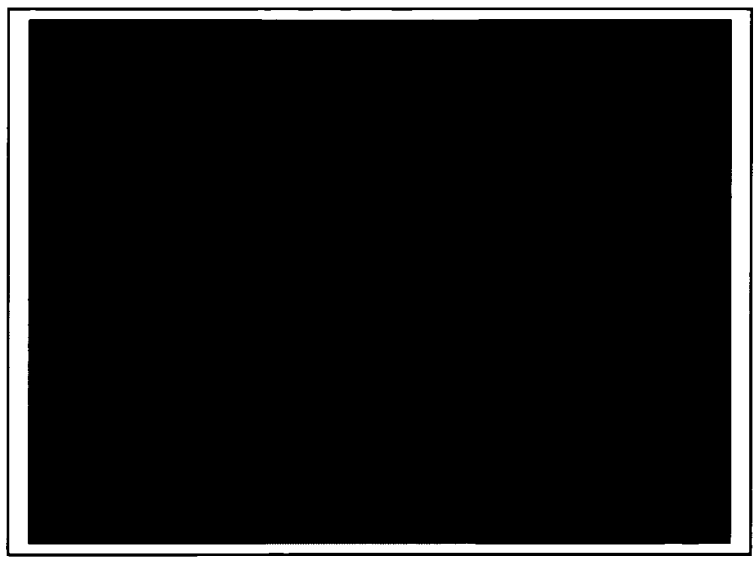

Figure 4.1: Photomicrograph of Alginate-Chitosan-PEG-PLL-Alginate (ACPPA) microcapsules in physiological solution. Capsule size: 450±30 μm. (Magnification: 10X)

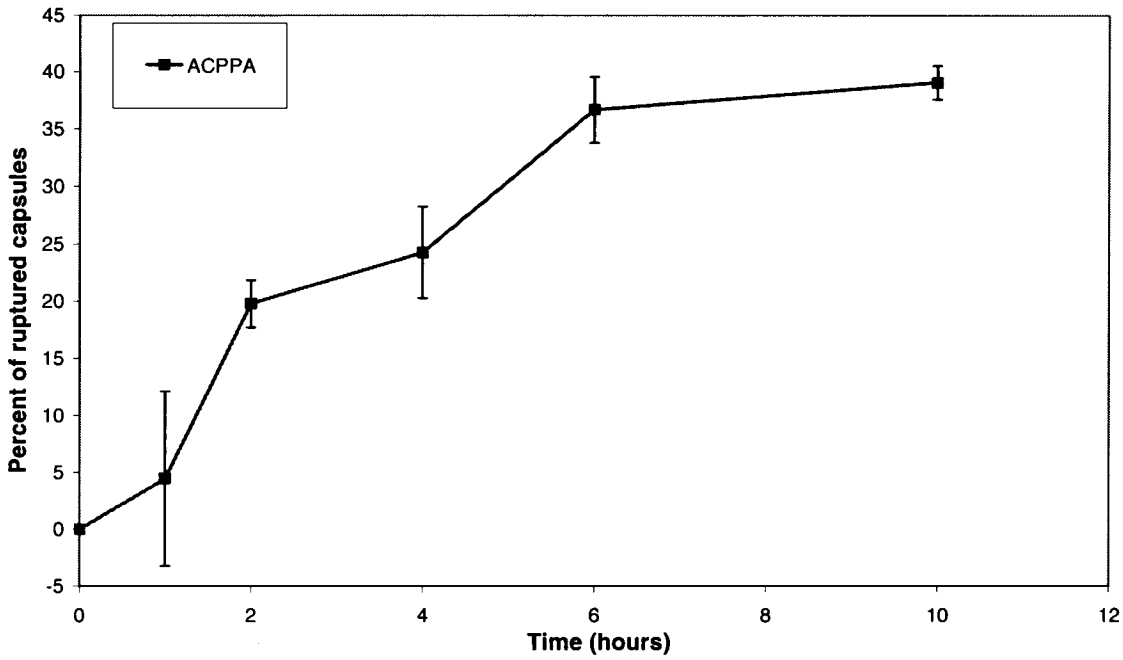

Figure 4.2: The effect of mechanical impact of rotation at 150 rpm and 37°C on Alginate-Chitosan-PEG-PLL-Alginate (ACPPA) microcapsule integrity.

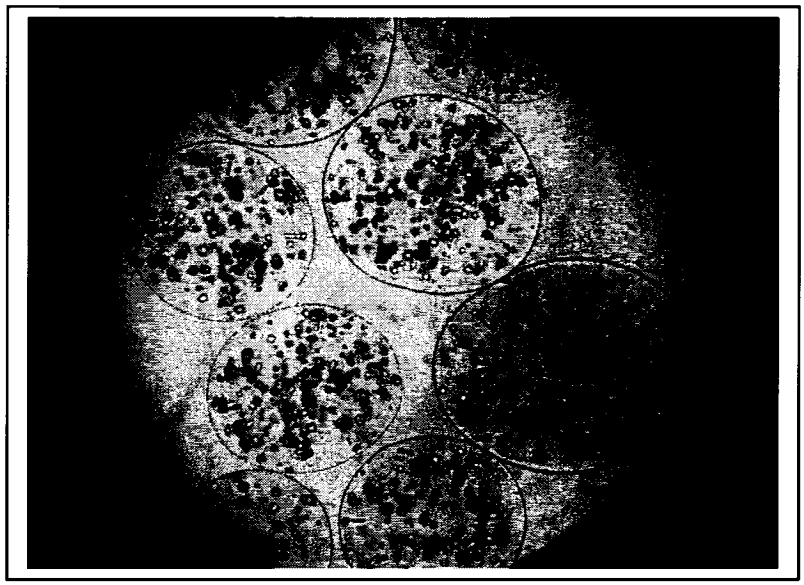

Figure 4.3: Photomicrograph of HepG2 cells encapsulated in Alginate-Chitosan-PEG-PLL-Alginate (ACPPA) microcapsules in MEM media 5 days after coating. Cells were encapsulated at 1.5 $x10^6$ cells/mL. Capsule size: 450±30 μm. (Magnification: 10X)

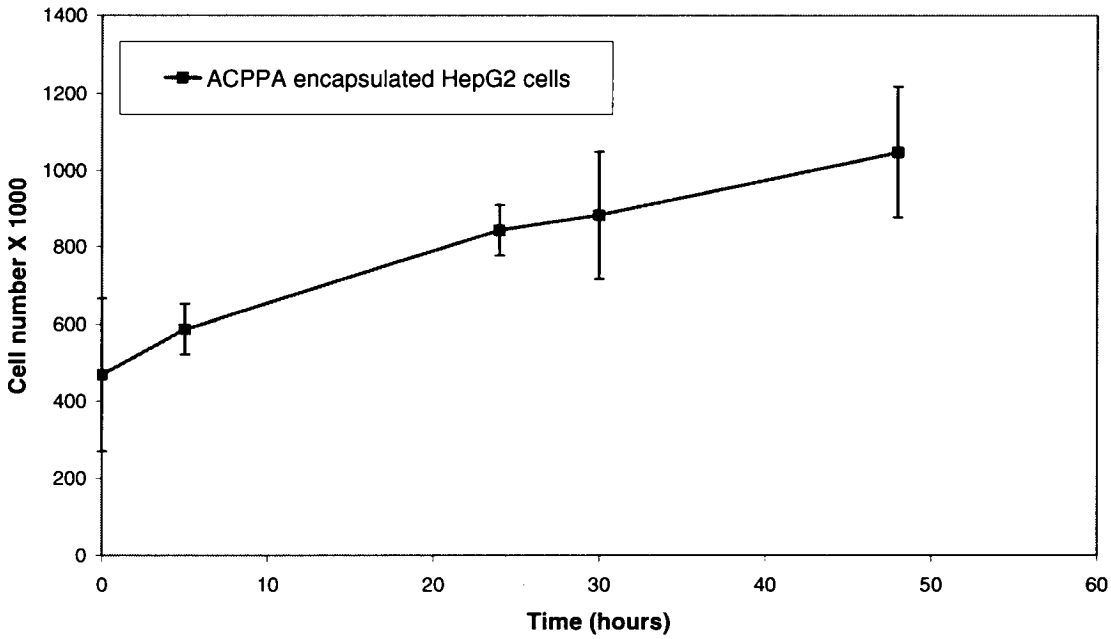

Figure 4.4: Viability of HepG2 cells encapsulated in Alginate-Chitosan-PEG-PLL-Alginate (ACPPA) microcapsules.

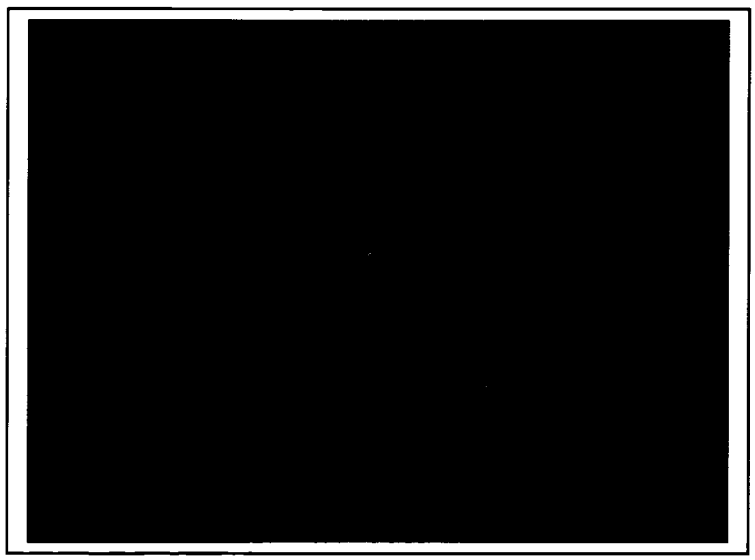

Figure 4.5: Photomicrograph of encapsulated HepG2 cells in Alginate-Chitosan-PEG-PLL-Alginate (ACPPA) microcapsules grown in the presence of lymphocyte leukemia cells for 3 days. Magnification 10X.

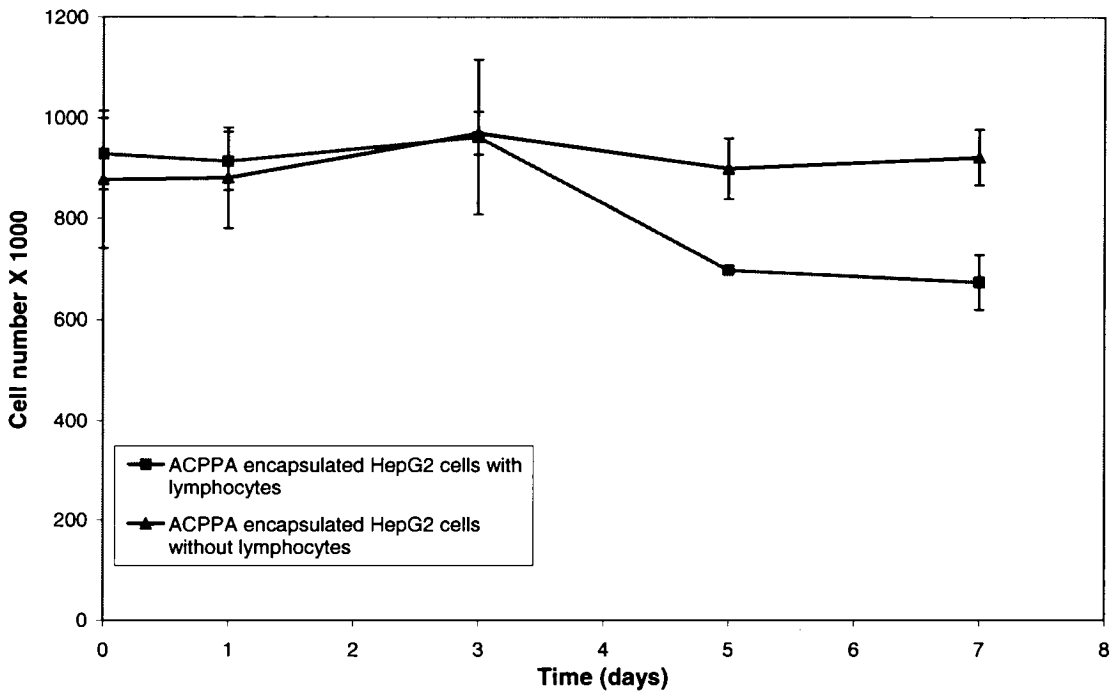

Figure 4.6: Viability of encapsulated HepG2 cells in Alginate-Chitosan-PEG-PLL-Alginate (ACPPA) microcapsules grown in the presence of lymphocyte cells.

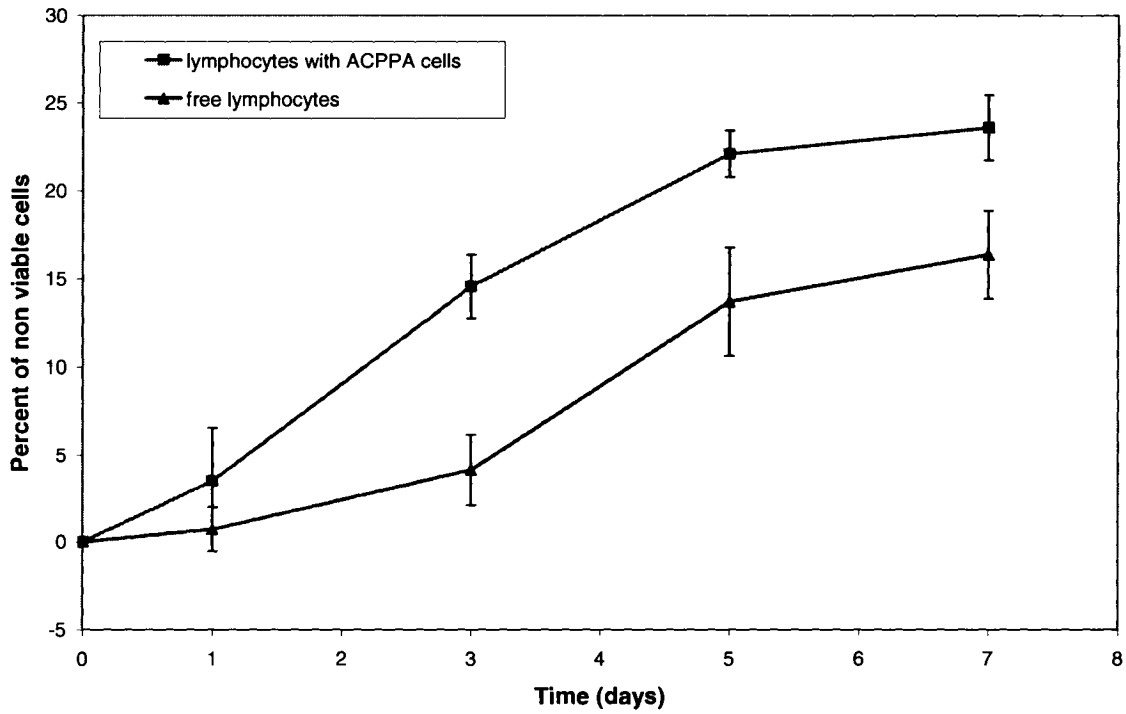

Figure 4.7: Viability measurement of lymphocytes grown with and without the presence of encapsulated HepG2 cells in Alginate-Chitosan-PEG-PLL-Alginate (ACPPA) microcapsules.

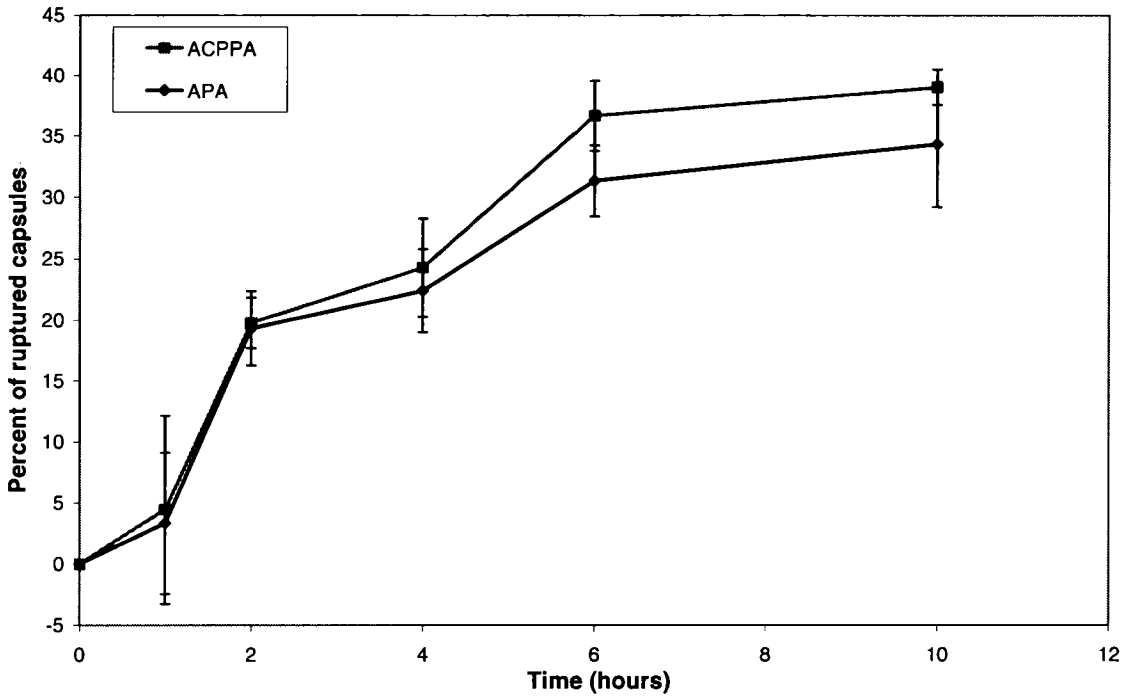

Figure 4.8: Comparison between Alginate-Chitosan-PEG-PLL-Alginate (ACPPA) and Alginate-PLL-Alginate (APA) membranes on the effect of mechanical impact of rotation at 150 rpm and 37°C on microcapsule integrity.

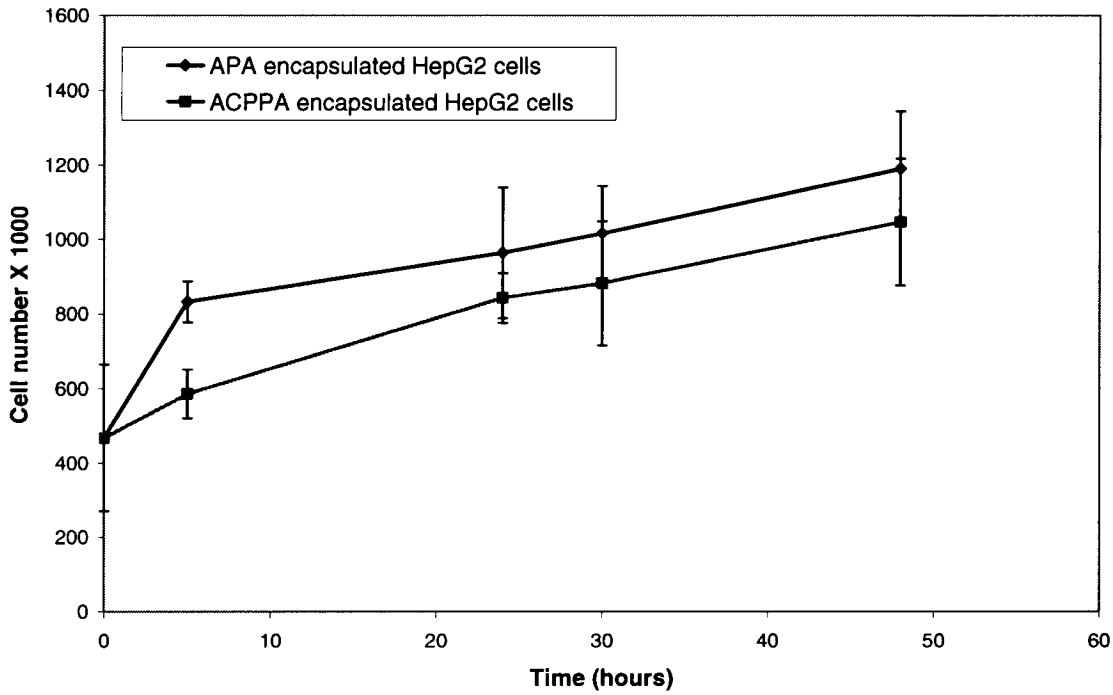

Figure 4.9: Comparative study between the viability of HepG2 cells encapsulated in Alginate-Chitosan-PEG-PLL-Alginate (ACPPA) and Alginate-PLL-Alginate (APA) membranes.

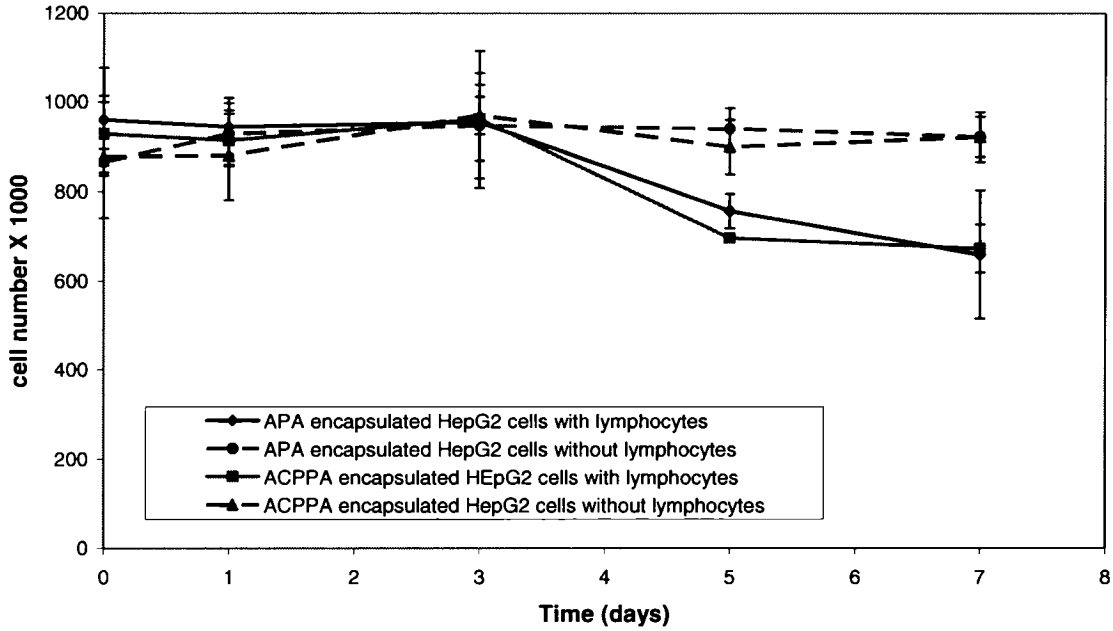

Figure 4.10: Comparative study of viability of HepG2 cells encapsulated in Alginate-Chitosan-PEG-PLL-Alginate (ACPPA) and Alginate-PLL-Alginate (APA) microcapsules grown in the presence/absence of lymphocyte cells.

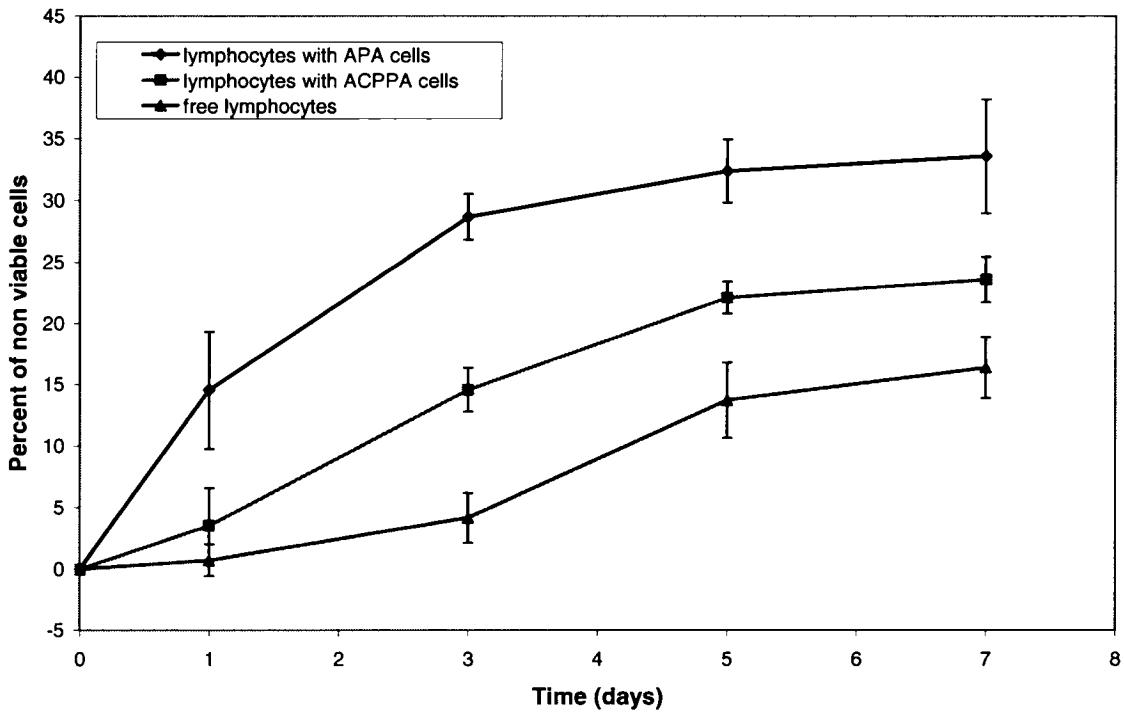

Figure 4.11: Comparative study of lymphocytes in the presence of Alginate-Chitosan-PEG-PLL-Alginate (ACPPA) and Alginate-PLL-Alginate (APA) microcapsules encapsulating HepG2 cells.

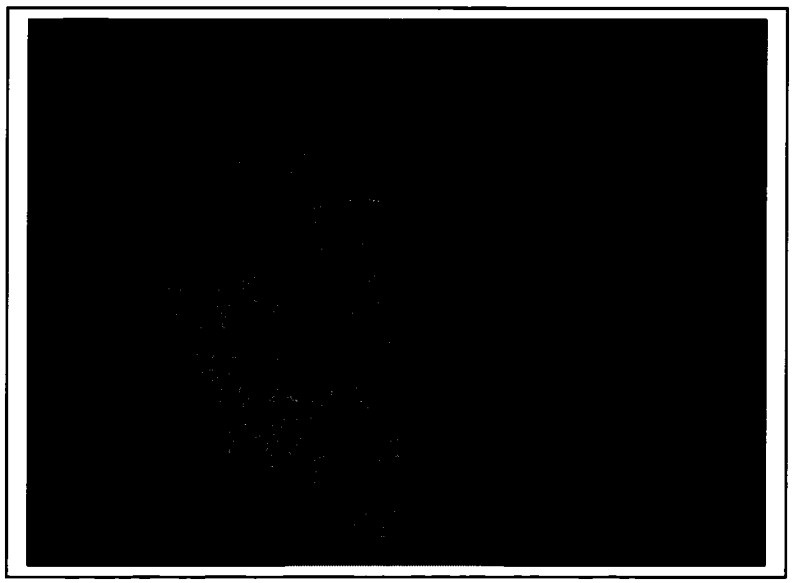

Figure 4.12: Photomicrograph of liquefied Alginate-Chitosan-PEG-PLL-Alginate (ACPPA) microcapsules after citrate treatment. Diameter: 450±30μm. Magnification 10X.

Chapter 5:

Superior Cell Delivery Features of Poly-ethylene glycol Incorporated Alginate, Chitosan and Poly-l-lysine Microcapsules

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Mol. Pharm., **2** (1), 29 -36, 2005. ***Published***

Chapter 5: Superior Cell Delivery Features of Poly-ethylene glycol Incorporated Alginate, Chitosan and Poly-l-lysine Microcapsules

5.1 Abstract:

Microencapsulation is an emerging technology in the development of bioartificial organs for drug, protein, and delivery systems. One of the advancements in establishing an appropriate membrane material for live cell and tissue encapsulation is the incorporation of polyethylene glycol to the widely studied alginate microcapsules. The current study investigates the properties of integrating polyethylene glycol to microcapsules coated with poly-l-lysine and chitosan as well as a novel microcapsule membrane which combines both poly-lysine and chitosan. Results show that microcapsules containing PEG can support cell viability and protein secretion. The addition of PEG to PLL and chitosan coated microcapsules improves the stability of microcapsules when exposed to a hypotonic solution. We also compared the novel microcapsule with other two previously used microcapsules including alginate-chitosan-PEG and alginate-PLL-PEG-alginate. Results show that all three membranes are capable of providing immuno-protection to the cells and have the potential for long term storage at -80 ^{o}C. The novel membrane containing PEG, chitosan and PLL, however, revealed the highest cell viability and mechanical strength when exposed to external rotational force, but it was unable to sustain osmotic pressure. The study revealed the potential of using poly-ethylene glycol incorporated alginate, chitosan and poly-l-lysine microcapsules for encapsulating live cells producing proteins and hormones for therapy.

Keywords: Microencapsulation, alginate, poly-ethylene-glycol, chitosan, transplantation, immuno-isolation, crypreservation.

5.2 Introduction:

Microencapsulation of live cells and tissues within a protective membrane is being widely studied as a method of eliminating the problems associated with immune rejection during allogenic and xenogenic transplantation [14,22,31,32,34,38]. The microcapsules provide a large surface area to volume relationship which allows the rapid diffusion and

passage of oxygen, nutrients, metabolites and waste products. However, the polymeric semipermeable membrane blocks the exchange of leukocytes, antibodies and tryptic enzymes across the microcapsules which prevent any direct contact between the entrapped cells and its surrounding environment. The concept of microencapsulation may therefore eliminate the requirement for immuno-suppressants when used in transplantations [18,20,24,31,34,38].

Several polymers have been studied for encapsulating biologically active materials including synthetic and naturally occurring polymers. The preparation of synthetic polymers such as poly (HEMA-MMA) copolymer and acrylamide can require exposure to organic solvents, toxic monomers, high temperatures and irradiation which may be undesirable for some applications [53]. On the contrary, naturally occurring polymers such as alginate provide suitable biocompatibility and mild preparation procedures. Calcium alginate beads have been widely researched and utilised for the encapsulation of animal tissues. The mechanical strength of alginate microcapsules is often increased by the addition of a poly-l-lysine (PLL) coating to form alginate-PLL-alginate microcapsules (APA) [8,11,18,65]. Although this membrane has been shown to support tissue growth, previous research has demonstrated that it may induce necrosis of encapsulated cells and fibrotic tissue growth around the membrane surface is observed when transplanted directly in rat models [6,21,38]. Chitosan, a naturally occurring polysaccharide is an alternative to the conventionally studied APA microcapsules, and has been studied for the encapsulation of drugs [56,68,75,79]. More recently, the addition of poly-ehtylene-glycol (PEG), a water soluble polymer, has been suggested as a means of increasing biocompatibility of microcapsules [48,51,53,61,82].

Improvements to the APA membrane as well as the design of new microcapsule membranes continue to be studied in order to implement the technology of microencapsulation. This study investigates the feasibility of using PEG integrated into poly-l-lysine and chitosan coated microcapsules for cell encapsulation therapy.

5.3 Material and Methods:

Chemicals:

Sodium Alginate (viscosity 2%), Polyethylene glycol (PEG) (MW 10000), MTT (Thiazolyl blue), Poly-l-lysine hydrobromide (MW 27,400) sodium citrate and Bradford Reagent were purchased from Sigma Chemicals (St. Louis MO). Chitosan 10 was obtained from Wako Chemicals, Japan.

Microcapsule Preparation:

To prepare alginate microcapsules, 50 mL of a 1.5% low viscosity sodium alginate solution in deionized H_2O was sterile filtered through a 0.22 μm filter. The solution was extruded through an INOTECH microencapsulator using a 60 mL syringe and 300 μm nozzle. The gelation process took place in a 0.1M $CaCl_2$ solution for 10 minutes. APA microcapsules were prepared by immersing the alginate capsules in a 0.05% poly-l-lysine solution dissolved in 0.45% NaCl for 10 minutes and then recoating with a layer of 0.1% alginate for 5 minutes after washing twice with physiological solution. PEG was incorporated into APA microcapsules to form alginate-poly-l-lysine-PEG-alginate (APPA) microcapsules by subjecting the microcapsules in a 0.5% solution of PEG dissolved in 0.45% NaCl for 10 minutes after being coated with PLL and washed with physiological solution. After washing, a final layer of 0.1% alginate was added for 5 minutes. Alginate-chitosan (AC) microcapsules were prepared by coating the alginate beads with a 0.5% chitosan solution dissolved in dilute acetic acid at a pH of 5.2 for 30 minutes. The AC microcapsules were then exposed to a solution of PEG to form Alginate-chitosan-PEG (ACP) microcapsules. The novel microcapsule (ACPPA) was prepared by coating the alginate capsules with a 0.5% chitosan solution. The capsules were washed twice with physiological solution and immersed in the 0.5% solution of PEG for 10 minutes. The microcapsules were then washed once and transferred to a solution of 0.05% PLL solution for 10 minutes. After being washed with physiological solution, the microcapsules were finally coated with 0.1% alginate for 5 minutes. All microcapsules stored in physiological solution prior to being used for testing.

Microcapsule Stability Tests:

The mechanical stability of the microcapsules was determined using an osmotic pressure test and a rotational stress test. The osmotic pressure test was performed by

subjecting 100±5 microcapsules which were previously stored in 0.85% saline solution to 2 mL of deionized water in 35x10 mL petri dishes. The effect of osmotic pressure on the microcapsules membrane was observed microscopically and the number of damaged capsules was recorded. To assess the microcapsules ability to sustain the mechanical stress of rotation, cell-free microcapsules were treated with a 0.05 M sodium citrate solution for 4 minutes to dissolve the alginate core. 200±10 capsules were placed in 25 mL volumetric flasks containing 3 mL of physiological solution. The flasks were rotated in an ENVIRON shaker at a speed of 150 rpm at 37 °C. The number of damaged capsules was observed and counted under a light microscope at various time intervals.

Cell lines and Growth conditions:

Cell lines HepG2 and lymphocyte leukemia cells were purchased from ATCC and routinely sub-cultured in MEM (minimum essential eagle media) supplemented with 10% FBS and 1% penicillin-streptomycin obtained from Sigma Aldrich. The cells were grown in 75 cm^2 tissue culture flasks and incubated at 37 °C with an air atmosphere of 5% CO_2 in a Sanyo MCO-18M multi-gas incubator. HepG2 were epithelial hepatocellular carcinoma tissues derived from human organisms. The cells were detached and subcultured every 10 days using Trypsin 0.53mM/EDTA (purchased from ATCC). Lymphocyte leukemia cells were derived from *mus-musculus* host and its cellular products consist of interleukin. Fresh media was added every 2 days for cell culturing.

Method for Cell Encapsulation:

HepG2 cells were encapsulated in alginate microcapsules using previously established procedures [14,17]. Briefly, HepG2 cells were trypsinized and then centrifuged at 1000 rpm for 10 minutes at 20 °C. The media was decanted and the cells were mixed with 0.5 mL of fresh media and 30 ± 10 mL of sterile filtered 1.5% alginate solution to attain a concentration of $1.5x10^6$ cells/mL. The encapsulation process followed the same procedure as described for alginate microcapsule preparation. The alginate capsules were coated to form APPA, ACP and ACPPA microcapsules which were stored in complete growth media used for culturing free cells at 37 °C and 5% CO_2. The media was changed once every 2 days. The entire procedure was performed under sterile conditions in a

Microzone Biological Containment Hood (Microzone Corporation ON, Canada) and all solutions were autoclaved with the exception of chitosan, PEG, PLL and the alginate solution which were 0.22 μm sterile filtered prior to usage.

Method of Testing Membrane Cytotoxicity and Metabolic Activity of Encapsulated Cells:

The microcapsule membranes were tested for cytotoxicity and suitability for live cell encapsulation using an MTT colorimetric assay. The MTT was also used to detect metabolic activity of cells within the microcapsules. Previously established procedures as described by Uludag [23] was used with some modifications. The MTT assay is based on the ability of mitochondrial dehydrogenase enzymes present in viable cells to convert MTT into insoluble, purple formazan crystals. The amount of formazan formed can be quantified and used to detect the level of cellular activity [91]. Approximately 30±2 capsules were incubated with 100 μL of media and 25 μL of an MTT solution (1% MTT in PBS) for 24 hours in 96 well plates. The media and MTT solution were removed from the wells and the microcapsules were washed once with physiological solution. The formazan crystals formed by the conversion of MTT was dissolved in 100 μL of DMSO. After 30 minutes of incubation, the absorbance was measured using a multiwell spectrophotometer at a wavelength of 570 nm. The cell number was obtained using a calibration curve correlating cell quantity with absorbance.

Protein Secreted by Encapsulated HepG2 cells:

The ability of the microcapsule membrane to permit the outflow of necessary proteins produced by liver cells was determined by monitoring albumin secretion using a Bradford Assay. The Bradford Assay is based on the ability of Brilliant Blue G dye to form complexes with proteins which causes a shift in colour depending on the quantity of protein present. The amount of protein can then be quantified using an absorbance reader. Approximately 100±5 microcapsules encapsulating HepG2 cells were washed 3 times with physiological solution to remove any traces of media remaining on the membrane surfaces. The capsules were placed in 96 well plates and 100 μL of serum-free media was added. The serum-free media contained no proteins in order to ensure that any protein

detected in the media were solely from the entrapped cells. The microcapsules were incubated for 48 hours prior to being tested. The quantity of proteins secreted by the cells was measured by taking 15 μL of the media in which the microcapsules were stored and adding 0.3 mL of Bradford reagent into 96 well plates. The absorbance was measured at 595 nm after 20 minutes of incubation. The protein concentration was attained using a calibration between albumin concentration and absorbance.

Method for Testing Immunogenicity of Cells:

To investigate the ability of the PEG integrated membranes to provide cell required immuno-protection, approximately 200±5 APPA, ACP and ACPPA microcapsules containing HepG2 cells were grown in 1 mL of media consisting of $9X10^4$ ± 500 cells/mL of lymphocytes in 24 well plates. Samples were withdrawn at every 48 hours for a period of 7 days. The volume in each well was kept constant by adding fresh media after taking the sample. The viability of the HepG2 cells was determined using an MTT assay. The microcapsule membrane was analyzed microscopically using a light microscope.

Cryopreservation Studies:

The possibility of long terms storage of encapsulated HepG2 cells in the membranes was tested. Approximately 150±10 capsules were washed with media and placed in a 2 mL cryovial containing 1 mL of complete growth media supplemented with 0.10 mL DMSO. The vials were placed at -20 ^{o}C for 1 hour prior to being stored at -80 ^{o}C. After 30 days of storing, the microcapsules were thawed by immersing the vials in a 37 ^{o}C water bath and re-cultured in media. The MTT assay was used to obtain the quantity of viable cells remaining.

5.4 Results:

Morphological Studies:

APPA, ACP and ACPPA microcapsules were prepared. The capsule diameter ranged from 450 ± 30 μm for each membrane. Optical microscopy of encapsulated

HepG2 cells revealed the hepatocytes to grow in clumps distributed within the membrane (figure 1).

Mechanical Stability test:

The microcapsule membranes ability to maintain integrity after being subjected to the mechanical impact of rotation was tested using a rotational stress test on sham capsules. The microcapsules were treated with citrate to dissolve the alginate core and the membrane integrity was monitored for all five membranes. Results are shown on figure 2. The AC and ACP membranes were unable to sustain the mechanical impact. Microscopic analysis revealed that the citrate treatment itself caused the membrane to weaken and lose its spherical shape. After 2 hours of rotation at 150 rpm, a total of 85 ± 1 % and 40 ± 2 % had ruptured for AC and ACP microcapsules respectively. After 10 hours of treatment a total of 100 % and 96 ± 1% of the AC and ACP membranes had ruptured. The addition of PEG to the chitosan coating therefore, seemed to slightly improve the membrane stability. The addition of PEG to the poly-l-lysine coating revealed contrary results. Within 2 hours, 34 ± 2 % of capsules were broken. At the completion of the 10 hour study, 48 ± 7 % were found broken. The membrane integrity was strongest for the APA and ACPPA membranes. Both membranes retained their spherical and uniform shape after citrate treatment. After 2 hours of exposure to rotational impact, 19 ± 3 % of APA microcapsules had ruptured while a total of 20 ± 2 % of the ACPPA membranes had broken. Within 10 hours, the number of broken capsules of APA and ACPPA membranes was 34 ± 4 % and 39 ± 1 % respectively.

Results for the membrane integrity after exposure to osmotic pressure were significantly different from the mechanical rotation test. The addition of PEG to PLL and chitosan coatings greatly improved the stability of microcapsules in hypotonic solutions. Results are presented in figure 3. Within the 60 minute analysis, it was observed that over 32 ± 0.3 % of chitosan coated microcapsules were broken. APA microcapsules displayed a higher stability with a total of 26 ± 1 % of broken capsules. In both cases, the addition of PEG greatly enhanced mechanical stability. PEG incorporated in the chitosan membrane resulted in all of the capsules to remain intact. Within 60 minutes, a total of 11 ± 0.7 % of APPA microcapsules were ruptured, which is a reduction of over 50 % of

broken capsules observed for the poly-l-lysine membrane without PEG. The ACPPA membranes were unable to sustain prolonged osmotic pressure. Within 5 minutes of exposure, 5 ± 0.3 % of microcapsules had ruptured. At the completion of the 60 minute analysis 95 ± 0.0001 % had broken.

Metabolic Activity and Cell Viability of encapsulated HepG2 cells:

To test if the PEG incorporated membranes can support liver cell proliferation and activity, HepG2 cells were encapsulated in ACP, APPA and ACPPA membranes and their cell viability was monitored for a period of 25 days using an MTT assay. Figure 4 represents the cell number for 30±2 capsules at various time intervals. All microcapsules revealed an increase in cell number and thereby metabolic activity within the first 5 days after encapsulating. Proceeding the 5 days, a decrease in cell number is observed. After 1 week of encapsulating, the ACPPA and APPA membranes once again display an increase in HepG2 activity resulting in a maximum cell number of $1.26x10^6 \pm 1.27x10^5$ and $1.13x10^6 \pm 5.65x10^4$ respectively at 9 days. After the 9th day of encapsulating, a gradual decrease in cell activity is observed for the remainder 25 day study. The ACP membrane continue to result in a decrease in cell number and HepG2 activity until the 11th day after encapsulating where a slight increase in cell number is observed. A maximum cell number of $1.11x106 \pm 9.16x10^4$ is attained after the fourth day of encapsulation. All of the membranes, however, express viability for the complete 25 days studied.

Protein Secretion by Cells:

The amount of protein produced and secreted by the encapsulated cells to the surrounding media was tested using the Bradford Assay. Results are presented in figure 5, which presents the protein secreted by 100±5 microcapsules at various time intervals. No significant difference is observed for the microcapsules studied. A peak in protein secretion is apparent between the 5th and 11th day after which a decrease is observed implying reduced functionality of the HepG2 cells. A maximum concentration of 1.30 ± 0.006 mg/mL is attained for the APPA microcapsules. The protein secreted by the ACP encapsulated cells was slightly lower at 1.28 ±0.05 mg/mL. HepG2 cells encapsulated in the ACPPA membranes showed a different pattern of protein expression. Although the

total concentration of protein secreted was slightly lower, the quantity remained consistent for a longer period of time ranging from the 5th to 17th day. A maximum protein concentration of 1.22 ± 0.04 mg/mL was attained.

Immunogenic properties of membranes:

The ability of the membranes to provide immunogenic protection to the encapsulated cells was tested by growing HepG2 microcapsules in media containing lymphocytes derived from mouse origin. Results are presented in figure 6. The APPA and ACPPA show similar behaviour. No change between the cells grown with lymphocytes and those grown without is apparent within the first 3 days. Following the third day a slight decrease in HepG2 activity is noted. There were no significant changes in the cell count for encapsulated HepG2 cells grown without lymphocytes. Throughout the 7 day analysis, the cell count for APPA encapsulated HepG2 cells showed a decrease in cell number from $7.51x10^5 \pm 8.18x10^4$ to $6.01x10^5 \pm 6.50x10^4$. ACPPA encapsulated HepG2 cells decreased from $9.29x10^5 \pm 7.13x10^4$ to $6.73x10^5 \pm 5.30x10^4$. The ACP membranes revealed different results. Encapsulated HepG2 cells in ACP membranes grown in the presence of lymphocytes showed no significant changes in cell viability.

Cryopreservation Studies:

The possibility of the membranes to be used for long term storage of liver cells was analyzed at –80 °C. The viability of the cells was only slightly reduced for the APPA, ACP and ACPPA membranes. All of the membranes reveal positive metabolic activity after the freeze-thaw procedure (fig. 7)

5.5 Discussion:

APA microcapsules are the most commonly studied membrane for live cell encapsulation. They provide sufficient immunogenicity and support cell viability. However, due to the problems associated with direct transplantation resulting in cell necrosis, an alternate membrane continues to be studied [15.21.83].

Results from the current study investigated the potential of adding PEG to APA and AC membranes. The osmosis test showed the addition of PEG can greatly improve the

integrity of both the APA and the AC microcapsules. These results are counter to expectations since PEG tends to increase the hydration and swelling characteristics of microcapsules due to hydrophilic segments in PEG which interacts with water. As a result, PEG generally would lead to greater swelling of the microcapsules and hence eventual rupture [48]. The observed increased stability may be a result of the interpenetrating network formed by PEG near the microcapsules membrane surface. It is also suggested that PEG incorporates directly into the alginate matrix which may be a possible reason for the improvement in integrity observed for microcapsules exposed to hypotonic solutions [51,53]. The novel membrane, ACPPA, however, was unable to sustain osmotic pressure. Although not investigated in the current study, this may be due to the interaction between the PLL and chitosan. Incorporating PLL to chitosan which is generally hydrophobic increases the hydrophilicity of the polymer [93]. The osmotic pressure causes rapid swelling of the membrane resulting in rupture.

The ACPPA membrane, in terms of strength, was similar to the APA in the rotational stress test. The addition of PEG to the chitosan membrane slightly improved the strength of the AC membrane however, reduced the strength of the APA membrane. The integration of PEG may therefore lead to improvements of the membrane to sustain exterior physiological changes however, does not improve mechanical strength of the microcapsules. The molecular weight and concentration of chitosan affects the mechanical properties of chitosan microcapsules [48,79,93]. Therefore, varying the concentration of chitosan used to coat the microcapsules may increase its strength.

The ACPPA membrane revealed the highest cell viability as apparent from the MTT study. The behaviour of APPA and ACPPA were similar, while that of ACP differed. The slight fluctuations in cell viability may be a result of mass transfer either of nutrients or the MTT itself. Since hepatocytes are anchorage dependent cells, the clusters of cells within the microcapsules can cause a delay or decrease in the rate of diffusion [23,34]. All of the microcapsules, however, retained metabolic activity and viability of cells for a prolonged period of time.

The three membranes allowed the outflow of secreted proteins from the cells to its surroundings. Despite the differences observed between the membranes in cell viability the amount of protein secreted did not differ significantly. The reason for this observation

is not evident from the current study. The novel membrane ACPPA resulted in the least amount of protein secretion; however, the duration was longer which may be advantageous for cell transplantation. The reduction may be due to slower diffusion as a result of the additional layers [34,47]. This however will require further investigations. On the other hand, the use of chitosan and PEG may lead to improved permeability, better cell attachment, increased strength and reinforced immuno-protection in comparison to the widely investigated APA microcapsules. This, in turn, would lead to a better microcapsule for cell encapsulation techonology.

It has been previously shown in literature that PEG can reduce protein adhesion and cell adhesion. As a result, the addition of PEG may minimize the interaction between the surrounding lymphocytes with the microcapsules leading to better immuno-protection [53,61]. Results show that the chitosan containing membrane provides the greatest protection against foreign cells. The hydrophobic nature of chitosan may be what causes the HepG2 cells within the microcapsules to be unaffected by the surrounding lymphocytes [48,53].

5.6 Conclusion:

Results from the current study reveal that the incorporation of PEG may be a potential improvement to microcapsule membranes for use in cell encapsulation. PEG incorporated into alginate microcapsules coated with either or both PLL and chitosan provides a new direction for live cell encapsulation which can be used for therapy. The novel membrane, (ACPPA), in comparison to the ACP and APPA microcapsule resulted in the highest cell viability. In order to test its full potentials, however, further detailed studies on the biocompatibility and cell delivery features of the membrane in-vivo remain to be tested.

5.7 Acknowledgements:

This work was supported by research grants (to Prakash) form the Canadian Institute of Health Research (CIHR). NSERC post graduate scholarships to Haque, Chen, and Martoni are acknowledged. Ouyang acknowledges Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT) Post Doctoral Fellowship.

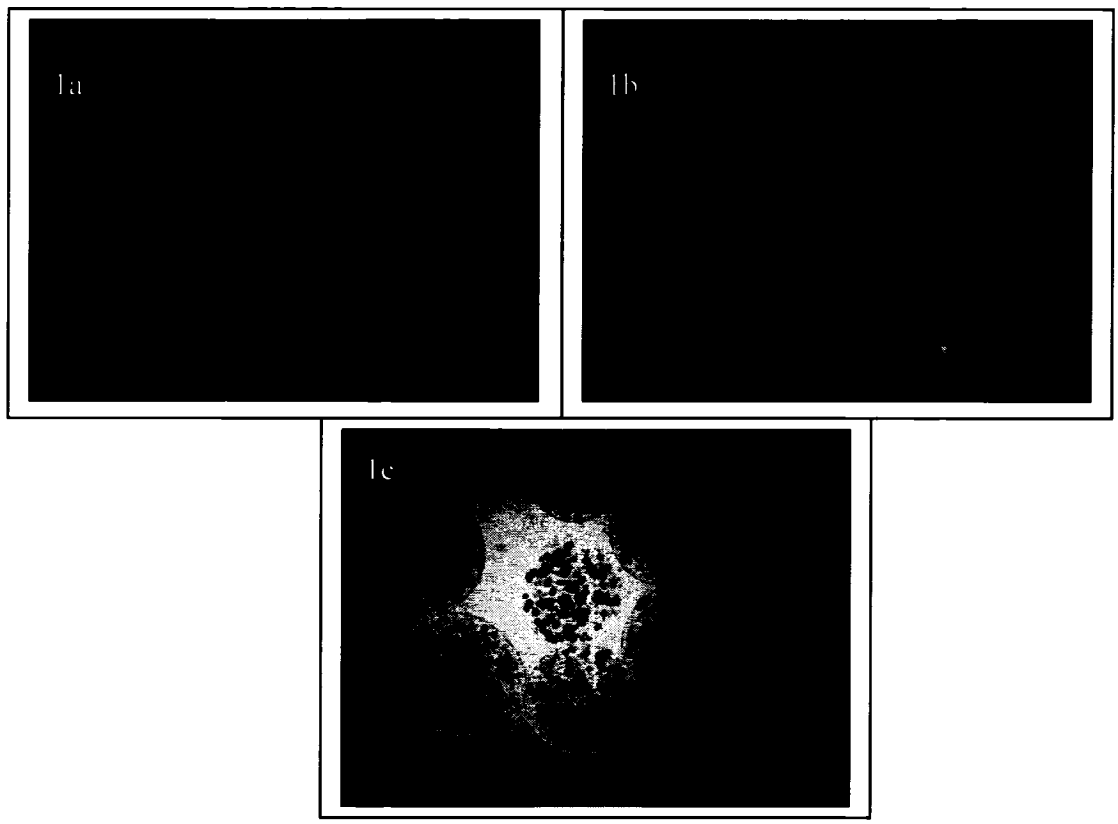

Figure 5.1: Photomicrograph encapsulated HepG2 cells in Alginate-Chitosan-PEG, (ACP), Alginate-PLL-PEG-Alginate (APPA) and Alginate-Chitosan-PEG-PLL-Alginate (ACPPA) microcapsules. 1a) APPA microcapsules 1 day after encapsulating; *magnification: 10X, size: 450±30 μm.* 1b) ACP microcapsules 1 week after encapsulating; *magnification: 10X, size: 450±30 μm.* 1c) ACPPA microcapsules 2 weeks after encapsulating; *magnification: 6.5X, size: 450 ±30 μm.*

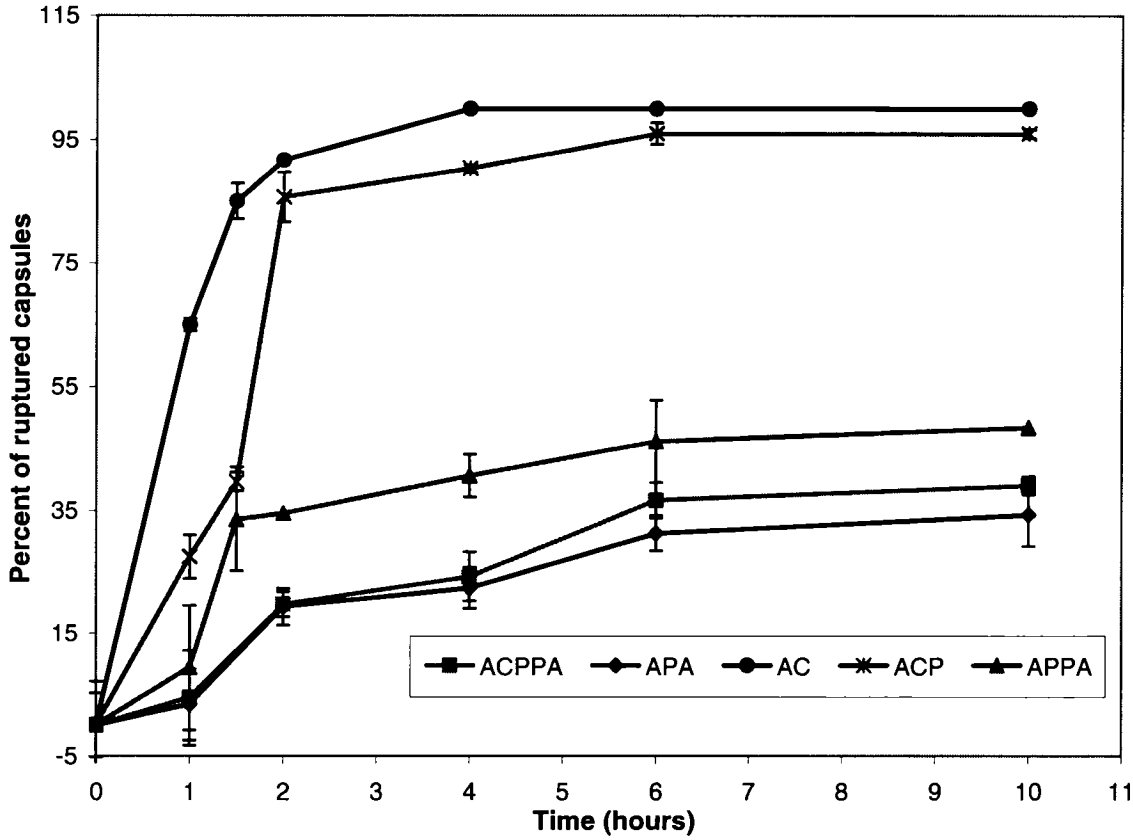

Figure 5.2: The effect of mechanical impact of rotation at 150 rpm and 37 °C on microcapsule integrity.

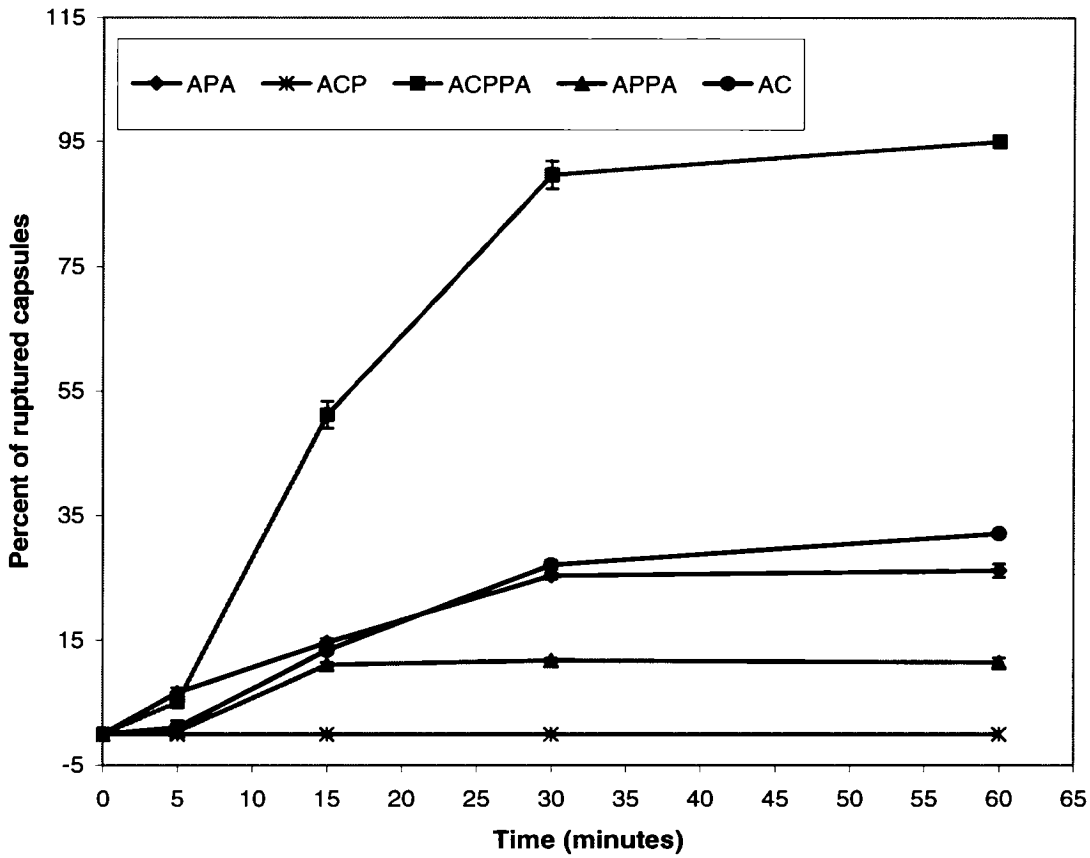

Figure 5.3: The effect of osmotic pressure on microcapsule integrity when exposed to a hypotonic solution.

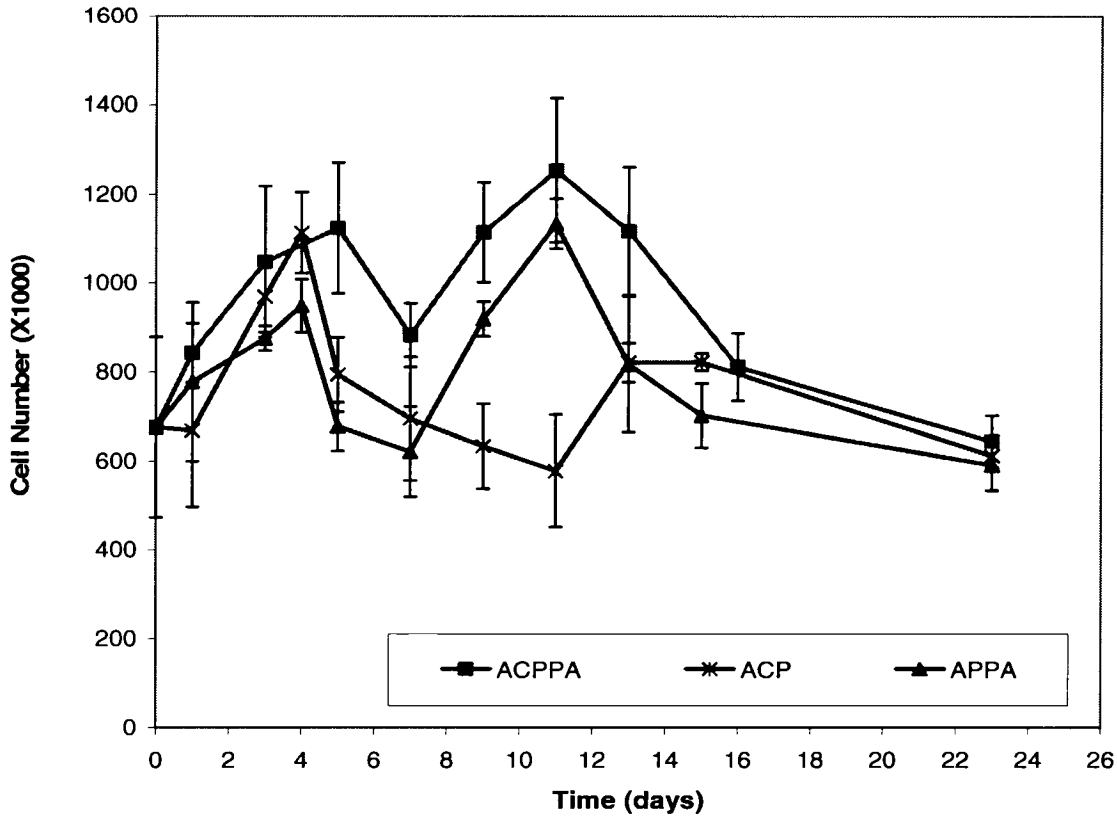

Figure 5.4: Cell Viability of HepG2 cells encapsulated in Alginate-PLL-PEG-Alginate (APPA), Alginate-Chitosan-PEG (ACP), and Alginate-Chitosan-PEG-PLL-Alginate (ACPPA) microcapsules.

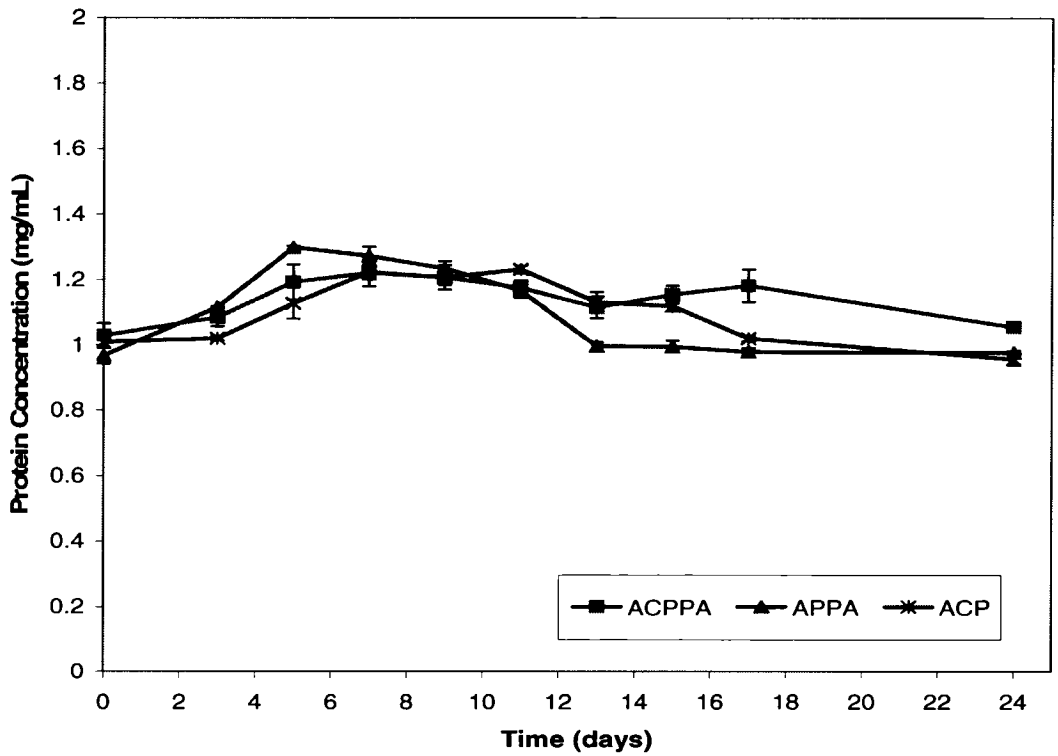

Figure 5.5: Protein released from encapsulated HepG2 cells in Alginate-PLL-PEG-Alginate (APPA), Alginate-Chitosan-PEG (ACP), and Alginate-Chitosan-PEG-PLL-Alginate (ACPPA) membranes.

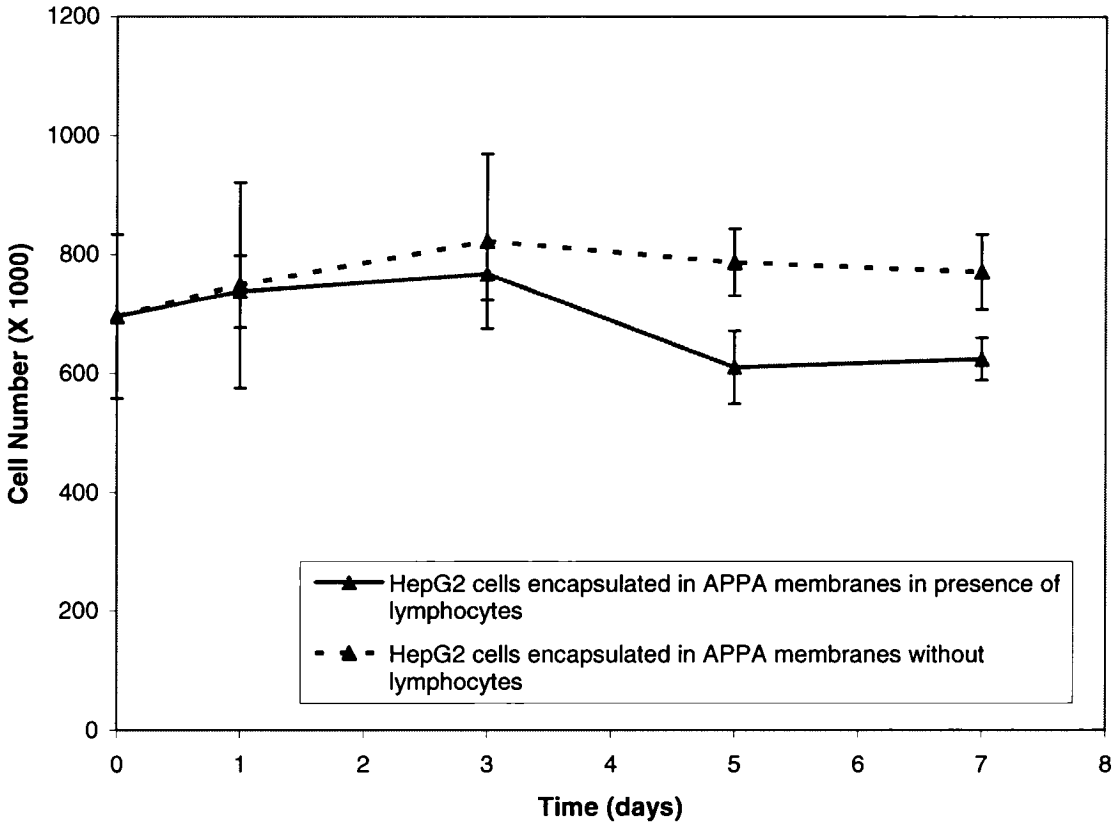

Figure 5.6a: Viability of encapsulated HepG2 cells in Alginate-PLL-PEG-Alginate (APPA) microcapsules grown with and without the presence of lymphocytes.

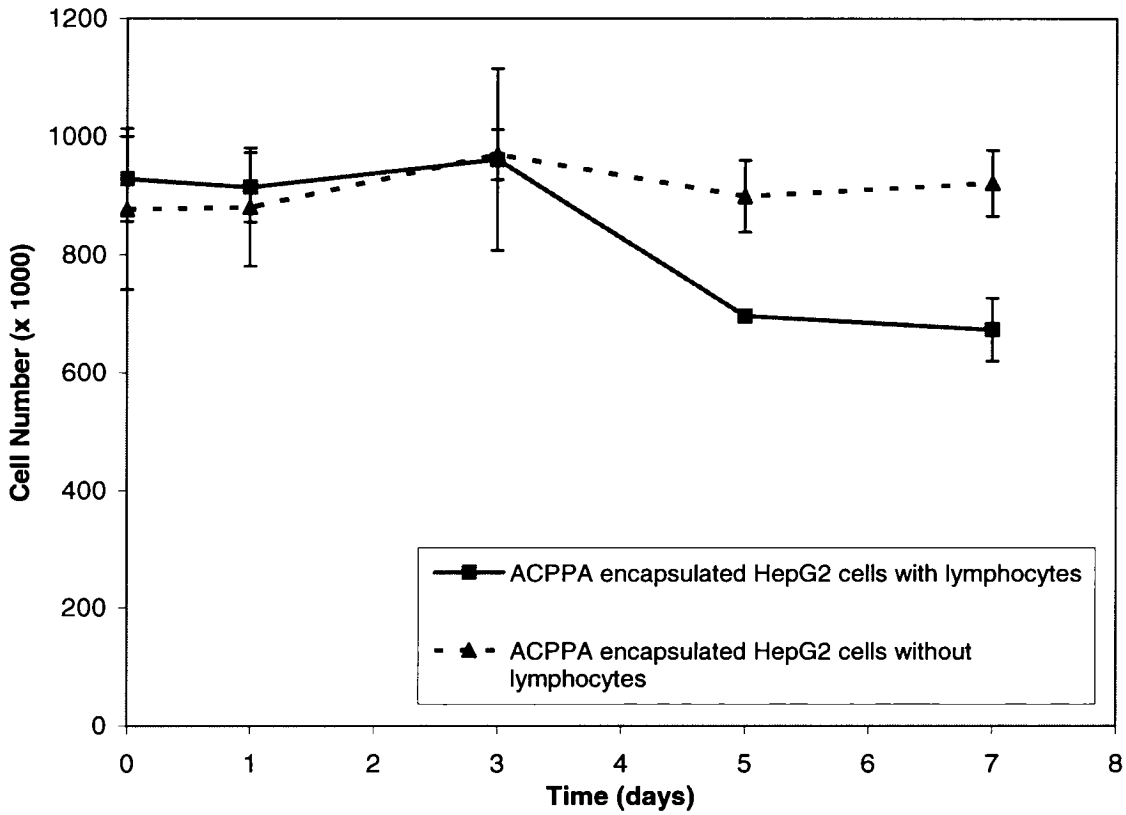

Figure 5.6b: Viability of encapsulated HepG2 cells in Alginate-Chitosan-PEG-PLL-Alginate (ACPPA) microcapsules grown with and without the presence of lymphocytes.

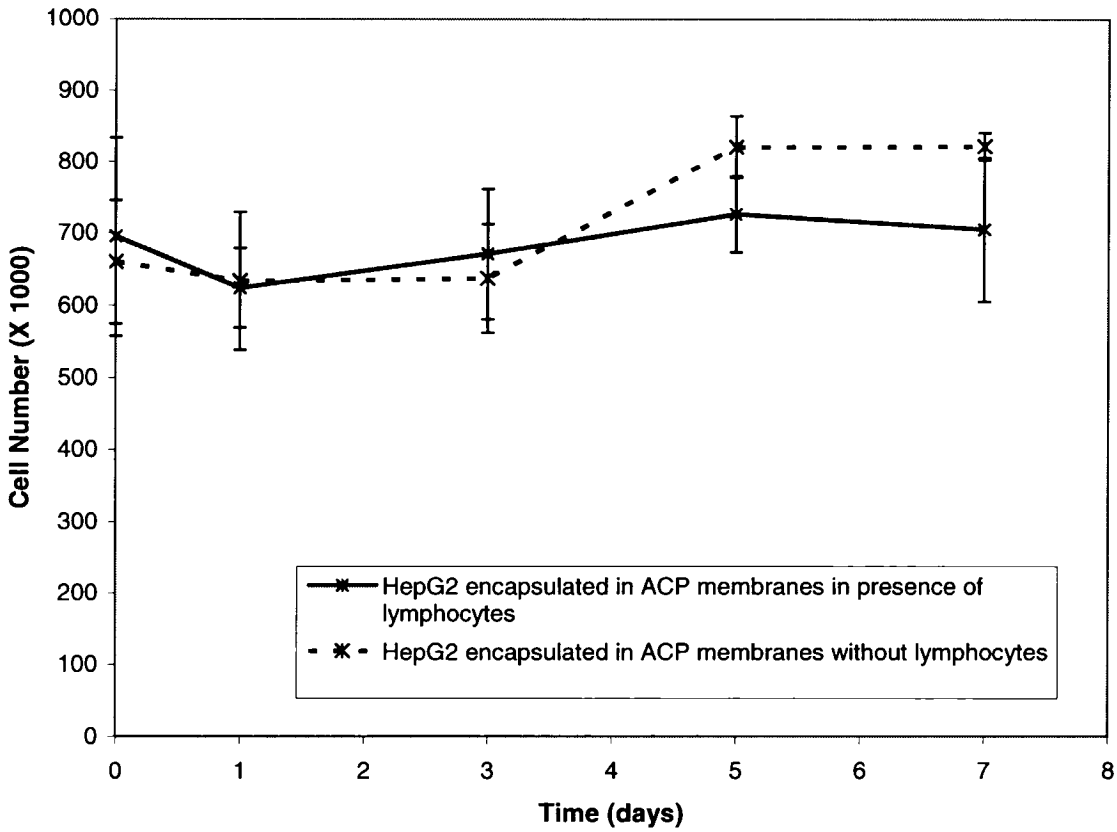

Figure 5.6c: Viability of encapsulated HepG2 cells in Alginate-Chitosan-PEG (ACP) microcapsules grown with and without the presence of lymphocytes.

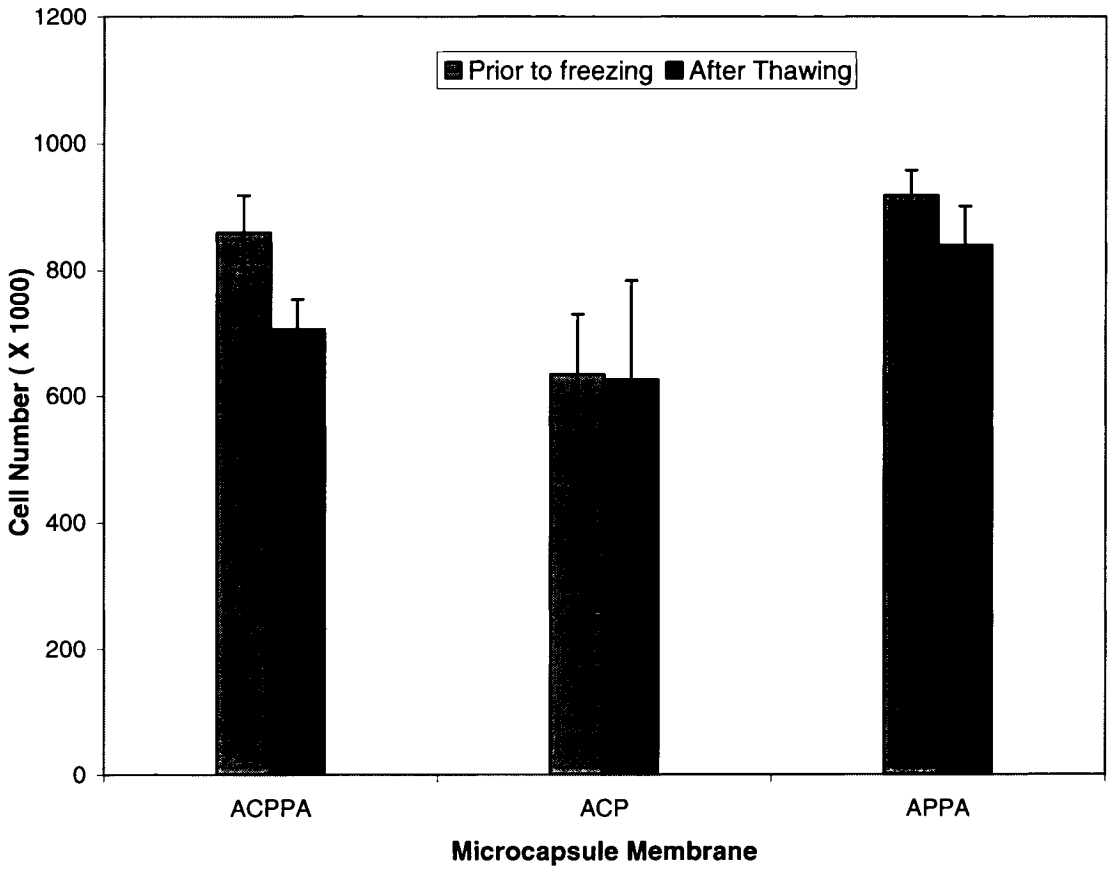

Figure 5.7: Cryopreservation Study: Cell number before and after storing at -80 ^{o}C for 30 days.

6.0 Summary of Observations

The study analyzed five different microcapsule membranes for the application of liver cell encapsulation. Previously established membranes, APA (alginate-poly-l-lysine-alginate) and AC (alginate-chitosan) were compared and the feasibility of incorporating polyethylene glycol (PEG) to these membranes was also analyzed. A novel membrane combining alginate microcapsules coated with chitosan, polyethylene glycol (PEG) and poly-l-lysine was designed and prepared. The study can be summarized as the following:

1) Design and Preparation of the Novel Microcapsule:

A novel membrane (ACPPA), composed of alginate capsules coated with chitosan for 30 minutes followed by additional coatings of PEG for 10 minutes, PLL for 10 minutes, and a final layer of dilute alginate for 10 minutes was prepared. The chitosan was added to this novel membrane in order to allow better cell attachment and increase immuno-protection and capsule strength. PEG was incorporated to improve biocompatibility and the PLL was added to reinforce immuno-protection. The final alginate layer was added to allow optimum biocompatibility. Upon microscopic analysis, smooth, spherical microcapsules with a diameter of 450±30 μm were obtained using this combination and order of layers. (fig. 4.1 and 4.3) The novel membrane was capable of supporting cell proliferation and did not exert any toxicity to the encapsulated liver cells.

2) Mechanical Stability and the Impact of Rotational Stress:

The ACPPA and the APA microcapsules revealed the strongest mechanical strength when exposed to a mechanical rotational stress test with the ACPPA microcapsules having strength similar to the APA. At the completion of 10 hours of exposure to the mechanical impact of rotation at 150 rpm, a total of 34 ± 4 % APA and 39 ± 1 % of ACPPA capsules had broken. The AC and ACP microcapsules had the weakest stability. After the citrate treatment and rotational impact, a total of 100 % and 96 ± 1% of the AC and ACP membranes ruptured. At the completion of the 10 hour study, 48 ± 7 % of the APPA microcapsules were found broken (fig. 5.2).

3) Mechanical Stability and the Impact of Osmotic Pressure:

The novel membrane was unable to sustain osmotic pressure from exposure to a hypotonic solution. At the completion of the 60 minute analysis 95 ± 0.0001 % had broken. The addition of PEG to the APA and AC microcapsules greatly enhanced stability (fig. 5.3). All of the ACP microcapsules remained intact after 60 minutes of exposure to a hypotonic solution compared to a total of 32 ± 0.3 % AC microcapsules that were found broken. Within 60 minutes, a total of 11 ± 0.7 % of APPA microcapsules were ruptured, which is a reduction of over 50 % of broken capsules observed for the APA membrane without PEG which had a total of 26 ± 1 % microcapsules that had ruptured.

4) Cell Viability:

Results indicate that all of the five membranes, APA, AC, APPA, ACP and ACPPA, do not exert any toxicity to the cells and cell viability and proliferation is apparent for the duration of the 25 day study. APA microcapsules provide the highest cell viability and metabolic activity of encapsulated liver cells followed by the ACPPA microcapsules membrane. A maximum cell number of $1.40x10^6 \pm 1.02x10^5$ and $1.26x10^6 \pm 1.27x10^5$ are obtained after the ninth day of encapsulating for both respectively. A maximum cell number of $1.13x10^6 \pm 5.65x10^4$ was observed for the APPA microcapsules. The AC and ACP microcapsules followed a slightly different pattern of proliferation reaching a peak of $1.21x10^6 \pm 8.80x10^4$ and $1.11x10^6 \pm 9.16x10^4$ respectively on the 4^{th} day after encapsulating (fig. 3.2 and 5.4). Results indicate that alginate microcapsules containing chitosan and PEG can be used to support cell proliferation.

5) Protein Secretion:

Results show that although the cell viability varied for the 5 microcapsules, no significant difference is apparent for the protein secreted by the encapsulated HepG2 cells. A peak in protein secretion is observed between the 5^{th} and 11^{th} day after which a decrease is observed implying reduced functionality of the HepG2 cells. A maximum of 1.31 ± 0.030 mg/mL is attained for the APA microcapsules and 1.30 ± 0.006 mg/mL is

attained for the ACP microcapsules. The maximum concentration of proteins secreted by the AC encapsulated HepG2 cells was 1.29 ± 0.044 mg/mL. The proteins secreted by the ACP encapsulated cells were slightly lower at 1.28 ±0.05 mg/mL. HepG2 cells encapsulated in the ACPPA membranes showed a different pattern of protein expression. Although the total concentration of protein secreted was slightly lower, the quantity remained consistent for a longer period of time ranging from the 5th to 17th day. A maximum protein concentration of 1.22 ± 0.04 mg/mL was attained (fig. 3.3, 5.5).

6) Immunogenicity:

The microcapsules supported the proliferation of encapsulated HepG2 cells grown in the presence of lymphocytes in the surrounding culture media, however, a reduction in HepG2 viability is observed. Even though the reduction was greatest for the APA microcapsules, this was also observed for the ACPPA and APPA membranes. Results show that membranes containing chitosan provide the highest protection against foreign cells since the viability of AC and ACP encapsulated HepG2 cells remained unaffected by the presence of lymphocytes. HepG2 viability within the ACPPA capsules decreased slightly after the third day of exposure to media containing lymphocytes. A reduction from $9.28x10^5 \pm 7.1x10^4$ to $6.73x10^5 \pm 5.4 X10^4$ HepG2 cells is observed and 24 ± 0.02% lymphocytes had lost viability (fig. 3.4a, 3.4b, 4.11, 5.6a, 5.6b and 5.6c).

7) Cryopreservation:

The possibility of the microcapsules to be used for long term storage of liver cells was analyzed at –80 °C. Results showed that the APA membrane had the greatest reduction of cell viability after re-growing. The cell viability had reduced from $9.47x10^5 \pm 1.0 \times 10^4$ per 30 microcapsules to $5.07x10^5 \pm 5.5 \times 10^4$ per 30 capsules after storage at –80 °C for 30 days (fig. 3.5). The cell viability was slightly reduced for the remaining membranes which was most likely due to strain from the handling and the freeze-thaw process. Morphological studies of the APA and APPA membranes revealed the membranes to lose their spherical and uniform shape.

7.0 Conclusions

APA microcapsules have been found to have several disadvantages including the promotion of fibrotic overgrowth, protein adhesion and cell necrosis during transplantation. In the present project alternative microcapsule membranes for the encapsulation of liver cells for cell therapy was investigated. A novel membrane formulation using alginate capsules coated with chitosan, PEG and poly-l-lysine was designed as one of the potential alternatives. It was hypothesized that using these materials, a stronger and more biocompatible material may be designed for cell transplantation. The objectives of the project was to assess the stability, biocompatibility and cryopreservation properties of various microcapsule membranes using alginate, PLL, PEG and chitosan in order to propose an alternate direction to consider for cell encapsulation therapy.

After reviewing the results, the following conclusions can be drawn:

1) APA microcapsules provide the best cell viability, and good mechanical strength, however, their immunogenicity and cryopreservation properties are poor.

2) Microcapsules containing chitosan may be an alternative microcapsule for liver cell encapsulation. Chitosan does not exert any toxicity to the cells and provides excellent immuno-protection to the encapsulated live cells. However, its cell viability is lower than the APA membrane and it has poor mechanical strength.

3) The addition of PEG to chitosan and APA microcapsules greatly improves mechanical stability when subjected to osmotic pressure. PEG added to chitosan membranes does not alter cell viability and functional properties. PEG added to APA microcapsules result in reduced viability, however, better immunogenic and cryopreservation properties are observed.

4) The novel microcapsule (ACPPA) had the highest cell viability following the APA microcapsules. The membrane can support cell proliferation and metabolic function, as well as provide immunogenic protection and cryopreservation potentials. However, its stability when exposed to a hypotonic solution is poor and will require improvements which may be attained by modifying the chitosan content.

5) The current study demonstrates that chitosan and PEG containing microcapsules have several advantages; however, the results are drawn only from in vitro analysis. In order to attain a more complete understanding of the microcapsule properties, permeability studies of the membranes, SEM and FTIR studies on the interaction between the microcapsule layers and the effect of modifying the concentration and molecular weights of chitosan and PEG to improve mechanical stability need to be investigated. In addition, in vivo studies will need to be performed to verify their full potentials.

8.0 Recommendations and Future Applications

1) The liver is an important organ in the body where a number of metabolic functions are regulated and protein secretion is performed. Inherited hepatic disorders and liver failure are often treated with a complete liver transplant. The shortage of healthy liver donors as well as immunosuppressant requirements makes this type of treatment very limited [5]. Transplantation of encapsulated hepatocytes may be an alternative treatment for liver failure. The microencapsulated cells provide immuno-isolation from the host thereby eliminating the requirement of immunosuppressants as well as problems associated with immuno-rejection [22]. Although APA microcapsules have been tested for this purpose, it has previously been shown that despite being able to support liver function, after prolonged period of time, the microcapsule fails [21]. The current study has shown the feasibility and potential advantages in terms of immuno-protection for membranes containing PEG and chitosan for the application of liver cell transplantation. Microcapsules containing chitosan were least affected by the presence of lymphocyte cells indicating better immunogenic properties and possibility for xenogenic transplantation applications.

2) Hemoperfusion is a method by which blood is detoxified by passing through a reactor column containing biologically active materials that can supply the necessary functions of a failed organ. Although hemoperfusion is usually performed using adsorbents such as charcoal, a more complete bioartificial organ can be simulated by the use of live encapsulated cells [1,22]. The current study has shown the possibility of applying five types of microcapsules membranes to this system. Alginate coated with various combinations of chitsoan, PLL and PEG are capable of supporting liver cell proliferation and function. Their potential in hemoperfusion will require further investigation by incorporating microcapsules in a packed bed bioreactor and testing their integrity as well as their potential to purify toxins and supply proteins to a flowing media.

3) Cell microencapsulation is an emerging technology which can be applied to controlled drug delivery systems, oral administration of probiotics as a protection from gastric fluids

and immuno-isoloation of cells during transplantation [80]. The implementation and advancement of this technology however requires the necessity to acquire an adequate biomaterial for encapsulating. The current study reports the formulation of a novel membrane which revealed high cell viability, mechanical strength, immuno-isoloation properties as well as potential for cryopreservation. Although the study investigated its potential for liver cell encapsulation, the membrane design can be tested for various other applications including oral or drug delivery.

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