Hollow microparticles as superior carriers for encapsulation and delivery of biomolecules

Kharel, Sharad

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**HOLLOW MICROPARTICLES AS SUPERIOR CARRIERS
FOR ENCAPSULATION AND DELIVERY OF
BIOMOLECULES**

SHARAD KHAREL

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2017

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FOR ENCAPSULATION AND DELIVERY OF
BIOMOLECULES**

SHARAD KHAREL

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2017

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

5 April 2018

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Date

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Sharad Kharel

Abstract

Bioactive molecules like peptides and proteins have gained significant attention in the field of therapeutics due to their exquisite specificity to their targets resulting in greater effective potency, reduced therapeutic dose and side effects. However, their low systemic stability and short half-life limits their potential. To overcome these problems, biodegradable polymer microparticles composed of polyester such as PLGA have been extensively used as carriers for delivery. However, they suffer from major limitations such as incomplete release and loss in bioactivity of the encapsulated biomolecules. Therefore, the overall objective of this thesis is to develop a method to fabricate hollow microparticles through an easy one-step method and validate the hollow microparticles as superior carriers for encapsulation and delivery of biomolecules. A method was developed wherein emulsion solvent evaporation technique was modified by adding osmogen into the oil phase before emulsification to fabricate hollow microparticles. The results obtained show conclusive evidence that by simply altering the amounts of osmogen in the formulation, the shell thickness and volume space of hollow cavity could be easily controlled. This was found to be an effective means to control and fine-tune the release kinetics of biomolecules to overcome the issue of incomplete release as observed in solid microparticles. It was found that by changing the particle morphology to hollow, the accumulation of polymer degradation products could be significantly reduced. This helped in reduction of acidic microenvironment in the microparticles, which decreased polymer-biomolecule adsorption. Consequently, the release of biomolecules from the microparticles was improved over solid microparticles. Furthermore, the bioactivity of encapsulated biomolecules was also preserved better in the hollow microparticles. It was thus established that hollow microparticles are superior over solid microparticles for delivery of biomolecules.

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Abbreviations

ACN	Acetonitrile
BSA	Bovine Serum Albumin
BSE	Back-scattered Electrons
CD	Circular Dichroism
CL	Cathodoluminescence
CEHMT	Coaxial Electro Hydrodynamic Microbubbling Technique
DCM	Dichloromethane
EDX	Energy Dispersive X-ray Spectroscopy
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GLP-1	Glucagon Like Peptide-1
GPC	Gel Permeation Chromatography
HPLC	High Pressure Liquid Chromatography
HTRF	Homogenous Time Resolved Fluorescence
h-MPs	Hollow Microparticles
LBL	Layer-by-layer
MF	Melamine Formaldehyde
MFT	Microfluidic Fabrication Technique
MPs	Microparticles
MCF-7	Michigan Cancer Foundation-7
MALDI TOF MS	Matrix Assisted Laser Desorption Ionization Mass Spectroscopy
PBS	Phosphate Buffered Saline
PNIPAM	Poly(N-isopropylacrylamide)
PLGA	Poly(D,L-lactide-co-glycolide)
PLLA	Poly(L-lactic-acid)
PVA	Poly(vinyl alcohol)
RP-HPLC	Reversed Phase High Pressure Liquid Chromatography
SE	Secondary Electrons
SEM	Scanning Electron Microscopy

SEI	Secondary Electron Images
s-MPs	Solid Microparticles
TFA	Trifluoroacetic Acid
THF	Tetrahydrofuran

Chapter 1

Introduction

*This chapter begins with a general introduction of the current market of biomolecules in therapeutic applications, followed by the attributes of biomolecules such as short half-life and low bioavailability due to rapid clearance from the body when they are administered without any protective delivery carriers. As such, the significance of biodegradable polymer microparticles (MPs) in their encapsulation and delivery is highlighted. However, the MPs delivery system, although widely used for delivery of biomolecules, is still lacking in efficacy due to the drawbacks such as incomplete release and loss in bioactivity of the encapsulated biomolecules over the release period. The chapter progresses to discuss these limitations of MPs system, and thus, introduces the objectives that includes developing a facile, one step technique to fabricate hollow MPs and validating these MPs of hollow morphology as an effective approach to address those issues. An overview of each of the chapters is presented in **Section 1.5** and the chapter is concluded after highlighting the novelty of this research.*

1.1 Background

In the year 1982, the first human protein therapeutic, human insulin (Humulin®) was created at Genetech^[1] using recombinant DNA technology and approved by the US Food and Drug Administration (FDA).^[2] It was used to replace its natural counterpart that can be deficient in diabetes mellitus. This marked the beginning of a new era of therapeutic ‘peptides and proteins’- hereinafter referred to as ‘biomolecules’ in this thesis. With the advancement in protein engineering and biotechnology, at present, there are more than 200 approved therapeutic proteins and over 100 peptides in the market accounting for more than 10% of the pharmaceutical market valued at \$40 billion per year.^[3] This significant growth of interest and investment in these therapeutic biomolecules is attributed to their ability to bind with exquisite specificity to in vivo targets, resulting in greater effective potency. This reduces the therapeutic dose, side effects and toxicity.^[4]

Still, the commercial potential of biomolecules as therapeutics is significantly hampered from two major drawbacks: 1. low oral bioavailability and 2: short half-lives, due to their rapid degradation by proteolytic enzymes in the blood plasma and digestive system.^[5, 6] To overcome this, biomolecules are therefore encapsulated into particles composed of biodegradable polymers (e.g. polyesters, polyanhydrides, etc.) to protect them from the proteolytic enzymes. At the same time, encapsulation also provides the means to control their release.^[7-9] Poly(L-lactide-co-glycolide) (PLGA), in particular, has been a popular choice of encapsulation material owing to its excellent biocompatibility, tunable degradation rate and the relative ease in designing them for controlled and sustained delivery.^[10, 11] In addition, it is approved by the FDA for parenteral human use.^[11, 12] Upon administration, these polymer particles become hydrated and undergo mainly bulk degradation,^[11] through the hydrolysis of their ester bonds. Subsequently, the polymer matrix becomes more porous, aiding in a faster rate of diffusion and release of the encapsulated peptide.^[13]

1.2 Statement of problem

The limitations of using biodegradable polymer particles for delivery of biomolecules, however, starts manifesting during the release period in the physiological environment. Biomolecules release is often characterized by an initial burst release, followed by a sluggish and often incomplete release.^[14, 15] This undesirable, incomplete biomolecules release, as reported in the literature,^[16-19] can be attributed to the interactions between the biomolecules and the encapsulating polymer. When the biomolecules-loaded particles are delivered to a physiological environment, hydrolysis of the polymer occurs and shorter polymer chains or oligomers, with carboxylic acid-terminated end-groups, are generated. The microenvironment within the particles therefore becomes acidic over time that results in an auto-catalytic degradation effect.^[20, 21] In addition, as many biomolecules contain positive charges, or transition from negative to positive charges at low pH,^[22] electrostatic interaction between a highly acidified (i.e. negatively-charged) polymer matrix and the encapsulated biomolecules^[23-25] will ensue. Also, in a low pH environment, acylation of the biomolecules with lactic and glycolic acids and their oligomers is catalyzed and polymer-peptide adducts are formed.^[26, 27] All these may influence release kinetics and possibly compromise the bioactivity of the biomolecules. Understanding such a phenomenon during release and developing a delivery system that circumvents these issues is therefore paramount.

This thesis therefore aims to fill the gap by developing a fabrication technique for hollow MPs that provides easy manipulation of the particle size and particle morphology such as shell thickness and volume space of hollow cavity. Since there are currently no papers that explore the use of MPs of hollow morphology as an approach to ameliorate the issue of incomplete release and loss of bioactivity of encapsulated biomolecules, it is therefore of interest to investigate the possibilities of this “hollow” particulate delivery system.

1.3 Objectives

The focus of this thesis is to address the issues of incomplete release and loss of bioactivity of the encapsulated biomolecules upon release through the polymer MPs, and the main objective is to investigate the use of hollow PLGA MPs instead of solid MPs as a potential solution. In fulfilling the objective, the research work is divided into three parts.

1. The first part is to conduct an in-depth study to develop a novel technique for the fabrication of hollow MPs with controllable particle size, shell thickness and volume space of hollow cavity. The effects of different process parameters on the final microparticle morphology are investigated and a correlation is established between the process parameters and the hollow morphology obtained. From these results, the formation mechanisms are subsequently proposed.
2. The second part is to validate the fabricated hollow MPs as a potential delivery vehicle for biomolecules. A hydrophobic model biomolecule is encapsulated and the cumulative release properties of the hollow MPs with the alteration of shell thickness and volume space of hollow cavity is compared with that of solid MPs. The biomolecule release result is explained by investigating the rates of hydrolytic degradation and morphological differences of these MPs.
3. The third part is to validate the superiority of hollow MPs over solid MPs as carriers for biomolecules. The cumulative release and the level of bioactivity of the encapsulated hydrophilic biomolecule is assessed, and thorough investigation is done to elucidate the superior results shown by hollow MPs.

At the end of the thesis, it is then determined if the use of the hollow MPs as carriers for biomolecules can provide better release kinetics and better preservation of the bioactivity of biomolecules.

1.4 Scope

In this work, poly(D,L-lactide-co-glycolide) (50-50) (PLGA) will be used as a representative biodegradable polymer for the formation of MPs. This polymer was chosen due to its wide application in the drug delivery field. The hollow MPs will be fabricated through an osmogen mediated emulsion solvent evaporation technique. Effects of the process parameters, such as initial polymer concentration, stirring speed, amount of osmogen and type of osmogen, on the morphology of the particles will be investigated. A hydrophobic biomolecule “bovine insulin” and a hydrophilic biomolecule “Glucagon Like Peptide-1” will be used as model biomolecules to assess the versatility of the carriers developed.

This study focuses on addressing two major drawbacks of using biodegradable polymer PLGA MPs as carriers for delivery of biomolecules viz: inability of the PLGA MPs to achieve complete release of encapsulated biomolecules and gradual loss of bioactivity of the biomolecules during the release period. The focus is on addressing these issues that are observed only after the particles are exposed to the release medium over the duration of release. The delivery of biomolecules by polymer MPs of hollow morphology instead of the more common solid morphology is presented as an effective approach to address those issues.

1.5 Dissertation overview

The thesis addresses the challenges faced in the encapsulation and delivery of biomolecules upon using biodegradable polymer MPs as carriers.

Chapter 1 provides a rationale for the research to contextualize the thesis, and outlines the objectives, scope and the novelty of the work.

Chapter 2 provides a review of the literature concerning the potentials and challenges of

the delivery of biomolecules for therapeutic application. It then discusses on the current use of PLGA MPs for delivery of biomolecules and their drawbacks as carriers for biomolecules, paving the way for a new method to fabricate hollow MPs to encapsulate and deliver biomolecules.

Chapter 3 discusses on the materials and equipment used in the study. The rationale behind choosing the equipment and their working principles are explained while also explaining the rationale behind using those methods and equipment.

Chapter 4 presents the results of the studies and discusses the results in correlation to each other. The first section elaborates the major set of results obtained in the development of a new technique to fabricate hollow MPs. The second section elaborates the results obtained for validating the suitability of the fabricated hollow MPs in encapsulation and delivery of therapeutic biomolecule. The third section elaborates the results to validate that hollow MPs are superior to solid MPs for encapsulation and delivery of therapeutic biomolecules.

Chapter 5 draws together the conclusions of the overall results obtained from the studies encompassed by this thesis and reflects on the extent of fulfillment of the objectives that was set at the inception of the thesis. Finally, the chapter ends by painting a brief picture of opportunities and strategies for future studies that can be further built upon the work presented in this thesis.

1.6 Novelty

There are two novel aspects of this thesis.

1. Development of a one-step easy technique of fabricating hollow MPs to be used in encapsulation and delivery of biomolecules:

Hollow MPs are fabricated in a single-step fabrication process utilizing the osmotic property of osmogen. Developing a technique by capitalizing on the relationship between the osmotic pressure of the osmogen and its corresponding effect on the precipitation rate of the polymer to produce such hollow particles by single oil-in-water (O/W) emulsion has yet to be reported. Achieving this would provide a robust, yet facile technique of fabricating hollow MPs with fully controllable particle parameters.

2. Proposing and validating the hollow MPs as superior delivery alternative over solid MPs.

By conducting a comparative study of different aspects of the hollow MPs and solid MPs as carriers for biomolecules, it has been shown that this can be a simple yet effective approach to use polymer MPs of the hollow morphology as carriers of biomolecules, instead of the traditionally used solid morphology, to overcome the limitations of using them in the delivery of biomolecules.

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Chapter 2

Literature Review

This chapter provides a detailed review of the literature concerning the potential and challenges of the delivery of biomolecules for therapeutic application. It begins with acknowledging the potential of biomolecules in therapeutics. The chapter progresses with discussions on the attributes of the biomolecules which makes them widely popular in therapeutics, while also discussing the attributes that limits their potential if administered without any delivery carrier protecting them from the physiological environment, thus defining the need for a delivery platform for the biomolecules. This is followed by discussions on the current use of poly(D,L-lactide-co-glycolide)(PLGA) microparticles (MPs) as protective delivery carriers for biomolecules and highlights its properties to elucidate on the wide market of PLGA based biomolecule formulations. Further, the chapter discusses the issues of incomplete release and loss in bioactivity of encapsulated biomolecules in the PLGA MPs, paving the way for a new method to fabricate hollow MPs to efficiently encapsulate and deliver biomolecules.

2.1 Biomolecules in therapeutics

Biomolecules such as proteins and peptides are the most abundant and important constituents of biological cells. Enzymes, hormones, structural elements and immunoglobulin are all nothing but proteins. Structurally, peptides and proteins are the chains of amino acids joined together by peptide bonds. While clear distinction between peptides and proteins is somewhat arbitrary and never has been resolved,^[1] peptides consist of less than 20 amino acids whereas proteins contain 50 and more.^[2] It can also be defined in terms of molecular weight, whereby peptides are considered to have a molecular weight of less than 5000 Da, while proteins have more than that.^[3]

Figure 2.1 Figure showing a structural progression from amino acids to protein.

With the discovery of numerous proteins and peptides' application in biopharmaceuticals and patho-physiology of human diseases, they have received much attention in modern times.^[4] A rarely used subset of medical treatments once, the number and frequency of use have increased dramatically since the introduction of the first recombinant protein therapeutic, human insulin, 35 years ago.^[5] These biomolecules based therapeutics are now well accepted in medical practices and research activities due to their significant role in integration of life processes and several advantages over small-molecule drugs.^[6] To start with, as the action of biomolecules are highly specific, there is often less chances for them to interfere with normal physiological process and cause adverse effects.^[5] The highly complex and specific set of functions peculiar to the biomolecules cannot be

mimicked by simple chemical compounds. Since many of the biomolecules that are used in therapeutics are naturally produced by the human body, they are often well tolerated and are less likely to elicit immune responses. Also, for diseases in which a gene is mutated or deleted, biomolecule therapeutics can provide effective replacement treatment without the need for gene therapy.^[7] Furthermore, in comparison to small-molecule drugs, these biomolecule therapeutics may have faster clinical development and FDA approval time.^[8] This is possible as these therapeutic biomolecules are also naturally produced in human body resulting in less chances of complication. Thus, the trial period for approval of the drug can be made shorter. Lastly, companies can have comprehensive patent protections of these biomolecules produced due to their unique structure and functions. From a financial perspective, these last two advantages make biomolecules very attractive in therapeutics.^[5, 9]

In 1922, the first life-saving insulin was purified from bovine and porcine pancreas.^[5] However, the industrial scale production and market development was constrained by the limited availability of the organs, the immunological reaction and the huge costs of production. Scarcely in the 70's, the recombinant DNA technology opened the new age of pharmaceutical biotechnology. With advancement in knowledge and technology in molecular biology and genetic engineering, large scale production of polypeptides is now possible by employing recombinant DNA and hybridoma technology.^[11] Thus, most of the therapeutic biomolecules are now produced and purified from a wide range of organisms.^[12, 13] Over the decades, several therapeutic biomolecules-based drugs such as Lupron, Zoladex, Copaxone, Sandostatin have made their way into the commercial market, each generating more than \$1 billion in annual sales.^[14, 15] There are currently over 500 peptide drugs in pre-clinical trials, 140 in clinical trials and 60 Food and Drug Administration (FDA)-approved peptide drugs in the market (**Figure 2.2**).^[16] With the approval rate of peptides being twice as high as small molecules, the peptides drug market is therefore growing twice as fast.^[17, 18] In 2015, the global peptide therapeutics market was valued at \$21.3 billion and is estimated to reach \$46.6 billion in 2024.^[19] Likewise, the global bioengineered protein drugs market is valued at \$177.7 bn in 2016 and predicted to become worth \$336.9 bn by 2025.^[20]

Figure 2.2 Bar graph showing the number of FDA approved therapeutic proteins by year (a), Pie chart showing the distribution by drug class (b), Pie chart showing the distribution by therapeutic area (c) Pie chart showing the distribution of secondary therapeutic area for oncology drugs (d) (January 1, 2011, through August 31, 2016) Adopted from Zuben et al.^[10]

2.2 Limitations of unencapsulated biomolecules in therapeutics applications

The most commonly used parenteral routes for delivery of therapeutic biomolecules are intravenous, intramuscular and/or subcutaneous.^[13] Most therapeutic biomolecules that are administered in the native form and without any protection are susceptible to biodegradation resulting in the loss in bioactivity, have low membrane permeability, or can be rapidly eliminated from the blood circulation.^[21] The pharmacokinetic properties including their plasma half-lives, which influences distribution and excretion, are key to the efficacy of biomolecule therapeutics. But, most of the biomolecules used in therapeutics have a molecular mass less than 50 kDa and a very short half-life, except for whole antibodies and Fc fusion proteins.^[22] Several mechanisms, such as, peripheral blood-mediated elimination by proteolysis, renal and hepatic elimination and receptor mediated-endocytosis promotes the clearance of therapeutic biomolecules from the body.^[23] These mechanisms are briefly discussed below.

2.2.1 Proteolysis

Proteolysis is the breakdown of proteins into smaller polypeptides or amino acids by hydrolysis of the peptide bond by utilizing the cellular enzymes called proteases, and also by intra-molecular digestion.^[24] The elimination of these therapeutic biomolecules can either be limited to a specific organ or tissue or can be non-specifically nearly everywhere in the body. The rate of proteolysis is generally dependent on the molecular weights of the therapeutic proteins/peptides and increases with decreasing molecular weight from large to small.^[25] It is also affected by other factors like its secondary and tertiary structure and the level of glycosylation. The injected therapeutic proteins can be metabolized in the liver, kidneys or gastrointestinal tissues and blood. While for orally administered therapeutic proteins, gastrointestinal tract is the major site of metabolism by proteolytic enzymes, parenterally administered proteins may also be metabolized in the intestinal mucosa.

2.2.2 Renal protein metabolism^[26-28]

The bioavailability of the injected therapeutic proteins is also significantly affected by the kidneys as they serve as a major site of protein metabolism by glomerular filtration, specifically for the smaller sized proteins approximately below 50 kDa.^[22] Proteolytic activities for these protein in other body regions determine the renal contribution to overall elimination of the proteins. While the elimination by glomerular filtration can be negligible in the presence of unspecific degradation throughout the body, their contribution to total clearance can be significantly high when the metabolic activity is low in other tissues. After glomerular filtration, small linear peptides undergo intraluminal metabolism, whereas, larger peptides and proteins get actively reabsorbed in the proximal tubes via endocytosis followed by lysosomal degradation. Additionally, peritubular extraction from post glomerular capillaries with subsequent intracellular metabolism also contributes in renal elimination of proteins.

Figure 2.3 Renal elimination processes of peptides and proteins: glomerular filtration followed by either (a) intraluminal metabolism or (b) tubular reabsorption with intracellular lysosomal metabolism, and (c) peritubular extraction with intracellular lysosomal metabolism.^[29]

2.2.3 Hepatic protein Metabolism^[26, 27]

The bioavailability of the injected therapeutic protein is also substantially affected by the Liver. The specific amino acid sequence in the protein largely determines the rate of elimination of protein by the hepatic metabolism.^[30] The primary step in this pathway of metabolism of protein is the uptake into the hepatocytes. While peptide with sufficient hydrophobicity is taken up by hepatocytes by passive diffusion through the membrane, uptake of larger biomolecules requires various carrier or receptor mediated energy dependent transport processes.

2.2.4 Mediated protein Metabolism

This mode of elimination of protein therapeutics can be facilitated by unspecific cell surface receptors as well as specific receptors that are pharmacological targets of protein therapeutics. While this mechanism doesn't contribute much in the elimination of conventional small molecule drugs, it significantly affects the pharmacokinetic profile of therapeutic protein by binding and elimination through receptor mediated uptake and intracellular metabolism. This serves as an initial step in the degradation and elimination by mediating the uptake of protein therapeutic into hepatocytes in the liver, tubular cells in the kidney and endothelial cells through the body as well as other cell types with target receptors.^[26] This mechanism also causes lack of dose proportionality in pharmacokinetics because of the limited number of receptors, saturation of their binding and the related drug uptake within therapeutic concentrations. Thus, systemic clearance for many therapeutics at concentration below the saturation of binding is much higher than at higher concentration than the saturation of binding.

The biomolecule therapeutics to be effective in treatment of diseases, however, requires that a therapeutic effective concentration is maintained over a longer period. Hence, multiple or high doses of biomolecules injection becomes necessary to reach the desirable concentration in the cell, which can cause significant discomfort to the patients and potentially result in side effects too. Thus, it becomes imperative to protect such

therapeutic biomolecules from direct exposure to the physiological environment, typically by using drug delivery carriers to improve their uptake at desired biological site. The carrier acts as a depot for high concentrations of therapeutics and provides a solubilizing and protective environment, either for local delivery where it is retained at the site of delivery, or for systemic delivery where it circulates through the vasculature and should increase the circulation lifetime.^[31]

2.3 Biodegradable polymeric MPs for protecting biomolecules for therapeutic efficacy

Biodegradable polymers are the polymers of synthetic or natural origin that degrade in-vivo, either enzymatically or non-enzymatically or both, to produce biocompatible, toxicologically safe by-products which are further eliminated by the normal metabolic pathways.^[32] Originally developed for biomaterial application, they had received considerable attention in drug delivery during the 1970s.^[33] After the development of Polyesters as the first successful biodegradable polymers as suture materials,^[34] there has been continuous growth in the varieties of polymers and copolymer compositions for controlled drug delivery. The market value of these biodegradable polymers reached 2.1 billion pounds in 2016 and is expected to reach 5.6 billion pounds in 2021. Medical applications for biodegradable polymers is expected to grow from £120 million in 2016 to £650 million in 2021.^[35]

2.3.1 Poly(lactide-co-glycolide) (PLGA) as one of the best biodegradable polymers

Among all the biodegradable polymers available, poly(lactide-co-glycolide) -a product of random copolymerization of polylactide (PLA) (both L-and D, L-lactide forms) and polyglycolide (PGA), is the most investigated polymer for drug delivery application.^[36] It is the best defined biomaterial (Food and Drug Administration, FDA, approved) available

for drug delivery with respect to design and performance and is considered “gold standard”^[37] of biodegradable polymers for controlled delivery.

Figure 2.4 Chemical structure of PLGA in 2D (left image) and in 3D (right image)

Figure 2.5 Hydrolysis of PLGA forming the degradation products

In comparison to other polymers that have been investigated for controlled release, PLGA is relatively easy to process into different device morphologies such as injectable micro-/nanospheres.^[38] It can be processed into any shape and size, and can encapsulate biomolecules of any size. The physical properties of PLGA have been shown to depend on the initial molecular weight of the monomers, the ratio of monomers, the exposure time to water and storage temperature.^[39] It can be dissolved in a wide range of organic solvents, including chlorinated solvents, tetrahydrofuran, acetone or ethyl acetate unlike the low solubility of its homopolymers.^[40, 41] The rate of water uptake is faster than the rate of hydrolysis for PLGA. Thus, it undergoes bulk degradation through random hydrolytic scission of its ester linkages in the presence of water.^[32] Several factors, such

as the ratio of lactide and glycolide, crystallinity and molecular weight determine the degradation rate of PLGA.^[42] PLGA 50:50 is fully amorphous as a result of disorder and poor packing of constituent PLA and PGA. This high free volume encourages the water uptake, thus enhancing hydrolytic scission of the ester linkage in PLGA 50:50. The degradation products with carboxylic end-groups would further enhance the hydrolysis, giving rise to an autocatalytic degradation. In vivo, lactic and glycolic acids are metabolized and eliminated from the body as carbon dioxide and water via tricarboxylic acid cycle.^[43] PLGA is reported to have Tg (glass transition temperature) above the physiological temperature of 37 °C and hence are glassy in nature, thus exhibiting fairly rigid chain structure.^[32] Tg of PLGA decreases with a decrease of lactide content in the copolymer and a decrease in molecular weight.^[44] Commercially available PLGA polymers are usually characterized in terms of intrinsic viscosity, which is directly related to their molecular weights.

2.4 MPs of PLGA polymer best suited for encapsulation and delivery

Although several forms of delivery platform have been introduced, PLGA carriers in the form of “microparticles” remain one of the most popular and widely accepted carrier of delivery of biomolecules. Small spherical-shaped particles, ranging from 10 to 1000µm in size are classified as MPs.^[48] Since 2000, around 200 papers are published annually on biodegradable polymeric MPs for peptides and proteins.^[1] **Table 2.1** below shows some of the PLGA MPs based commercially available biomolecule formulations. One of the main advantage of polymeric MPs system is that it provides protection and stability of labile peptides which would otherwise be cleared out in vivo.^[48] Other is, it also provides the flexibility of changing their shapes and sizes, internal and external morphologies with easy manipulation of fabrication process parameters.^[49] This would mean very easy manipulation of the release kinetics of encapsulated biomolecules as per the requirement. Thus, they are extremely efficient alternatives to avoid the inconvenient surgical insertion of large implants for controlled-release of biomolecule drugs. Drugs formulated in such polymeric devices are released either by diffusion through the polymer barrier, or by

erosion of the polymer material, or by a combination of both diffusion and erosion mechanisms.^[32] The MPs for biomolecules delivery can be prepared by several methods with the most widely used techniques being, (i) microfluidics (ii) phase separation-coacervation (iii) spray drying and (iv) solvent evaporation method (single or double emulsion)^[50] They are briefly discussed in the sections below.

Table 2.1 PLGA MPs based commercially available injectable biomolecules formulation.^[1, 45-47]

Trade name	Polymer	Indication	Methods	Company
Lupron Depot ® (Leuprolide acetate)	PLGA	Palliative treatment of advanced prostatic cancer	Emulsion	Takeda
Sandostatin ® (Octreotide acetate)	PLGA	Acromegaly, Carcinoid tumors, VIPomas	Emulsion	Novartis
Trelstar™ Depot (Triptorelin pamoate)	PLGA	Palliative treatment of advanced prostatic cancer	Spray-drying	Watson Pharma
Bydureon (Exenatide)	PLGA	Type II diabetes	Emulsion	Amylin/Eli/Lilly /Allkermes
Zoladex® (Goserelin acetate)	PLGA	Prostate cancer	-	Astra Zeneca Pharma
Nutropine Depot® (Recombinant human growth hormone)	PLGA	Growth hormone deficiency	-	Genentech-Allkermes
Decapeptyl® (Triptorelin)	PLGA	LHRH agonist	Emulsion	Debiopharm
Signifor LAR (Pasireotide pamoate)	PLGA	Acromegaly	-	Novartis
Somatuline® LA (Lanreotide)	PLGA	Acromegaly	-	Ipsen Biopharmaceuticals

2.4.1 Phase separation (coacervation)^[32, 51]

This technique is based upon liquid-liquid phase separation techniques. This process consists of decreasing the solubility of the encapsulating polymer by addition of a third component to the polymer solution in an organic solution. This basically involves two liquid phases viz: coacervated phase containing polymer and supernatant phase depleted in polymer. Firstly, the drugs (in solid or solution forms) are dispersed/emulsified with polymer solution in organic solvent. Then an organic medium is gradually added to the polymer-drug-solvent phase while stirring, which extracts the polymer solvent resulting in phase separation of polymer by forming a soft coacervate of drug containing droplets. This is followed by quickly quenching these microdroplets by dipping into a medium (organic or aqueous) in which the polymer is not soluble, finally yielding coarsened and hardened polymer particles which can then be collected, washed, freeze-dried and stored.

Methods	Schematic diagram	Advantages	Disadvantages
Phase separation (Coacervation)		<p>Simplicity in process</p> <p>Suitable for temperature sensitive compounds</p> <p>Suitable for both hydrophobic and hydrophilic compounds</p>	<p>Agglomerated particles</p> <p>Difficulty in mass production</p> <p>Large quantities of organic solvent required</p>
Microfluidics		<p>Ultra-small quantities of reagents needed</p> <p>Precise control over drug release rate, drug loading efficiency, particle shell thickness, particle shape and size</p> <p>Multiple components are easily generated using single-step emulsification</p>	<p>An extremely time-consuming method as single drops are generated one at a time</p>

Spray drying		<p>Can encapsulate wide range of moieties into MPs without significant loss</p> <p>Final drying step not required</p> <p>One step and reproducible</p> <p>Atomizers (nozzles) eliminate the need for complicated pre-preparation processes and enable continuous manufacture by utilization of liquid feeds via two separate channels</p> <p>Adhesion of MPs to inner walls of the spray-dryer</p> <p>Not suitable for temperature-sensitive compounds</p> <p>Difficult to control particle size</p> <p>Low yield, agglomeration of sticky particles</p>
Single (o/w) emulsion		<p>Solvent residuals</p> <p>Low yield, agglomeration of sticky particles</p> <p>Simplicity</p> <p>Suitability for temperature-sensitive compounds</p>
Double (w/o/w) emulsion		<p>Control of particle size</p> <p>Multiple steps</p> <p>Difficult to encapsulate higher concentration of hydrophilic drugs</p>

Figure 2.6 Methods for producing PLGA based MPs for sustained-release formulations: Advantages and disadvantages. Adapted from Mare et al.^[38, 52]

2.4.2 Microfluidic fabrication^[52]

This technique uses electrostatic forces to control the size and shape of the particles for enhanced tuning of drug release characteristics.^[53] Microfluidic systems have been employed for fabrication of complex drug carriers with precise size and composition leading to a predictable and tunable release profile.^[54, 55] Two continuous and immiscible streams (i.e., oil phase-polymer dissolved in organic phase, and water phase (surfactant dissolved in water)) are infused via two separate inlets.^[56] Monodisperse droplets are generated at the junction where the two streams meet due to the high shear stress. Surfactant dissolved in the water phase prevents coalescence of the droplets in the collecting flask. The droplet sizes are in the range 20–100 μm and 100–300 nm.^[57] The collection flask is immediately mounted on a rotary evaporator and the organic solvent is removed by evaporation under reduced pressure.

2.4.3 Spray drying^[51, 52]

In this technique, the solid-in-oil dispersion or water-in-oil emulsion is sprayed in a stream of heated air to rapidly evaporate the volatile organic solvent giving drug loaded solid MPs. The choice of solvent to be used in the process is decided by the nature of drug (hydrophobic or hydrophilic) to be encapsulated. This, together with the temperature of the solvent evaporation and the feed rate affects the final morphology of the microspheres.

2.4.4 Solvent Evaporation method ^[51]

2.4.4.1 Single emulsion process

This method is suitable for water insoluble emulsified with the larger volume of water containing a surfactant (e.g. PVA) (water phase) under continuous stirring. This is known as oil-in-water emulsification process. With the gradual evaporation of the organic solvent from the polymer solution droplet, polymer precipitates out and forms a hardened polymer

particle with drug crystals encapsulated within its matrix. The resultant solid MPs are then collected, washed and lyophilized to finally give a dry polymeric particles formulation.

2.4.4.2 Double emulsion process

Water-in-oil-in-water are best suited to encapsulate water soluble drugs.^[58, 59] This technique involves dissolving the drug in small volume of deionized water first and mixing with the organic phase with vigorous stirring to yield a water-in-oil emulsion. Next, this emulsion is added to larger volume of PVA aqueous solution and stirred continuously. Then the particles are finally collected in the same manner as in oil-in-water emulsion technique.

The emulsion solvent evaporation technique is a one-step process that gives MPs with higher yields, uniform layer thickness and a controllable particle size within a range of 20 μ m–1000 μ m. Only simple and economical lab apparatus, such as an overhead stirrer, is required in this fabrication method. Through a one-step solvent evaporation method, biomolecule drugs can be dispersed in the polymer solution and do not necessarily have to be dissolved in the polymer solution.

2.5 Limitations of PLGA MPs in delivery of biomolecules

Despite the immense popularity of PLGA as the most suitable polymer for delivery of biomolecules, there still are some limitations that need to be addressed. Many encapsulation techniques to load biomolecules into PLGA particles, unfortunately, have inherent limitations. For example, most of these encapsulation techniques utilize the emulsion approach. Due to the creation of oil/water interface in emulsion approach, the encapsulated biomolecules tend to undergo conformational changes at the aqueous/organic interface.^[60] The localization of these biomolecules at the aqueous/organic interface is to minimize the free energy of the system during

encapsulation. This, however, has a detrimental effect on the structural integrity and bioactivity of these biomolecules.^[61-64]

Likewise, the release of biomolecules from PLGA particles is also reported to be rather inefficient.^[65-67] The release is often characterized by an initial burst release, followed by a sluggish and often incomplete release.^[66, 67] This undesirable, incomplete release, as reported in the literature,^[61, 68-70] can be attributed to the interactions between the encapsulated biomolecules and the encapsulating polymer. When the biomolecule-loaded particle is delivered to a physiological environment, hydrolysis of the polymer occurs and shorter polymer chains or oligomers, with carboxylic acid-terminated end-groups, are generated. The microenvironment within the particles therefore becomes acidic over time that results in an auto-catalytic degradation effect.^[71, 72] Auto-catalytic degradation occurs when the acidic degradation products themselves act as a catalyst for the same degradation reaction.^[73] In addition, as many biomolecules contain positive charges, or transition from negative to positive charges at low pH^[74] (shown in **Table 2.2**), electrostatic interaction between a highly acidified (i.e. negatively-charged) polymer matrix and the encapsulated biomolecules^[75-77] will ensue. Also, under a low pH environment, acylation of the biomolecules with lactic and glycolic acids and their oligomers is catalyzed and polymer-biomolecule adducts are formed.^[78, 79] All these may influence release kinetics and possibly compromise the bioactivity of the biomolecule. Understanding such a phenomenon during release and developing a delivery system that circumvents these issues is therefore paramount and the key aim of this paper.

To address these issues, a number of studies have focused on different approaches, including chemical modification of the biomolecules^[9, 80-83] and the addition of stabilizing excipients such as $\text{Mg}(\text{OH})_2$, ZnCO_3 , $\text{Ca}(\text{OH})_2$.^[84, 85] However, these approaches result in other problems, such as uncontrolled burst release,^[82] limited utility in biomolecule protection due to non-homogeneous distribution of such hydrophobic excipients and induction of formation of covalent aggregates (for strong alkali as $\text{Ca}(\text{OH})_2$ as excipient) as a result of alkali induced thiol-disulphide exchange.^[61, 86]

Another approach would be to encapsulate biomolecules in its solid microcrystal form rather than a solution form.^[87] This is because any inter-molecular biomolecule interaction is relatively weaker in its crystal form as compared to a dissolved solution form.^[88] This eliminates the possibility of covalent aggregation of biomolecules via the thiol-disulphide exchange reaction that is commonly observed in aqueous biomolecule solutions.^[89, 90] In addition, as crystallized biomolecules have reduced area of contact with organic solvent during the emulsifying process, the denaturing effect due to the organic solvent is also minimized.

Table 2.2 Therapeutic biomolecules with positive or variable net charge. Adapted from Balmer et al^[74]

Protein/peptide	Molecular Weight (kDa)	Isoelectric Point (pI)	Charge per kDa	
			pH 5	pH 3
CXCL 10 (IP-10)	8.6	10.7	+1.38	+1.95
CXCL12(SDF1 α)	8.0	10.3	+1.42	+1.87
bFGF	16.4	9.9	+0.88	+1.64
PDGF-BB (dimer)	24.6	9.3	+0.83	+1.51
TGF- β 1(dimer)	25.6	8.2	+0.59	+1.13
BMP-2 (dimer)	25.8	7.9	+0.67	+1.41
IL-12p40	34.7	5.3	+0.07	+1.13
GM-CSF	14.5	5.0	-0.00	+0.97
EGF	6.2	4.6	-0.20	+1.02
Exenatide	4.2	4.5	-0.30	+0.91
Enfuvirtide	4.5	4.1	-0.70	+0.62
Thymalfasin	3.1	4.0	-1.27	+1.14

Lastly, the lattice structure of biomolecule crystals efficiently inhibits the migration of biomolecules to the aqueous/organic interface during encapsulation that further reduces loss of bioactivity.^[87] However, loading them in their microcrystal form still does not totally resolve the issue of incomplete release. In fact, with a reduced surface area of biomolecule crystals, this may reduce both dissolution and release rates. Also, biomolecule microcrystals embedded deep in the core of the particle would also be largely inaccessible to the release environment. Hence, developing a delivery system that circumvents these issues is therefore paramount and the key aim of this thesis.

It is thus hypothesized that encapsulating biomolecules in their microcrystal form into MPs of hollow morphology could address the above inherent problems encountered in biomolecules delivery. It is speculated that the accumulation of acidic degradation products can be significantly reduced through the use of hollow MPs which shall minimize polymer-biomolecule interaction. Also, loading crystallized biomolecules into hollow MPs could potentially reduce this interaction. This is especially so when the polymer degrades, as this would lead to the destabilization and non-covalent aggregation of acid-labile biomolecules. Biomolecules encapsulated within hollow MPs would also have shorter diffusion distance, thus promoting their release.

2.6 Current techniques of fabrication of hollow MPs

In the literature, hollow particles made of metals ceramics or polymers have been frequently reported. The template-based technique, i.e. coating the surfaces of colloidal templates with thin layers of desired material (or its precursor), followed by selective removal of the templates by means of calcination or chemical etching, has been the most widely used approach to obtain hollow particles. Based on the properties of templates, the approach can be categorized as: soft templates (micelles, block copolymer, aggregate etc.) and hard templates (silica, polymer nanoparticles, gold particles etc.).^[91-94]

Alternatively, hollow microparticle fabrication methods can be categorized as emulsion/interfacial polymerization, self-assembling, phase separation and spray drying.^[95] Thermo-sensitive poly(N-isopropylacrylamide) (PNIPAM) hollow MPs were prepared by interfacial polymerization at a temperature below the lower critical solution temperature of PNIPAM. The NIPAM monomer-contained W/O emulsion droplets, obtained by Shiras porous-glass (SPG) membrane emulsification technique, were polymerized at 20 °C at the W/O interface by UV irradiation to prepare hollow PNIPAM microcapsules.^[96]

Self-assembly of block copolymers followed by shell cross-linking and subsequent core degradation is frequently used to prepare shape and size invariant hollow spheres. This technique relies upon the micellization of diblock copolymers by self-assembly into spherical particles of core-shell morphology, followed by network formation selectively throughout the shell layer, and then degradation and extraction of the core material to leave the membrane-like shell as the hollow particles.^[97]

Hollow microcapsules for ultrasound echogenic contrast agents could be prepared by spray drying. A volatile core material such as ammonium bicarbonate was encapsulated with a polymer using the scalable encapsulation technique of spray drying, followed by freeze drying to decompose and evacuate the ammonium bicarbonate.^[98] Layer-by-layer (LBL) technique using alternate depositions of oppositely charged species on various templates and subsequent sacrificing core can produce diverse hollow spheres.^[99] Hollow microcapsules built by LBL assembly were prepared by dissolving the melamine formaldehyde (MF) templates coated with six double layers of poly(sodium 4-styrene sulfonic acid) (PSS) and poly(ethylene imine) (PEI) using hydrochloric acid.^[100]

Microfluidic fabrication technique (MFT) and the coaxial electro hydrodynamic microbubbling technique (CEHMT) have also been reported for hollow microparticle fabrication.^[101, 102] In MFT, three coaxial nozzles system produced a smooth coaxial jet comprising a carrier, annular shell and core streams, which was acoustically excited to break up into core-shell droplets to fabricate hollow MPs. Selective dissolution of the core

material is performed to obtain the hollow particles.^[101] Likewise in CEHMT, solution of water-insoluble polymethylsilsesquioxane was perfused through the outer needle of a co-axial needle arrangement while air is passed simultaneously through the inner needle, with both needles placed in an electric field, eventually collecting the microbubbles in the distilled water in hollow core-shell morphology.^[102]

2.7 Disadvantages of current fabrication techniques of hollow microparticles

Most of the currently available techniques to fabricate hollow MPs suffer from either of these drawbacks viz: 1. multiple steps needed, time consuming, 2. requirement of complex and costly equipment. For example, in the template-based fabrication technique, the core template over which the desired polymer had been coated earlier has to be removed by additional dissolution process. The dissolution process used can be deleterious to the encapsulated agents. Moreover, sometimes the polymer shell coating over the template has to be cross linked in order to withstand this dissolution process to retain the morphology. As such, the process becomes inherently laborious and time consuming. Similarly, the MFT technique also requires an additional, selective dissolution of the core material to achieve a hollow cavity. In CEHMT, there are many parameters that affect the electrohydrodynamic process, such as viscosity, density, electrical conductivity, relative permittivity along with processing parameters such as flow rates, applied voltage etc. With these, the successful fabrication of such MPs becomes rather complicated.

2.8 Reported effects of osmogen in particle morphology

Compounds that can create osmotic drive of water molecules towards themselves are known as osmogen. The effect of introducing osmogen in the particle fabrication process has been reported in several instances.^[103-105] Notably, for particles fabricated by water-in-oil-in-water emulsion solvent evaporation technique, T.Kissel et al. reported that the

addition of salt in the internal and/or external aqueous phases had significant impact on the overall morphology of the particles.^[103] The effect of salt on the final morphology of the particle was attributed to the osmotic pressure gradient and consecutive water influx into the system due to the inherent osmotic property of the salt.^[103] The porosity, specific surface area, particle size and surface morphology showed conspicuous variation with the changing amount of salt.

Several papers report addition of salts in the external water phase as a simple and efficient method to encapsulate drugs with high encapsulation efficiency and improved particle morphology. Ge Jiang et al. reported reduced protein burst release, sustained release profile and retained integrity of the protein with introduction of salt/sugar to the external water phase as a result of dense particle formation with reduced porosity.^[104] Pean et al. reported remarkable reduced burst effect when he used 10% NaCl in the extraction medium to prepare nerve growth factor (NGF)-loaded PLGA MPs.^[105]

Likewise, Thomas P. Russel et al. showed that the origin of the process of self-emulsification is the osmotic drive created by the salt impurities present within the amphiphilic block copolymers used for emulsion stabilization. They further reported utilizing this osmotic property of the salt impurities to tailor the emulsion droplets morphology.^[106]

These reports agree that introduction of salt/sugar or any osmogen into particle fabrication by the emulsion technique affects the morphology due to the influx of water. Thus, controlling the osmotic drive can be an ingenious method of controlling the internal and external morphology of the particles produced. While, the effects of osmogens are briefly reported on multiple occasions, the literature still lacks robust explanation on the relationship between the used osmogen and the achieved morphology of the particle. Thus, in this thesis, the aim is to understand this phenomenon and use the knowledge to fabricate hollow MPs by simply controlling the effects of osmogen on the particle morphology.

2.9 Summary

Biomolecules such as proteins and peptides have immense potential in therapeutics due to their several advantages over small-molecule drugs, such as exquisite specificity and low toxicity. However, their short biological half-lives, delicate structures, unique physiochemical properties and tendency to be rapidly eliminated by the body significantly limits their potential. As such, development of steady state delivery systems which can protect the biomolecules is mandatory to capitalize on the potential of biomolecules. Biodegradable polymeric MPs of PLGA have been a popular and widely used delivery system for biomolecules due to its long proven clinical history and desirable physical and chemical properties. Several fabrication methods such as solvent evaporation, microfluidics, spray drying, phase separation etc. have been used in producing PLGA MPs to encapsulate biomolecules. These MPs undergo degradation by hydrolysis of ester linkage into non-toxic biodegradable monomers, lactic acid and glycolic acid, during release of biomolecules. However, the accumulation of these acidic degradation products in the MPs have been frequently reported to cause problems due to the creation of acidic microenvironments. Consequently, the release of the biomolecules is incomplete due to the polymer-biomolecules interaction and bioactivity is compromised due to the high acidity. Several approaches revolving around chemical modification of the biomolecule or addition of stabilizing excipients have been developed to address this issue but with limited success. Encapsulating biomolecules in microcrystal form into particles of hollow morphology instead of solid morphology could potentially reduce the accumulation of acidic degradation products. Currently available methods to fabricate hollow MPs are either inherently time consuming and the setup required is rather not simple. As such, developing a simple and effective method to fabricate hollow MPs using the osmotic property of osmogen could be an interesting approach to encapsulate the biomolecules.

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Chapter 3

Experimental Methodology

This chapter presents the materials and the experimental methodologies used in this study. The methods used are explained and the rationale of using specific equipment are elucidated. The first section reveals the technique developed for fabricating hollow microparticles (MPs) and the process parameters tested to develop a correlation between the parameters and the morphology. The second section includes the methods applied to analyze the particle morphology by Scanning Electron Microscopy (SEM), evaluate encapsulation efficiency and release profiles by Reversed Phase High Performance Liquid Chromatography (RP-HPLC), structural integrity by Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI TOF MS) and Circular Dichroism Spectroscopy; and bioactivity of bovine insulin using cell proliferation assay. The last section reveals the methods and equipment used to evaluate the efficacy of hollow MPs over solid MPs by evaluating the release profile, intraparticle pH measurements, mass loss, molecular weight changes, polymer-biomolecule adsorption, structural integrity and bioactivity of Glucagon Like Peptide-1.

3.1 Emulsion solvent evaporation technique

The emulsion solvent evaporation technique is a one-step process that gives MPs with higher yields, uniform layer thickness and a controllable particle size within a range of 20 μm –1000 μm . Only simple and economical lab apparatus, such as an overhead stirrer, is required in this fabrication method. Through a one-step solvent evaporation method, drugs can be dispersed in the polymer solution and do not necessarily have to be dissolved in the polymer solution. Thus, this method of fabricating MPs has been frequently used for fabrication of polymer MPs loaded with small molecule drugs or biomolecules such as proteins and peptides. Briefly, this technique requires dissolving a biodegradable polymer in a volatile organic solvent which is subsequently emulsified with aqueous phase containing a surfactant. The drugs to be encapsulated should be dispersed in the organic phase before emulsifying the polymer containing organic phase with the surfactant containing organic phase. The surfactant helps in stabilizing the droplets of organic phase formed upon application of shearing force by the overhead stirrer. Gradual removal of volatile organic solvent with continuous stirring finally leads to fabrication of solid hardened MPs with drugs encapsulated within the polymer matrix.

Although this technique of particle fabrication is simple and straightforward, it provides plenty of parameters that could be optimized to achieve particles of different morphologies. As the focus of this thesis is in proposing and validating the use of hollow MPs as superior carriers over solid MPs for encapsulation and delivery of biomolecules, our interest was in modifying this technique to produce hollow MPs. As such, it is hypothesized that this fabrication technique can be modified to produce hollow MPs in a single step by introducing osmogen into the organic phase of the solution before emulsifying with the external aqueous phase. This hypothesis was put to test by loading the hollow MPs with biomolecules (bovine insulin and GLP-1) and several attributes are analyzed and compared with that of solid MPs to validate the superiority of the hollow MPs.

3.2 Materials

Poly(D,L-lactide-co-glycolide, 50:50) (PLGA) (intrinsic viscosity (IV): 1.18 and 0.2, Purac) was purchased from Purac Biochem, and used without further purification. Poly(vinyl alcohol) (PVA) (MW: 30–70 kDa, Sigma–Aldrich), trifluoroacetic acid (TFA), sodium chloride (NaCl), potassium chloride (KCl) and sucrose was purchased from Sigma Aldrich. High performance liquid chromatography (HPLC)-grade deionized water (Millipore, 0.22 μm), dichloromethane (DCM) and acetonitrile (ACN) and tetrahydrofuran (THF) (Tedia Co., Inc.) were used as solvents. The therapeutic biomolecules bovine insulin and Glucagon Like Peptide-1 (GLP-1) were purchased from Sigma Aldrich and Prospec respectively and used as received without any further purification. HTRF active GLP-1 assay kit was purchased from Cisbio Bioassays.

For cell culture study, insulin sensitive human breast cancer cell line MCF-7 was obtained as a gift from the Agency for Science, Technology and Research (A*STAR) (Singapore). Phosphate-buffered saline (PBS) (Gibco), dulbecco's modified eagle medium (DMEM) with and without phenol red (Gibco), fetal bovine serum (FBS) (Gibco), penicillin-streptomycin (Gibco), L-glutamine (Gibco) and 0.25% trypsin–EDTA (Gibco) were purchased from Life Technologies Holdings Pte. Ltd. Cell counting kit-8 was purchased from Dojendo Molecular Technologies, Inc. All other reagents were of analytical grade and were used as received.

3.3 Fabrication of MPs

Hollow MPs were fabricated using an (oil/water) emulsion solvent evaporation technique^[1] with a simple but important modification to the fabrication process, i.e. the inclusion of an osmogen at an optimized amount. Briefly, 0.2 g of PLGA (6.67% (w/v)) and 5 mg of NaCl (2.5% (w/w)) were added to 3 mL of dichloromethane (DCM). The content was put under magnetic stirring for 3.5 hours and vortexed in between to ensure that the polymer was completely dissolved, and finer salt particles were uniformly

distributed within the polymer solution. Subsequently, the polymer/salt solution was poured into 50 mL of polyvinyl alcohol (PVA) aqueous solution (3% (w/v)) and emulsified using overhead stirrer (Calframo BDC 1850-220) for 3.5 hours at 500 rpm at room temperature (25 °C). This amount of PVA aqueous solution was carefully chosen based on solubility limit of DCM in water (2% (v/v)). At this oil to water ratio (o/w: 0.06), the slow diffusion of DCM into the external aqueous phase led to slower precipitation of the polymers. In this work, DCM was chosen as the solvent and PVA was chosen as the surfactant because of their Food and Drugs Administration (FDA) approval.^[2-4] Moreover, DCM is less toxic than other common solvents such as chloroform.^[4] Likewise, PVA is also known as non-toxic and widely used in the solvent evaporation method.^[5] Finally, the hardened MPs were then centrifuged (3000 rpm, 5 mins) and washed three times with de-ionized water to get rid of the PVA. These MPs were dried overnight in a freeze-dryer and stored in -20 °C until further use. Removal of the water from the particles by freeze drying finally produced dry hollow MPs. **Figure 3.1** below shows the schematics of the fabrication process.

Figure 3.1 Schematic illustration of the process of hollow MPs fabrication

MPs of different sizes and morphologies were also prepared by altering the starting polymer solution concentration, type and amount of osmogen, different polymer and stirring speed. **Table 3.1** summarizes the parameters that were used in this study. To fabricate biomolecules-loaded MPs, biomolecules crystals were dispersed in the polymer solution together with or without the osmogen.

Table 3.1 Process parameters used in the fabrication of the hollow MPs by osmogen dispersed O/W emulsion solvent evaporation technique

	Polymer solution conc. (% w/v)	Oil-to-water ratio	Osmogen		Stirring speed (rpm)	Polymer	
			Type	Amount (mg)		Type	Amount (mg)
Reference hollow microparticle	6.67	0.06	NaCl	5	500	PLGA	200
Effect of polymer solution concentration	3 10	0.06	NaCl	5	500	PLGA	200
Effect of amount of osmogen	6.67	0.06	NaCl	40 20 10 2.5	500	PLGA	200
Effect of type of osmogen	6.67	0.06	NaCl KCl Sucrose	5 5 10	500	PLGA	200
Effect of stirring speed	6.67	0.06	NaCl	5	1000 500	PLGA	200

3.4 Morphological analysis of MPs by Scanning Electron Microscopy (SEM)

SEM is one of the most popular techniques used to characterize the morphology of different kinds of samples.^[6-8] The surface and internal morphologies of the fabricated MPs of each formulation were analyzed using a scanning electron microscope operated at

5 KeV (SEM JEOL 6360A). This is a type of electron microscope which uses a focused beam of high-energy electrons to scan the surface of the sample to produce images. The electron beam is first emitted from an electron gun fitted with a tungsten filament. This beam is focused by one or two condenser lenses to a spot of 0.4nm to 5nm in diameter. The beam then passes through pairs of scanning coils in the electron column, typically in the final lens, which deflects the beam in x and y axes so that it scans in a raster fashion over a rectangular area of the sample source.^[9] Upon interaction of these primary electron beams with the sample, significant amount of kinetic energy is dissipated as a variety of signals. This happens within a teardrop shaped volume^[10] (Figure 3.2b) of the specimen known as interaction volume that extends from less than 100 nm to approximately 5 μm in into the surface.^[11]

Figure 3.2 Digital image of an actual SEM machine (a), interaction volume showing the regions of various electron-specimen interactions^[11] (b) and schematics of a SEM machine (c)

The various types of signals produced are secondary electrons (SE), back-scattered electrons (BSE), characteristic X-rays and light (cathodoluminescence) (CL), absorbed

current (specimen current) and transmitted electrons.^[12, 13] SE is the mostly used signal in imaging for showing morphology and topography of the sample.^[14] The secondary electrons are collected by a detector (scintillator-photomultiplier system) and is displayed as a two-dimensional intensity distribution that can be viewed and photographed on an analogue video display or can be displayed and saved as digital image by analog-to-digital-conversion.^[9] The schematics of SEM is shown in **Figure 3.2c**. SEM analysis is considered to be "non-destructive", that is no volume loss of the sample, so it is possible to analyze the same materials repeatedly.^[10, 15]

Energy Dispersive X-ray Spectroscopy was also used in conjunction with SEM. This is a chemical microanalysis technique which uses characteristic X-rays emitted from the sample to characterize the elemental composition of the sample. The EDX detector creates a charge pulse proportional to the energy of detected x-ray, then converts to a voltage signal which is further processed. Finally, upon evaluation of the spectrum of x-ray energy versus counts, elemental composition of the sampled volume is determined.^[16]

Samples were prepared by uniformly dispersing freeze-dried MPs onto metal stubs and cross-sectioning approximately at the center line with a surgical grade razor blade. Cross-sectioned MPs were finely coated with gold using sputter coater model SPI-Module and inspected using the SEM for morphology. However, for EDX analysis, a Carbon Coater was employed to coat the samples with carbon instead of gold. The coated samples were analyzed at 15 keV to obtain the EDX spectrum. Finally, by comparison of the spectrum obtained from the sample and the controls, results were obtained.

3.5 Particle size analysis

ImageJ, a public domain, Java based image processing program was used to analyze the SEM images for particle size distribution. For each batch, an average of 100 random MPs were measured. The mean diameter of the MPs was taken to be the number average of the diameters of the particles captured on the SEM image.

3.6 Biomolecules for encapsulation

In this thesis, the MPs are tested as carriers for the delivery of a hydrophobic and a hydrophilic biomolecule. This is to assess the versatility of the MPs in the encapsulation and delivery of biomolecules of wider spectrum in terms of hydrophilicity/hydrophobicity. Bovine insulin is used as a model hydrophobic biomolecule and Glucagon Like Peptide-1 is used as a model hydrophilic biomolecule. **Figure 3.3** below shows the amino acid structure of these biomolecules.

Figure 3.3 Amino acid structure of bovine insulin (left) and GLP-1 (right)

3.7 Encapsulation efficiency and in vitro release study

It is important to obtain the encapsulation efficiency and the release profiles of the encapsulated biomolecules for the delivery systems designed. With these knowledge, necessary optimization of the system would be performed to make the delivery system more efficient.

3.7.1 Encapsulation efficiency

The percentage of drug that is successfully encapsulated into the particles is given by the encapsulation efficiency. It is one of the preliminary characterization that is imperative to determine the efficacy of the MPs in entrapping the drugs. Thus, our study also involves determination of encapsulation efficiency of various batches that are fabricated for further

testing. For insulin loaded batches, 10 mg (n=3) of each formulation were dissolved in 0.5 mL dichloromethane. After the particles were dissolved, 1 mL of 0.01 N HCl was added for extraction of the insulin. Likewise, for GLP-1 loaded batches, (10 mg, n=3) of each formulation were dissolved in 0.2 mL acetonitrile and 1 mL of DI water was added to the solution. The solutions were vortexed vigorously, allowed to settle and centrifuged (5000 rpm, 5 minutes). Finally, the amount of insulin/GLP-1 in the upper supernatant was determined by using a reversed-phase High Performance Liquid Chromatography. The encapsulation efficiency was calculated by using the equation^[17] give below.

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Actual drug loading}}{\text{Theoretical drug loading}} \times 100$$

All the measurements were carried out in room temperature.

3.7.2 In vitro release study

For insulin loaded MPs, 20 mg of MPs (n=3) were suspended in 2 mL of Phosphate Buffer Saline (PBS) solution (pH 7.4) in Eppendorf tubes and incubated at 37 °C. Likewise, the release study of GLP-1 loaded MPs were carried out by incubating 20 mg of MPs in 1.5 mL PBS with 0.01% BSA in Eppendorf tube and kept on a rotor-shaker at 37 °C. At regular time interval, the tubes were centrifuged, and 1 mL of the supernatant was withdrawn for analysis by HPLC and replenished with 1 mL of fresh medium.

The cumulative amount of biomolecules released at time, t, was calculated from the equation^[18] below and was plotted over time.

$$\begin{aligned} &\text{Cumulative amount of insulin release at time t (\%)} \\ &= \frac{\text{amount released at time t}}{\text{total amount released at time infinity}} \times 100 \end{aligned}$$

3.7.3 Reversed-phase High Performance Liquid Chromatography

Figure 3.4 Schematic representation of the HPLC chromatography^[19] (a), schematic representation of binding of a peptide and a protein, to an RP-HPLC silica-based sorbent (b) and an actual HPLC machine (c)

This technique involves separation, identification and quantification of molecules in a mixture on the basis of their hydrophobicity. The technique involves pumps eluting a moderately polar liquid solvent under high pressure. The sample solution is injected and carried by the flowing solvent through a column filled with a solid adsorbent material (the sorbent) attached with immobilized non-polar hydrophobic ligand (**Figure 3.4a-b**). The elution can be isocratic (the concentration of organic solvent is constant) or gradient (the

amount of organic solvent is increased over time). Due to the difference in hydrophobicity of each components in the sample, they bind differently with the immobilized hydrophobic ligand attached to the stationary phase in the column. This causes the difference in the flow rates for different components leading to the separation of components as they flow out of the column and get detected by a UV detector. The components are, therefore, eluted in order of increasing molecular hydrophobicity.

In our study, HPLC measurement was carried out using a BC-Zorbax SB-C18 Analytical (4.6 x 250 mm, 5 μ m) chromatographic column. The mobile phase, for detection and quantification of bovine insulin, comprised of ACN and water (30:70) containing 0.1% TFA. Sample injection volume was 100 μ L, the mobile phase was eluted at a flow rate of 1.5 mL/min and effluent was monitored at 214 nm. Freshly prepared insulin solutions of known concentrations (0.01-1 mg/mL) in triplicates were used each time to prepare the calibration curve for quantification of the concentration of insulin in the sample. The obtained R^2 value of 0.997 ensured that the calibration curve prepared was reliable.

Likewise, for GLP-1, the mobile phase comprised of eluent A (20 % ACN; 80% water, 0.05% TFA) and eluent B (50% ACN and 50% water, 0.05% TFA) eluted at a flow rate of 2 mL/min in gradient mode. The gradient used for separation was 50% A from 0 to 4.5 min, followed by a linear gradient that increased the concentration of B from 50% to 60% from 4.5 to 7.5 min, and 60% to 100% for additional 0.5 min and then returned to starting condition for 2 minutes. Sample injection volume was 100 μ L and effluent was monitored at 200 nm. Freshly prepared GLP-1 solutions in PBS of known concentrations (0.001-0.1 mg/mL, n=3) were measured and plotted against the total peak area of the biomolecule to prepare the calibration curve of GLP-1.

3.8 Hydrolytic degradation of the MPs

PLGA MPs undergo hydrolytic degradation during the release. This has effects on the release kinetics of the encapsulated biomolecules and on the microenvironment of the

particles. Hence, hydrolytic degradation study of the MPs was carried out to determine the change in the molecular weight of the polymer MPs under degradation.

10 mg of GLP loaded MPs (n=3) of each formulation were suspended in 1 mL PBS in Eppendorf tubes and incubated at 37 °C in a shaking incubator. At predetermined time points, MPs were centrifuged (5000 rpm, 5 minutes), washed with DI water and lyophilized overnight. The samples were analyzed for the change in molecular weight by Gel Permeation Chromatography (GPC) using Agilent 1100 Series LC System.

3.8.1 Gel Permeation Chromatography

This is a technique which separates molecules such as polymers or proteins based on differences in their molecular weight. While the technique is mostly like HPLC, the separation principle is different because of the different type of column used in this technique. The column consists of a hollow tube tightly packed with extremely small polymer beads that have tiny pores of different sizes. As the eluent carrying the samples travels down the column, some components of the sample that are smaller in size diffuse freely into the pores and their movement through the column is slowed, while the larger ones cannot enter as many pores. Larger molecules simply pass by the pores and therefore the larger the size of the compound, the faster is the retention time. Hence molecules are separated in order of decreasing molecular weight, with the largest molecules eluting from the column first (**Figure 3.5**).

In our study, the MPs were dissolved in 1 mL of THF. This polymer solution was then analyzed at 30 °C with THF as eluting solvent, using a refractive index detector on an Agilent 1100 Series LC System. The flow rate was maintained at 1 mL/min. Calibration of the average molecular weight was determined using polystyrene standards with molecular weights ranging from 162 to 47,190 g/mol (Polystyrene Easycal Vial, Agilent).

Figure 3.5 Schematics of the GPC separation process for a polymer sample containing mixture of polymer A and polymer B^[20]

3.9 MALDI TOF MS for analysis of molecular mass to detect degradation of biomolecules

MALDI TOF MS stands for Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry. This is a powerful tool used primarily for determination of molecular mass of proteins and peptides. In this technique, a laser light is used in conjunction with a chemical matrix to ionize sample by imparting charges to the sample in question. The schematics of the working principle MALDI TOF machine is shown in **Figure 3.6a**. The sample is usually mixed in a solution of an ultraviolet absorbing compound. It is recommended to use α -Cyano-4-hydroxycinnamic acid (α -CHCA) solution as matrix compound for detection and analysis of proteins and peptides of molecular size less than 10 kDa. The matrix needs to be carefully chosen based on the molecular size of the sample of interest so that no issues are faced regarding the detection of molecular mass of the sample. Upon evaporation of the liquid, the sample consists of dry crystals of the sample mixed with the matrix. When this mixture is irradiated with a laser, the matrix assists in volatilization and ionization of the samples. These charged ions are then accelerated through a flight tube to the detector due to the potential difference applied between the sample slide and the ground. The velocity attained by the attracted ions is

determined by the law of conservation of energy. Lighter ions with a smaller m/z value and more highly charged ions move faster through the tube until they reach the detector. As such, depending upon the mass-to-charge ratio (m/z) value of ions, the time of flight differs accordingly from ions to ions. Thus, the time-of-flight (TOF) is directly proportional to the mass of the molecule and has mass resolution of approximately 0.03%. Finally, a chromatogram is generated by presenting intensity (y-axis) versus m/z (x-axis). The peak intensities are expressed as a percentage of the most intense signal (the base peak). **Figure 3.6b** shows the picture of an actual MALDI TOF MS machine.

Figure 3.6 Schematic representation of the working principle of the MALDI TOF MS for analysis of molecular mass^[21] (a) and digital picture of the MALDI TOF MS machine (b)

This instrument was used in our study to analyze the encapsulated biomolecules to detect the fragments of biomolecules that might have formed due to degradation of biomolecules in the microparticle matrix. This was used at two occasions 1. to analyze the bovine insulin extracted from solid MPs and hollow MPs and 2. to analyze Glucagon Like Peptide-1 extracted from solid and hollow MPs. The rationale behind using this instrument is to determine if the change in the morphology of the MPs have different

effects if any, on the degradation of the encapsulated biomolecules by analyzing the molecular mass detected by the equipment. For this, the extracted solutions of the biomolecules were mixed with the matrix consisting of solution of α -cyano-4-hydroxycinnamic acid as these are considered suitable for the proteins and peptides less than 10 kDa in mass. The mixture was then applied on a clean sample plate and air dried after which it was analyzed using MALDI-TOF instrument (Shimadzu, Japan).

3.10 Analysis of structural integrity of biomolecules by Circular Dichroism Spectroscopy

The structural integrity of the entrapped insulin in the MPs were analyzed using Circular Dichroism (CD). This is an excellent tool to probe into the changes in the secondary structure of biomolecules to assess its structural conformity or stability.^[22] This technique basically involves passing of a monochromatic light through a Photo Elastic Modulator (PEM). The linear polarized light is converted into alternating left and right handed polarized light by PEM. These two polarized lights are differently absorbed by the sample molecule and the difference in absorption is detected with a Photo Multiplier Tube (PMT) which is translated into a CD plot.^[23] This is depicted in **Figure 3.7a**.

Thus, proteins with regular structure would result in spectrum having both positive and negative signals while the absence of regular structure results in zero CD intensity.^[25] This is predominantly based on the excitation of electronic transitions in amide groups of proteins. CD spectra between 250 and approximately 190 nm (far-UV spectral region) is studied for the different secondary structural types such as alpha helix, parallel and antiparallel beta sheet, turn, and other. The peptide backbone forms characteristic secondary structures such as α -helices, β -pleated sheets, turns, and disordered sections with specific Φ , Ψ dihedral angles and H-bond patterns affecting the CD spectrum.^[26, 27] **Figure 3.7c** shows the characteristic CD spectrum for different types of secondary structures of proteins.

Figure 3.7 Picture showing the principle behind CD spectroscopy^[24] (a), an actual CD spectroscopy machine (b) and the characteristic CD spectra of secondary structures of proteins^[25] (c)

Insulin remaining in the hollow MPs was extracted after 21 days to be examined by CD spectroscopy. Briefly, 100 mg ($n=3$) of hollow MPs were incubated in PBS at 37 °C for 21 days. The samples were centrifuged (5000 rpm, 5 minutes) and washed three times with deionized water to wash away the insulin released in the PBS during the period. Finally, the insulin entrapped in the MPs was extracted with the same method as described earlier. The extracted insulin was then analyzed using CD spectroscopy. Measurements were performed using 1 cm path cuvette at 100 nm min⁻¹ scanning speed, with a step size of 0.5 nm at 25 °C and wavelength range of 190-260 nm (far UV). CD spectrum of freshly prepared insulin solution and denatured (heated for 24 hours) insulin were also obtained to serve as controls. Each spectrum is the average of 10 runs.

3.11 Assessment of bioactivity using human breast cancer cell line MCF-7 proliferation assay

The bioactivity assessment would provide insight into whether the biomolecule has lost its therapeutic value due to loss in its potency as a result of degradation/denaturation or not. Insulin sensitive human breast cancer cell line MCF-7 was used to assess the bioactivity of the bovine insulin. Briefly, after 21 days of incubation in PBS at 37 °C, the remaining insulin extracted from the MPs was quantified using reversed phase HPLC as described earlier. Cells were seeded at 10000 cells per well in 96 well plates in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 1% penicillin and 1% L-glutamine. When the cells had reached 70% confluency, the cells were serum starved for 12 hours and cultivated for 4 days in serum free DMEM medium supplemented with 10 µg/mL of extracted insulin from the hollow MPs (n=10). Medium supplemented with 10 µg/mL of freshly prepared bovine insulin served as a positive control and medium (without insulin and FBS) served as a negative control (n=10). Serum free conditions were employed so that the proliferative effect can be attributed to the insulin which can then be correlated to the bioactivity of insulin. Finally, cell number was quantified using Cell Counting Kit-8 following the protocol supplied by the manufacturer (Dojendo Molecular Technologies).

3.12 Mass loss study

A mass loss study was performed to quantify the loss in the mass of the particles before and after the particles are suspended in the release medium over a longer duration. A weighed mass (W_{initial}) of dry particles was suspended in PBS under rotary shaker at 37 degrees. At predetermined intervals, the particles were collected and freeze dried. The dried particles were weighted again to determine the new mass (W_{new}). Finally, the mass loss was calculated based on the difference between the W_{new} and the W_{initial} . The values were normalized by dividing with their initial weights, expressed in terms of percentages and were plotted against time. The equation below was used for the calculation.

$$\text{Mass Loss (\%)} = \frac{W_{\text{initial}} - W_{\text{new}}}{W_{\text{initial}}} \times 100$$

3.13 Measurement of biomolecule adsorption to polymer by bulk concentration measurement

A simple method was used wherein blank polymer MPs are dispersed in appropriate media and mixed with biomolecule solution allowing enough interaction time.^[28] MPs are then removed by centrifugation and the supernatant is measured for the biomolecule concentration. The adsorption of biomolecule to the polymer was measured in two conditions 1. varying particle concentration and 2. varying pH of the medium. For the first experiment, 25 mg of MPs was suspended in 0.5 mL PBS adjusted to different pH values (7, 4 and 2). Then GLP-1 solution was added to the MPs suspension to achieve the final biomolecule concentration of 2.5 µg/mL. This suspension was then kept on a roto-shaker at 37 °C for 30 mins. The suspension was then centrifuged (5000 rpm, 5 mins) and 200 µL of clear supernatant was withdrawn and analyzed by HPLC for biomolecule concentration. Likewise, a study was also carried out with varying MPs concentration i.e. 0, 1, 10, 50 and 100 mg/mL in PBS adjusted to pH 2. All the samples were measured in triplicate and controls in the absence of the polymer MPs were included.

3.14 Measurement of change in intraparticle pH upon degradation of MPs

The average pH of the intraparticle microenvironment in both the hollow MPs and solid MPs was studied using a previously described technique^[29, 30] that is based on the total aqueous volume of hydrated MPs. A calibration curve was prepared by obtaining a correlation between the known pH of lactic acid monomers in PBS, and lactic acid monomer in the mixture of polymer, PBS and DI water. Briefly, 10 mg of MPs (n=3) for each formulation were incubated in 1 mL of PBS in 1.5 mL Eppendorf tubes on a rotor-

shaker at 37 °C. At predetermined time points, the tubes were centrifuged, and the supernatant was removed. 800 µL of acetonitrile was then added to the centrifuged polymer pellet to dissolve it. To this, 200 µL of DI water was added and vortexed vigorously and measured for pH with a pH small probe (Mettler Toledo, Columbus, OH). The pH probe is reported to behave satisfactorily in organic solvent-water mixtures up to 90 wt% of organic solvent.^[30] Hence the readings obtained are considered reliable. Also, the pH of the supernatant was measured.

3.15 Bioactivity assessment of GLP-1 using HTRF active GLP-1 assay kit

The bioactivity of GLP-1 was assessed using a HTRF active GLP-1 assay kit. This kit uses a sandwich immunoassay involving two monoclonal antibodies, one labelled with Lumi4Tb-Cryptate (Donor) and the second with d2 (Acceptor).^[31] The principle of detection is based on HTRF® technology. The two antibodies bind to the active GLP-1 and upon the excitation of the donor with a light source, Fluorescence Resonance Energy Transfer (FRET) towards the acceptor is triggered (**Figure 3.8a**) which in turn fluoresces at 665 nm wavelength. This fluorescence signal intensity is proportional to the antigen-antibody complexes formed and therefore to the active GLP-1.

The MPs of both the formulations from release study were collected at day 7 and the GLP-1 that was still entrapped into the particles was extracted by a similar method discussed earlier and analyzed for degradation and measured for concentration by HPLC. The extracted GLP-1 from the formulations were then diluted with DI water with 0.05% Triton X100 and 0.04% of Tween 20 to normalize the concentration to 1000 pg/mL. These solutions were analyzed for bioactivity using a HTRF active GLP-1 assay kit from Cisbio following the protocol provided with the kit (**Figure 3.8b**). Briefly, 5 µL each of the HTRF conjugates (d2-conjugate and cryptate conjugate) were premixed and added to 10 µL of the sample in White 384-well, low volume plate. The plates were centrifuged at 4000 rpm for 5 minutes and then incubated overnight at room temperature. Finally, the

plates were scanned by a HTRF compatible cell plate reader and the data was analyzed. All the samples were measured in triplicates.

Figure 3.8 Principle of the active GLP-1 sandwich assay (a) and representation of working protocol at a glance (b)^[32]

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Chapter 4

Developing an osmogen mediated fabrication technique to produce hollow PLGA microparticles

This chapter presents the results pertaining to the development of fabrication technique for poly(D,L-lactide-co-glycolide) microparticles (PLGA MPs) of hollow morphology, and development of a correlation between the process parameters and the morphology of MPs. Emulsion solvent evaporation technique was modified by adding osmogen in the formulation to yield MPs of hollow morphology. The first section of this chapter presents the results obtained by varying different parameters of the fabrication process such as, initial polymer solution concentration, amount of osmogen, type of osmogen and stirring speed. The role of osmogen in the fabrication of hollow MPs and the effects observed upon altering the fabrication process parameters are then discussed together in the second section to develop an understanding of how these parameters affects the final morphology of the MPs. This understanding would allow for a controlled fabrication of hollow PLGA MPs through a single-step osmogen mediated emulsion solvent evaporation technique.

*Most of the texts and results in this chapter have been published and reprinted from a. Journal article. **Kharel S**, et al. "Osmogen-Mediated One-Step Technique of Fabricating Hollow Microparticles for Encapsulation and Delivery of Bioactive Molecules." *Macromolecular bioscience* 17.4 (2017) (Copyright 2017, with permission from Wiley Online Library), b. Patent. Loo SC, **Kharel S**, Lee WL. "Method of preparing hollow microparticles and hollow microparticles prepared thereof." **PCT/SG2015/050483** filed on 2 December 2016.

4.1 Fabrication of hollow MPs and characterization using SEM

A reference set of hollow MPs was first fabricated using the osmogen mediated emulsion solvent evaporation technique described in section 3.1. Briefly, PLGA polymer and osmogen were added to dichloromethane (DCM). The content was put under magnetic stirring for 3.5 hours and vortexed in between to ensure that the polymer was completely dissolved, and finer salt particles were uniformly distributed within the polymer solution. Subsequently, the polymer/salt solution was poured into polyvinyl alcohol (PVA) aqueous solution and emulsified using an overhead stirrer for 3.5 hours at 500 rpm at room temperature.

Figure 4.1 Scanning electron micrograph (SEM) showing an overall size distribution of reference MPs (a), cross section of a reference MPs (b) and particle size distribution histogram of reference MPs (c)

The osmogen scattered in the polymer solution droplet created an osmotic drive withdrawing the water from the external aqueous phase into the polymer solution droplet which coalesced into the core while the polymer precipitated forming the shell of the MPs resulting in MPs with polymer shell and water filled core. Upon freeze drying, the water was removed resulting in the hollow MPs.

After fabricating the reference set of MPs, other batches of MPs were fabricated to study the effect of different processing parameters, as summarized above in **Table 3.1**, on the final morphology of the MPs. One parameter was changed at a time while keeping the other parameters constant to correlate the change in the morphology with the parameter that was changed. The SEM micrographs of the fabricated MPs and the overall size distribution of the reference batch are shown in **Figure 4.1**. The particle sizes were analysed using ImageJ software and determined to be $84 \pm 25 \mu\text{m}$. The MPs were observed to have a smooth external surface. Cross-sections of successfully cut particles was observed in each of the SEM images. The results revealed hollow morphology with a hollow core formation effectiveness estimated to be ~90%.

4.2 EDX analysis to determine fate of osmogen used in fabrication

EDX analysis of the cross sectioned hollow MPs was performed to elucidate whether the salt (NaCl as osmogen) introduced in the organic phase during the fabrication process was retained in the particles or leached out from the particles upon dissolution by the water withdrawn from the external aqueous phase. The cross sectioned particles were coated with carbon and analysed at 15 keV. The result showed presence of the osmogen in the particles. The EDX spectrum revealed a strong peak for sodium and chlorine at 1.04 keV and 2.62 keV respectively (**Figure 4.2**). It is safe to conclude that these peaks for sodium and chlorine are from the NaCl that was introduced in the fabrication and thus, the salt introduced in organic phase is present in the MPs.

Figure 4.2 Energy Dispersive X-Ray (EDX) spectrum showing the presence of NaCl in the hollow MPs that was fabricated by the introduction of NaCl in the organic phase.

4.3 Study of effects of fabrication parameters on final morphology

In the past few decades, PLGA MPs of different sizes had been fabricated by emulsion solvent evaporation techniques. There could be direct implications of the difference in sizes of the particles on the medical applications. Thus, different process parameters, such as the initial polymer solution concentration and stirring speed had been manipulated to obtain the required sizes. It had been shown that an increase in the polymer solution concentration could lead to a larger particle size. This can be attributed to the increase in the viscosity of the polymer solution. The higher viscosity of the polymer solution presents a resistance against the shear force applied by the overhead stirrer during emulsification and thus decreases the chance of breaking the polymer solution into smaller droplets. As a result, particles of larger sizes are usually fabricated. Similarly, a

decrease in initial polymer solution concentration would result in a decrease in the size of the particles fabricated for the same reason. Likewise, particles size has been shown to be affected by increasing or decreasing the stirring speed of the overhead stirrer. The rationale behind this is same as that of effect of initial polymer solution concentration. The increase in stirring speed of the overhead stirrer translates into an increase in shear stress applied on the emulsion. This larger tangential stress leads to breaking the emulsion droplets into further smaller droplets. Also, the increase in stirring speed reduces the relative viscosity of the emulsion, which too aids in decreasing the size of the droplets formed.

While there are plenty of reports on the effect of changing those parameters in the fabrication process, these reports are generally for emulsion solvent evaporation technique to fabricate solid MPs. However, the technique, in this work, has been modified by introducing osmogen to fabricate hollow MPs. So, it is of interest to investigate whether changing those parameters would manifest similar effects when the technique has been modified by inclusion of additional component, i.e. osmogen. As such, experiments were carried out for a comparative study by fabricating particles keeping all the parameters constant except the one whose effect was to be investigated. Furthermore, as the osmogen is the key to producing the hollow morphology, it is imperative to investigate the effect of different amounts and types of the osmogen to have better understanding of the fabrication process itself. Thus, different batches were fabricated with variable amounts and types of osmogen. The results are illustrated in the sections below.

4.4 Initial polymer solution concentration

The effect of the initial polymer solution concentration was investigated by using organic phases of different initial polymer solution concentration in the fabrication process such as 3% (w/v) which is lower initial polymer solution concentration and 10% which is higher initial polymer solution concentration in comparison to 6.67% (w/v) of reference batch fabricated earlier. When the initial polymer solution concentration was decreased

to 3% (w/v), it was observed that the size of the MPs was reduced to $44 \pm 11 \mu\text{m}$, which was almost half of that of the reference batch MPs (**Figures 4.3a**). This, however, had no influence on the hollow morphology. Although the size decreased, the batch still retained its hollow morphology. SEM images showed uniform size distribution across the batch samples, and consistent hollow morphology. On the contrary, increasing the initial polymer solution concentration to 10% (w/v) resulted in larger MPs with wider size distribution. The hollow morphology was also not maintained in this batch (**Figure 4.3b**).

Figure 4.3 Scanning electron micrograph of the cross-section of hollow MPs fabricated with the same process parameters as the reference batch except, the polymer solution concentration of 3% (w/v) (a), and the polymer solution concentration of 10% (w/v) (b)

4.5 Stirring speed

To study the effect of stirring speed on final morphology of the MPs, a batch with higher stirring speed of 1000 rpm in comparison to the 500 rpm of the reference batch was fabricated. Only the stirring speed was altered while the rest of the process parameters remained the same. Increasing the stirring speed to 1000 rpm resulted in fabrication of smaller MPs. At this speed, the size decreased to $41 \pm 14 \mu\text{m}$ (**Figure 4.4a**), compared to $81 \pm 27 \mu\text{m}$ at 500 rpm (**Figure 4.4b**). The hollow morphology was still achieved at this higher stirring speed of 1000 rpm. Thus, the fabrication method allows the flexibility of

producing the particles of variable sizes while still achieving the hollow morphology. However, the stirring speed could not be higher than 1000 rpm as it disrupted the hollow morphology due to rapid precipitation of the polymer which would not allow sufficient time for the tiny water droplets to coalesce into a single larger droplet in the core. At the significantly higher stirring speed, smaller sized particles with significant porosity can be fabricated due to the entrapment of tiny water droplets scattered within the core because of faster precipitation of polymer.

Figure 4.4 Scanning electron micrograph of the cross-section of hollow MPs fabricated with the same process parameters as the reference batch except, with a stirring speed of 1000 rpm (a), and with a stirring speed of 500 rpm (b)

4.6 Amount of osmogen

Osmogen is the key parameter in our fabrication process to produce hollow MPs. As the whole fabrication process relies on the inclusion of the osmogen in the organic phase, altering the amounts of osmogen would manifest a significant effect on the morphology achieved. Thus, to have better understanding of the effects of the amount of osmogen, particles were fabricated with 1.25% (w/w), 5% (w/w), 10% (w/w) and 20% (w/w) of NaCl while keeping the rest of the parameters constant.

Figure 4.5 Scanning electron micrograph of the cross-section of hollow MPs fabricated with the same process parameters as the reference batch, except with 20% (w/w) (a), 10% (w/w) (b), 5% (w/w) (c) and 1.25% (w/w) NaCl (d)

Changing the amount of osmogen (NaCl) manifested its effect both in the shell thickness and in the volume space of the hollow cavity (**Figure 4.5a-d**). Increasing the amount of osmogen from 1.25% (w/w) resulted in a decrease in shell thickness and a corresponding increase in volume space of the hollow cavity. However, at osmogen amounts of 20% (w/w) and above, MPs with multiple internal pores instead of a single well-defined hollow core were obtained.

4.7 Type of osmogen

Figure 4.6 Scanning electron micrograph of the cross-section of hollow MPs fabricated with the same process parameters as the reference batch, except with NaCl as osmogen (2.5% w/w) (a), KCl as osmogen (2.5% w/w) (b) and sucrose as osmogen (5% w/w) (c)

At the beginning of this study, NaCl was used for the fabrication of the hollow MPs. Although, it was successfully utilized to change the morphology from solid to hollow, further evidence was required to elucidate that this effect is due to the osmotic property of osmogen and not due to other peculiar chemical or physical characteristics of NaCl. As such, other osmogens like KCl and sucrose were experimented with. The rationale was that if the hollow morphology was achieved due to the osmotic property of the NaCl, similar relatable effects would manifest upon usage of other osmogens too. Results

showed that the effect was indeed from the osmotic property of the osmogen incorporated into the organic phase. It was observed that the use of KCl as an osmogen at 2.5% (w/w) produced hollow MPs with a thicker shell and smaller hollow cavity (**Figure 4.6b**) when compared to the batch with an equal amount of NaCl (**Figure 4.6a**). Sucrose was the least effective, whereby twice the amount (5% (w/w)) was still not able to produce hollow MPs (**Figure 4.6c**). On the contrary, multiple pores were observed in the cross-sectioned particles. This showed that the fabrication process developed is dependent on the osmotic property of the osmogen which can be achieved by different kinds of osmogen. If the osmotic pressure of the osmogen introduced in the organic phase is strong enough to draw sufficient water from the external aqueous phase into the polymer solution droplets, then MPs with a shell surrounding a hollow cavity would form as in the case of KCl and NaCl, whereas in case of the osmogen such as sucrose which has significantly low osmotic property, the MPs couldn't achieve hollow morphology.

4.8 Discussions

A novel, one-step fabrication technique to produce hollow PLGA MPs for protein delivery is developed. This technique capitalizes on the relationship between osmotic pressure of the incorporated osmogen and its corresponding effect on the precipitation rate of polymer to produce such hollow particles through a single oil-in-water (O/W) emulsion. By careful addition of a chosen amount of osmogen in the oil phase (polymer dissolved in organic solvent) and emulsifying the oil phase with the external water phase (aqueous PVA solution), MPs with water filled core were first formed after the solvent has evaporated. Since the oil/water ratio and amount of osmogen are two key factors in altering the precipitation rate of the polymer^[8], they were carefully adjusted to maintain the precipitation rate of the polymer such that it was slow enough to allow the penetrating water droplets to coalesce into the core, thus reducing the interfacial area between the oil and inner water phases. After freeze drying, the water was removed to achieve hollow MPs.

Further study on the process parameters such as initial polymer solution concentration, amount of osmogen, type of osmogen and stirring speed showed that these fabrication parameters influence the size, shape and surface morphology of these MPs. For example, decreasing the initial polymer solution concentration to 3% (w/v) resulted in a significant size reduction, while maintaining the hollow morphology (**Figure 4.3a**). Lowering the polymer solution concentration decreased the viscosity of the organic phase which resulted in formation of smaller emulsion droplets under mechanical stirring.^[9] At 10% (w/v), consistent hollow morphology could not be achieved as a result of an excessively viscous organic phase (**Figure 4.3b**). This increased viscosity also resulted in formation of larger MPs and a wider size distribution.

Similarly, change in the stirring speed manifested its effect on the size of the hollow MPs produced. At higher stirring speed, more intense agitation disperses the polymer solution into smaller droplets. Hence, increasing the stirring speed to 1000 rpm reduced the size of the MPs by half (compared with 500 rpm), while still maintaining the hollow morphology (**Figure 4.4a-b**). It was observed that 3% (w/v) polymer solution concentration or 1000 rpm stirring speed produced similar sized MPs ($44 \pm 11 \mu\text{m}$ or $41 \pm 14 \mu\text{m}$ respectively) when the other processing parameters were kept same in both the batches. This size range easily falls in the preferable size range for different routes of delivery for clinical applications such as $<100 \mu\text{m}$ for subcutaneous delivery, $<150 \mu\text{m}$ for parenteral delivery and $<100 \mu\text{m}$ for intra-arterial chemo-embolization.^[10]

It was found that altering the amount of osmogen (i.e. NaCl) could achieve MPs of varying volume space of hollow cores and shell thicknesses. This arises from the magnitude of osmotic pressure obtained from different salt solution concentrations. In other words, osmotic pressure in the emulsion droplet is dependent on the amount of osmogen used in fabrication. High osmogen content drives a tremendous amount of water into the emulsion droplet. The volatile organic solvent DCM must first diffuses into the water and finally evaporates at the air/water interface^[11] resulting in gradual precipitation of polymer. Consequently, a higher amount of water drawn into the polymer solution droplet causes faster removal of DCM which significantly increases the precipitation rate

of the polymer. Hence, the polymer starts to precipitate and solidify faster, reducing the time for the inner water droplets to coalesce into a single core. The consequence is MPs with multiple large pores dispersed within the structure, without the formation of a single hollow core (**Figure 4.5a**). However, decreasing the amount of osmogen to achieve a slower, but desirable, precipitation rate subsequently facilitates the coalescence of the inner water droplets, giving a hollow core structure (**Figure 4.5b**). By further decreasing the amount of osmogen, the volume of this water-filled hollow core decreased, while shell thickness increased simultaneously, as less water is drawn into the o/w emulsion droplet (**Figure 4.5c-d**). Hence, by simply adjusting the amount of osmogen, shell thickness and volume space of hollow cavity can be controlled.

Similarly, this hollow core formation can also be elucidated by incorporating other osmogens, e.g. KCl and sucrose. The final morphology was found to be dependent on the inherent osmotic pressure of these different osmogens. The osmotic pressure (OP) of NaCl, KCl and sucrose in the polymer emulsion droplet, assuming the same initial condition for each osmogen-containing emulsion droplet, can be estimated by the relationship,

$$OP = iMRT$$

Where i = Van't Hoff Factor, M = Molar Concentration, R = Universal Gas Constant,
 T = Absolute temperature.

The osmotic pressure of these osmogens is in the order of $\text{NaCl} > \text{KCl} > \text{sucrose}$.^[12] KCl (5 mg) having 21.6% less osmotic pressure (from above equation) than that of NaCl (5 mg), produced MPs with comparatively thicker shells and smaller hollow cores (**Figure 4.6a-b**). Likewise, sucrose (10 mg), with 82.9% less osmotic pressure than that of NaCl, resulted in MPs with many tiny pores. The amount of sucrose used in fabrication could not generate sufficient osmotic pressure to drive sufficient amount of water into the polymer solution droplet that could coalesce into a single droplet in the core to achieve a well-defined hollow core (**Figure 4.6c**).

4.9 Summary

The emulsion solvent evaporation technique was easily modified by introducing osmogen in the oil phase to fabricate MPs of hollow morphology. The osmogen played the key role of withdrawing water from the external aqueous phase into the polymer solution droplet which would eventually coalesce into a single larger droplet in the core. Altering the amount of osmogen provided an easy control over the shell thickness and volume space of hollow cavity. Upon increasing the osmogen amount in the oil phase, the shell thickness decreased subsequently. However, adding excessive osmogen resulted in formation of MPs with larger pores scattered in the polymer matrix due to faster precipitation of the polymer. Thus, the manipulation of the shell thickness and volume space of hollow cavity is possible only within a specific range, beyond which the hollow morphology is disrupted.

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Chapter 5

Validation of the fabricated hollow microparticles as delivery vehicle for a hydrophobic biomolecule

*This chapter focusses on evaluating the suitability of the fabricated hollow microparticles (MPs) as carriers for a model hydrophobic biomolecule, bovine insulin. The hollow MPs are loaded with bovine insulin and several important attributes of the MPs as carriers are analyzed, and the results are compared with the traditional solid MPs loaded with the same biomolecule. This chapter, thus, presents the results pertaining to the encapsulation efficiency and release kinetics achieved from the hollow MPs of varying shell thickness, the hydrolytic degradation of the MPs, the structural integrity and the level of bioactivity of biomolecules retained by the MPs. A discussion taking all the results in totality is then presented in **Section 5.8**. The results showed that solid MPs suffered from incomplete release of the biomolecule, whereas the release from hollow MPs was significantly improved to achieve near complete release by decreasing the shell thickness. The assessment of the bioactivity by MCF-7 cell proliferation test showed no loss in the bioactivity of bovine insulin from both batches.*

*Most of the texts and results in this chapter have been published and reprinted from a. Journal article. **Kharel S**, et al. "Osmogen-Mediated One-Step Technique of Fabricating Hollow Microparticles for Encapsulation and Delivery of Bioactive Molecules." *Macromolecular bioscience* 17.4 (2017) (Copyright 2016, with permission from Wiley Online Library), b. Patent. Loo SC, **Kharel S**, Lee WL. "Method of preparing hollow microparticles and hollow microparticles prepared thereof." **PCT/SG2015/050483** filed on 2 December 2016.

5.1 Loading of bovine insulin in the MPs

After developing a facile one step osmogen mediated emulsion solvent evaporation technique to fabricate hollow MPs and having established an understanding of how the process parameters can be used to control the morphology of the fabricated hollow MPs, the focus was shifted to determining whether the fabrication method allows for effective encapsulation of biomolecules and subsequent release of the biomolecules in release medium. It is of interest to compare how the hollow MPs perform in comparison to the solid MPs as a carrier for biomolecules delivery. This would determine if the hypothesis that the poor release kinetics from the PLGA MPs could be improved by changing the morphology of the MPs from solid to hollow morphology holds true or not.

Figure 5.1 Scanning electron micrograph of the cross-sectioned MPs showing the insulin microcrystals embedded in the shell of MPs and (a) and in the hollow core (b)

Thus, bovine insulin microcrystals were loaded into MPs as a model hydrophobic biomolecule. The MPs were cross-sectioned and imaged using a SEM operated at 5 keV. **Figure 5.1a-b** shows the SEM micrographs of the cross-section of insulin-loaded MPs. No preferential localization of the microcrystals was observed from the SEM. The biomolecule crystals were found to be embedded in the shell as well as in the internal surface of the hollow core of the particles. It is often reported that for a monolithic polymer particle system, there is no layer localization of the encapsulated moieties and a

double layered configuration is often needed to accomplish layer localization. However, since the hollow MPs have a hollow cavity filled with water during the encapsulation process, and bovine insulin being a hydrophobic molecule, the possibility of the biomolecules localizing at the hydrophobic polymer layer rather than the hydrophilic environment at the core needed to be tested. Nonetheless, SEM analysis confirmed that no such preferential localization is possible in our system. This was further confirmed by loading hollow MPs with two different dyes viz: a hydrophilic dye rhodamine-6G and a hydrophobic dye coumarin-6. These two dyes have been previously shown to preferentially localize in the individual polymer layers when loaded in double layered MPs composed of outer PLLA shell and inner PLGA core. It is reported in literature that coumarin-6 preferentially partitioned to the PLLA shell due to its relatively higher hydrophobicity whereas the hydrophilic rhodamine-6G partitioned into the PLGA core.

Figure 5.2 Schematic figure showing the expected confocal image of the hollow MPs loaded with rhodamine-6G and coumarin-6 simultaneously (a), Confocal Laser Scanning Microscopy images showing red fluorescence for distribution of rhodamine-6G (b) and green fluorescence for coumarin-6 (c)

Thus, in our case, both of these dyes were introduced in the polymer solution to check if the hydrophilic dye would preferentially localize in the water filled core and hydrophobic dye coumarin-6 would preferentially localize in the polymer shell upon forming the MPs. **Figure 5.2a** shows the schematics of the expected layer localization of the dyes. The dye loaded MPs were then imaged using a Confocal Laser Scanning Microscopy (CLSM). The results revealed that both the dyes were localized in the shell of the microparticle

despite one dye being hydrophobic and the other being hydrophilic (**Figure 5.2b**), implicating that preferential localization of any encapsulated biomolecules in such hollow MPs of a single polymer is not achievable.

Figure 5.3 Energy Dispersive X-Ray (EDX) spectrum of a blank carbon tape (a), insulin microcrystals (b), region of hollow PLGA microparticle devoid of insulin microcrystals (c) and insulin microcrystals embedded in the polymer shell (d)

EDX spectrum analysis was used to confirm the observed embedded microcrystals. The EDX spectrum of the blank carbon tape (**Figure 5.3a**) and the regions void of the insulin microcrystals (**Figure 5.3c**) showed carbon and oxygen peaks, whereas the EDX spectrum of pure microcrystals of bovine insulin showed the presence of sulphur (**Figure 5.3b**). A strong signal at 2.3 keV can be assigned to the six sulphur atoms for each insulin

monomer.^[1] These sulphur atoms form the disulphide bonds between the three pairs of cysteinyl residues of insulin. Finally, the embedded microcrystals in the shell of cross-sectioned hollow MPs also showed the sulphur peak at 2.3 keV (**Figure 5.3d**). Comparing the peaks in **Figure 5.3b** and **Figure 5.3d**, it was evident that the microcrystals embedded in the shell were indeed bovine insulin microcrystals, i.e. the biomolecule was loaded successfully in the MPs without affecting the hollow morphology.

Table 5.1 Encapsulation efficiency of the MPs loaded with bovine insulin

Formulation batch name	Biomolecule for encapsulation	Physical state of biomolecule	Amount of osmogen used in formulation	Encapsulation efficiency (%)
Solid (S1)	Bovine insulin	Microcrystals	0	84 ± 2
Thick shell hollow (S2)	Bovine insulin	Microcrystals	2.5	83 ± 2
Standard hollow (S3)	Bovine insulin	Microcrystals	5	85 ± 1
Thin shell hollow (S4)	Bovine insulin	Microcrystals	20	81 ± 4
Porous (without salt) (S5)	Bovine insulin	Solution	0	84 ± 3
Porous (with salt) (S6)	Bovine insulin	Solution	10	82 ± 3

After characterization of the MPs by SEM and the confirmation of biomolecules loading by EDX analysis, the encapsulation efficiency for all the fabricated batches were evaluated. For this, equal amount of MPs from each batch were firstly dissolved in the organic solvent DCM. The insulin was then extracted by vigorously mixing the polymer solution with 0.01N HCl solution. The mixture was centrifuged, and the supernatant was analyzed by HPLC for determination of the encapsulation efficiency of the MPs. The

standard hollow MPs showed encapsulation efficiency of $84 \pm 2\%$ which was comparable to the encapsulation efficiency of $85 \pm 1\%$ for fully solid MPs. Encapsulation efficiency of the other batches (**Figure 5.5b-f**) with different amounts of osmogen was also similar as shown in the **Table 5.1** above.

5.2 Insulin release from MPs

Figure 5.4 HPLC chromatogram of the released insulin in SGF (red line) and PBS (blue line)

The cumulative in vitro release profiles of solid and hollow MPs with varying shell thicknesses were studied in two media a) SGF (pH 2) and b) PBS (pH 7.4) (**Figure 5.6**). Insulin (microcrystal form) loaded solid MPs (S_1), thick shell hollow MPs (S_2), standard hollow MPs (S_3), thin shell hollow MPs (S_4), and insulin (solution form) loaded MPs without salt (S_5) and with salt (S_6) (**Figure 5.5**) were used for the release study. The collected release samples were analyzed using HPLC to determine the amount of released insulin. For this, a calibration curve was generated first by plotting the area under the peak observed in the HPLC chromatogram against the known concentration of fresh insulin solutions. The insulin released in both the media was observed to be intact as no

degradation peak was obtained in the HPLC chromatograms over the entire release study period. For instance, **Figure 5.4** shows the representative HPLC chromatogram of released insulin in SGF and PBS at the same time point.

Figure 5.5 Bovine insulin loaded solid microparticle (S1) (a), thick shell microparticle (S2) (b), standard hollow microparticle (S3) (c), thin shell microparticle (S4) (d) in solution form (without salt) microparticle (S5) (e) and in solution form (with salt) microparticle (S6) (f)

The HPLC chromatogram revealed a single peak for the bovine insulin released in both media. However, the cumulative amount of insulin released at each time point was comparatively higher in SGF as compared to that in PBS. This can be attributed to a higher solubility of the protein and accelerated degradation of the polymer in an acidic environment.^[2] Likewise, in both release media (for the batches S₁, S₂ and S₃), the cumulative amount of protein released at each time point was higher with decreasing shell thickness (i.e. higher amount of osmogen used) (**Figure 5.6**). Batches S₅ and S₆ showed a massive burst release as a result of the high particle porosity.

Figure 5.6 Cumulative release profiles of the bovine insulin in (A) simulated gastric fluid (pH 2) and (B) phosphate buffer saline (pH 7.4), data presented as mean $\pm$ SD (n = 3)

5.3 In vitro hydrolytic degradation of insulin loaded MPs

A degradation study of the insulin loaded formulations (S₁-S₆) was conducted over a period of 21 days. At predetermined intervals of time the MPs were imaged using SEM to observe the degradation of the particles. The SEM micrographs of the MPs at the start (day 0) and end (day 21) of the study are shown in **Figure 5.7**. The particle morphology remained intact for all the batches over the period of study and no significant changes on surface morphology due to degradation was observed.

Figure 5.7 SEM images of the particles suspended in release medium at day 0 and day 21

5.4 Evaluation of possible degradation of encapsulated bovine insulin by MALDI TOF MS

MALDI TOF MS is one of the techniques that can be used in the analysis of the molecular mass of the biomolecules. Molecular mass analysis of the encapsulated bovine insulin can also provide insight into the fragments of the biomolecules that might have formed as a result of degradation of the biomolecules owing to the various stresses in the MPs. Thus, this could be one approach to elucidate on the structural integrity of the encapsulated biomolecules.

Figure 5.8 MALDI TOF MS spectra of bovine insulin extracted after 21 days from the hollow MPs (a) and solid MPs (b)

For this study, the insulin that was still entrapped in the hollow and solid MPs were extracted after 21 days of incubation in the release medium. Briefly, the particles were collected by centrifugation and washed twice with DI water. Then, the MPs were dissolved in acetonitrile, dried at room temperature under vacuum and reconstituted in 0.01N HCL solution. The concentration of the extracted solutions was measured by HPLC and the concentrations were normalised by dilution with DI water to match the lower concentration of bovine insulin solution obtained after extraction from hollow MPs.

Finally, aliquots of 2 μL of the prepared samples were then mixed with 6 μL of α -Cyano-4-hydroxycinnamic acid (α -CHCA) solution and 2 μL of this mix was loaded on a clean sample plate and left to dry at room temperature. After the samples were dried, the plate was then inserted into the mass spectrometer.

The MALDI TOF MS spectrum for the extracted biomolecules are shown in **Figure 5.8a-b**. Two major peaks were observed in the mass spectra for both bovine insulin extracted from hollow and solid MPs. The mass spectra showed one molecular peak at m/z 2873.3 and doubly charged molecular weight at m/z 5756.9, both of which are of intact bovine insulin molecule. Similar two peaks were observed in the case of solid MPs as well. However, both spectra did not show any other significant peaks in between these two major peaks that could point at the fragmentation of biomolecule into lower molecular mass segments. Thus, further investigation of the structural integrity of the encapsulated biomolecule with other techniques is necessary to corroborate the results obtained by MALDI TOF MS.

5.5 Assessment of structural integrity by CD Spectroscopy

Apart from the molecular mass analysis of the encapsulated biomolecule to correlate with the structural integrity of the biomolecule, the information on the secondary structure of biomolecules also could provide an insight on the structural integrity. Thus, CD spectroscopy was performed to analyze changes in the secondary structure of the entrapped insulin in hollow MPs and solid MPs after 21 days. In both the batches CD spectroscopy of the encapsulated protein showed two minima around 208 nm and 222 nm, which is typical of the predominant α -helix structure of proteins, in this case insulin.^[3] The profile of the insulin extracted from hollow and solid MPs (after 21 days) superimposed almost entirely to the profile of standard insulin (**Figure 5.9**). Evidently, the protein maintained its α -helical structure retaining its structural integrity over the period of residence in the MPs. This indicates that the structural integrity of insulin loaded

in the MPs was preserved over the period of 21 days, and thus further corroborated the observation made in the study of molecular mass of bovine insulin by MALDI TOF MS.

Figure 5. 9 Far-UV circular dichroism (CD) spectra of the bovine insulin extracted from the PLGA hollow MPs, freshly prepared insulin solution and denatured insulin (heated for 24 hours at 110 °C). The CD spectrum of the extracted insulin was very similar to the spectrum of freshly prepared insulin solution.

5.6 Assessment of bioactivity of encapsulated biomolecule by MCF-7 cell proliferation tests

To evaluate the level of bioactivity of the insulin that was entrapped in the MPs for 21 days, the proliferative effect of the insulin on insulin-sensitive breast cancer cell line MCF-7 was analyzed in comparison to the same concentration of freshly prepared standard insulin solution (as control). The extracted insulin from hollow MPs and solid MPs, as well as the freshly prepared insulin stimulated the growth in cell number of the

MCF-7 cells. After 4 days of culture, an increase in cell numbers (with good adherence and morphology) for wells treated with serum free medium supplemented with insulin extracted from the MPs as well as freshly prepared insulin (i.e. positive control) was observed. Furthermore, the results of cell proliferation quantified using Cell Counting Kit-8 clearly showed that the proliferative effect of the extracted insulin was almost equal to that of the freshly prepared insulin solution (**Figure 5.10**). For the negative control, i.e. wells treated with blank medium, a decrease in cell numbers was observed. These cells were observed to be round in shape and lacked adherence capabilities.

Figure 5. 10 Result of the effect of insulin extracted from the MPs after 21 days in the proliferation of MCF-7 cells determined by CCK-8 assay, data presented as mean $\pm$ SD (n = 3)

5.7 Discussions

Bovine insulin (a hydrophobic protein) was chosen as a model biomolecule to analyze the effectiveness of these particles as suitable carriers for biomolecule delivery. Insulin was

loaded in microcrystal form into these hollow MPs because of the following advantages: 1. overcoming its solubility limitation as a hydrophobic protein, 2. minimizing loss in bioactivity during fabrication, 3. inhibiting its migration to the aqueous/organic interphase. SEM images (**Figure 5.1a-b**) show that there was no preferential localization of insulin microcrystals in the hollow MPs. Insulin microcrystals were observed to be embedded in the shell and also scattered within the core. However, for non-hollow (i.e. solid) MPs, a significant portion of insulin was embedded in the center of the MPs. This core localization of insulin in solid MPs can possibly explain for its extremely slow (**Figure 5.6**) or incomplete release, due to a longer diffusion distance^[4] for the protein (**Figure 5.5**). Also, encapsulation efficiency of solid and hollow MPs batches was found to be similar. For hollow MPs batches, the osmogen aid in establishing an osmotic pressure that drives water from the continuous phase into the polymer solution droplets, which later coalesce to form a water-filled core. However, this doesn't produce any changes on encapsulation efficiency of hollow MPs when compared to solid MPs because of the hydrophobic nature of bovine insulin. In the brief moment of particle hardening along with water penetration into the particles, there is not much leaching of bovine insulin out of the particles owing to its hydrophobic nature. Hence, although the morphology changes to hollow core, EE of solid and hollow batches essentially remains similar. Although the encapsulation efficiency of the hollow MPs and solid MPs were comparable, solid MPs provided very little control over release kinetics, whereas hollow MPs could be tailored to have various desirable release kinetics as evident in **Figure 5.6**. In the release study, it was observed that the release could be tuned by just manipulating the amount of osmogen used. Increasing the osmogen content produced MPs with a thinner shell, correlating to a faster release kinetics as a result of shorter diffusion distance for encapsulated biomolecule. This can therefore be a facile and ingenious technique to tailor release kinetics and profiles for biomolecule delivery.

Release of encapsulated biomolecule occurs by either diffusion, polymer degradation due to hydrolysis, or both.^[3] Polymer degradation, however, results in the formation of lactic and glycolic acids that acidifies the microenvironment within these MPs. The exposure of acidic degradation products results in a loss of structural integrity and bioactivity of

biomolecules.^[5] So to understand the mechanism governing the release kinetics of the MPs, the in-vitro release data (in PBS medium) were fitted to the Higuchi equation which can be simply written as,

$$M=k(t)^{0.5}$$

Where M is the percentage of drug released at time t and k is a diffusion rate constant.

At Day 1, three of the batches (S₄, S₅ and S₆) suffered from significant burst release which can be attributed to the particle morphology as shown in **Figure 5.5**. The significantly thinner shell of S₄ gave rise to shorter diffusion distance of the drug, and thus a high burst release (~47%). Likewise, for the batches with insulin loaded in solution form with and without osmogen (i.e. S₅ and S₆ respectively), the burst release was as high as 75%. This increased burst release was due to the high particle porosity. During fabrication of batches S₅ and S₆, bovine insulin solution (in 0.01 N HCl) was first briefly emulsified with the polymer solution (in DCM) forming water-in-oil primary emulsion. This was then emulsified with aqueous PVA solution to finally produce water-in-oil-in-water secondary emulsion.

Table 5.2 Result of release profiles fitting Higuchi equation for different microparticle batches loaded with bovine insulin

microparticle batch	r ² value
Solid MPs	0.9556
Thick shell hollow MPs	0.9773
Standard hollow MPs	0.9943
Thin shell hollow MPs	0.9679
Insulin (solution form) loaded MPs without salt	0.7829
Insulin (solution form) loaded MPs with salt	0.7923

Absence of surfactant in the internal water phase (i.e. insulin solution) in primary emulsion droplets cannot stabilize the tiny droplets of the internal water phase. So, their tendency to connect with each other and also with the external aqueous continuous phase resulted in formation of interconnecting water channels and pores throughout the MPs as it hardened.^[6] This resultant particle porosity makes it easy for the release medium to permeate the particles, subsequently making easy for protein to dissolve and diffuse. After the initial burst release at Day 1, the release data for hollow MPs batches (S1-S4) at subsequent days significantly fitted to the Higuchi equation. The R^2 values in **Table 5.2** below elucidates that the biomolecule release mechanism for solid, standard hollow, thick shell hollow and thin shell hollow microparticle batches during the release study period was diffusion controlled.

However, the release data of the microparticle batches with protein loaded in solution form did not significantly fit to the model. This is because the porous morphology of these batches allows the release medium to rapidly penetrate into the particles allowing for faster dissolution and release of the biomolecule, eventually resulting in more than 70% of cumulative release by day 1 and plateauing in subsequent days. Hence, unlike the batches in which the biomolecule is loaded in solution form, the hollow MPs batches loaded with biomolecule microcrystals were optimized for different release profiles by changing the thickness of the shell by altering the amount of osmogen, which consequently changes the diffusion distance for the encapsulated biomolecule. This further elucidates the increased cumulative release at each time point for the batches in the order of solid MPs < thick shell hollow MPs < standard hollow MPs < thin shell hollow MPs; whereby hollow MPs provided a means to control the release of biomolecules, unlike solid MPs.

Subsequently, structural integrity and bioactivity was determined for the insulin that was extracted from the standard hollow MPs and solid MPs after 21 days to examine any effect of possible acidic microenvironment formed due to the degradation of polymer. Only these two batches were chosen for this study as almost 75% of encapsulated biomolecule already released by day 8 (**Figure 5.6**) for the batches with insulin loaded in solution form (i.e. insufficient amount of biomolecule was remained in these MPs).

Polymer degradation and a decrease in pH due to the degradation product in the MPs starts manifesting after a week.^[3] For this reason, the MPs were suspended in PBS and incubated at 37⁰ C for 21 days before the insulin was finally extracted for analysis of the loss in structural integrity and bioactivity. The extracted insulin from both the batches retained its structural integrity and bioactivity up to 21 days and potentially may retain even longer as determined by the CD spectroscopy analysis and MCF-7 proliferation assay. Substantial information about protein's secondary structure was provided by CD analysis and the obtained spectrum of the extracted insulin from hollow and solid MPs was in close agreement with the spectrum of the freshly prepared insulin solution (**Figure 5.9**). The biological activity of the extracted insulin was analyzed by examining the proliferative effect on MCF-7 human breast cancer cells under serum-free conditions as insulin is known to elicit mitogenic response in these cells.^[7, 8] Insulin extracted from both the batches showed similar levels of proliferation of MCF-7 cells. Hence, in our study no loss in the structural integrity and bioactivity of the insulin extracted from the solid MPs was observed as there was not any significant degradation of the polymer even by 21 days (**Figure 5.10**). Apparently, the effect of acidic microenvironment in these solid MPs may manifest upon even longer incubation of these particles in the release medium, or changing the PLGA used in the formulation to a lower molecular weight PLGA^[6] might be necessary to elucidate the loss in bioactivity due to acidic microenvironment in solid MPs compared to hollow MPs fabricated using our technique.

5.8 Summary

The hollow MPs were successfully loaded with the hydrophobic biomolecule and were shown to be easily optimized for different release kinetics. The release of biomolecules from the hollow MPs could be easily tuned and improved to obtain near complete release while the solid MPs suffered from incomplete release. Further, the encapsulated protein was found to be fully bioactive and structurally intact up to 21 days as shown by CD study and MCF-7 cell proliferation assay which validates the hollow MPs produced by this technique as an effective carrier for biomolecules.

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Chapter 6

Hollow microparticles as a superior delivery system over solid microparticles for the encapsulation of biomolecules

This chapter converges the focus on analyzing the attributes that contribute to the differences observed during the release of biomolecules from particles of different morphologies viz: hollow and solid. A hydrophilic biomolecule Glucagon-like-Peptide-1 (GLP-1) is encapsulated as a model biomolecule for this study. The chapter, thus, presents the result pertaining to the loading of GLP-1, release kinetics and the bioactivity of GLP-1 achieved from the microparticles (MPs) of the hollow morphology in comparison to solid morphology. The chapter then proceeds with the results of studies involving hydrolytic degradation of the MPs, mass loss study, molecular weight change study, intraparticle and supernatant pH measurement study, polymer-biomolecule adsorption study and finally, the active GLP-1 HTRF assay with the aim to find explanation of the key differences observed in using particles of the hollow morphology instead of solid. All the results are discussed in correlation to each other in the later part which conclusively showed that the hollow MPs are better suited than solid MPs to be used in the encapsulation and delivery of biomolecules.

*Most of the texts and results in this chapter have been taken from the manuscript submitted for peer-review in *Molecular Pharmaceutics*. **Kharel S**, et al. "Hollow Microparticles as a Superior Delivery System Over Solid Particles for the Encapsulation of Peptides", 2018.

6.1 Fabrication of GLP-1 loaded MPs

So far, an easy technique to fabricate hollow MPs has been shown. Also, the release kinetics of the encapsulated biomolecules have been shown to be optimised by altering the shell thickness and volume space of hollow cavity and it has been established that hollow MPs can avoid the issue of incomplete release which is prevalent in solid MPs. However, in regard to the level of bioactivity of the encapsulated biomolecules, both the MPs batches showed similar results. The polymer chosen did not degrade within the time frame of the study, thus the effect of degradation on the bioactivity could not manifest. So, in this part of study, the focus was shifted towards investigation of whether the MPs morphology (solid or hollow) will influence the bioactivity of the encapsulated biomolecules, if the particles degraded substantially. For this, PLGA polymer with lower average molecular weight and lower intrinsic viscosity (0.2 dL/g instead of 1.18 dL/g) was used. The rationale behind this is to have the particles degrade sufficiently over shorter period of time, so that the effects of degradation of polymer on the encapsulated biomolecules could be studied. Subsequently, the focus will be to explain the differences observed. Thus, the study will be converged to compare the attributes of just two batches; solid MPs and hollow MPs.

Table 6.1 Encapsulation efficiency of the hollow and solid MPs loaded with Glucagon Like Peptide-1 (GLP-1)

Formulation batch name	Biomolecule for encapsulation	Physical state of biomolecule	Amount of osmogen used in formulation (mg)	Encapsulation efficiency (%)
h-MPs	GLP-1	Microcrystals	5	51 ± 2
s-MPs	GLP-1	Microcrystals	0	55 ± 3

Microparticle batches having two different morphologies were fabricated for this study viz: solid (s-MPs) and hollow (h-MPs), with GLP-1 loaded in solid crystal form as

provided by the supplier. Both samples were fabricated using the emulsion-solvent evaporation technique, with a slight modification in the protocol to produce MPs with a hollow-core structure as discussed earlier.

Figure 6.1 Scanning electron micrograph (SEM) of (a) h-MPs and (b) s-MPs, showing overall size distribution (I), cross section of a particle (II) and particle size distribution histogram (III)

For both the formulations of h-MPs and s-MPs, a spherical and smooth exterior was observed (**Figure 6.1**). Cross-sectioning the MPs revealed a solid core for s-MPs and a hollow core for h-MPs. The average particle sizes measured were $127 \pm 23 \mu\text{m}$ and $103 \pm 20 \mu\text{m}$ for h-MPs and s-MPs respectively. The encapsulation efficiency of hollow MPs and solid MPs were $51 \pm 2\%$ and $55 \pm 3\%$ respectively as shown in **Table 6.1**. While the encapsulation efficiency did not seem to be affected with the inclusion or omission of osmogen during the formulation, relatively lower encapsulation efficiency was achieved for GLP-1 in comparison to that for bovine insulin shown in **Table 5.1**.

6.2 Study of mass loss and change in molecular weight of the polymer

Figure 6.2 Graphical plots showing the mass loss of hollow MPs vs solid MPs of PLGA at the different degradation time points (a), and the % change in average molecular weight of the polymer particles of hollow and solid MPs at different degradation time points (b), data presented as mean $\pm$ SD (n = 3)

During the release, the hydrolysis of the PLGA backbone produces oligomers that are carboxylic acid-terminated. The progressive degradation of the polymer leads to the accumulation of these acidic oligomers that builds up towards an acidic microenvironment

within the particle.^[1] Thus, to investigate this, mass loss and molecular weight change for the two batches were analysed. **Figure 6.2** plots the mass loss and average molecular weight of the MPs against time. The loss of polymer mass of the MPs is shown in **Figure 6.2a**, whereby no significant mass loss was observed for both formulations during the initial 7 days of hydrolysis. Furthermore, the mass loss for s-MPs was $36 \pm 3\%$ at day 11 that increased to $54 \pm 5\%$ at day 14, whereas h-MPs experienced only $6.7 \pm 8\%$ and $33 \pm 6\%$ mass loss at the same time points respectively. At day 7, the average molecular weights of s-MPs and h-MPs were $44 \pm 6\%$ and $88 \pm 8\%$ of the original molecular weights respectively (**Figure 6.2b**).

6.3 Characterization of hydrolytic degradation by SEM

The degrading particles were also analysed under SEM to have a visual inspection of the changes in their morphology due to the degradation. The particles were collected at day 0, day 3 and day 14 and freeze dried overnight to remove moisture from the sample. The dried particles were then spread on a carbon tape on a metal stub and imaged using SEM at 5keV voltage.

SEM images revealed changes in the surface morphology as early as day 3 (**Figure 6.3**). While the particles of both the batches had smooth surface at day 0, the surface was observed to be rough at day 3. Not much difference was observed in the surface morphology of hollow MPs from day 3 to day 7, whereas visible sign of increased degradation was observed for solid MPs at day 7. At day 14, due to sufficient degradation, the particles showed loss in structural integrity and both the batches were seen to have randomly fragmented particle debris. However, it was noticed that the fragments from solid batch showed higher internal porosity in comparison of hollow MPs. This could be attributed to the acid catalysed autocatalytic degradation of the polymer due to the acidic microenvironment created that formed as a result of entrapment of polymer degradation products.

Figure 6.3 SEM images showing the degradation of the hollow MPs (a) and solid MPs (b) at day 0, day 3, day 7 and day 14

6.4 Measurement of intraparticle pH

To validate the above observations, the intraparticle pH of both the formulations were measured. A calibration curve was first prepared by obtaining a correlation between the known pH of lactic acid monomers in PBS and lactic acid monomer in the mixture of polymer, PBS and DI water. The **Figure 6.4** shows the calibration equations used in the measurement of pH for this study.

The change in the intraparticle pH of the solid and hollow MPS with increasing duration of hydrolysis is shown in **Figure 6.5**. For both MPs, intraparticle pH was observed to decrease with hydrolysis, but with s-MPs having a significantly lower intraparticle pH (an average difference of 1.63 ± 0.65 as compared to h-MPs). Polymer hydrolysis forms acidic oligomers as by-products, leading to a correspondingly lower intraparticle pH (**Figure 6.5a**). On the other hand, although the average molecular weight of h-MPs decreased significantly after day 7 (**Figure 6.2b**), its intraparticle pH did not seem to be influenced by this. Despite the drop in molecular weight of h-MPs, the hollow morphology of the particle allowed for easy escaping of the acidic degradation products because of its thinner shell and volume space of hollow cavity.

Figure 6.4 Graph of the calibration curve for quantification of the intraparticle pH

Next, the pH values of the release medium for both degrading formulations were measured. The quantification of the pH of the release medium would provide information on how much of the acidic oligomers has leaked or diffused out into the physiologically-relevant environment. **Figure 6.5b** shows that the pH of the release medium for h-MPs decreased steadily over time. This is unlike s-MPs where its release medium only showed a significant decrease in pH after day 7. This sudden reduction in pH for s-MPs after day 7, coincides with its drastic mass loss at this time point (**Figure 6.2a**).

Figure 6.5 Graphical plot showing the intraparticle pH measurements for the hollow MPs and solid MPs (a), the corresponding supernatant pH measurements for the microparticle formulations (b), data presented as mean $\pm$ SD (n=3)

6.5 Stability of GLP-1 in release and storage conditions

Before the release studies can be carried out, it is important to assess the stability of the biomolecules in the release and the storage conditions. If the released biomolecules degrade in the medium before the time point set for measurement, then the measurement done may not be an accurate representation of the actual amount of biomolecules released.

Thus, it is imperative to validate that the biomolecules are stable in between the time points set for the measurement.

Figure 6.6 Graphical plots showing the amount of GLP-1 recovered in the solution as a percentage of the initial amount of the GLP-1 in the solution at day 0 for solution of concentrations at 0.005 mg/mL (a) and 0.05 mg/mL (b), data presented as mean $\pm$ SD (n = 3)

With this in mind, GLP-1 solution in PBS with 0.1% Tween 20 as a release medium was prepared at two different concentrations that are 0.05 mg/mL (low-GLP-1) and 0.005 mg/mL (high-GLP-1). For each concentration, the prepared solutions were kept in vials at 4 °C without agitation (denoted as chiller), 37 °C with continuous agitation using rotor-shaker (denoted as rotor) and 37 °C without agitation (denoted as still). Every day, the vials were analysed with HPLC to determine the change in the amount of GLP-1 in the solution over time. Graphs were plotted for the GLP-1 recovered (%) over days. The GLP recovered (%) denotes the total amount of GLP-1 detected in the solution given as percentage of the amount of GLP-1 in the solution at day 0. Important observations were made from the results obtained. 1. The GLP-1 was not very stable at 37 °C temperature. 2. Continuous agitations further decreased the recovery of GLP-1 in the solution. The loss in the recovery of GLP-1 is dependent on the concentration of the GLP-1 solution. By day 2, the amount of GLP-1 recovered had significantly dropped to 2 % of that of day 0 for low-

GLP-1 sample kept at 37 °C under continuous agitation. Without agitation at 37 °C, the recovered amount was 22% for the same. In contrast, the recovered amount of GLP-1 was significantly high at 94% for the one kept at 4 °C temperature and remained significantly high over the period of two weeks, dropping to 72% by day 14. However, for the samples at 37 °C with or without agitation, no GLP-1 could be detected from day 4 onwards (**Figure 6.6a**). A similar observation was made for the high-GLP-1 solution. While the samples at 37 °C with continuous agitation showed the least recovered amount, the samples without agitation at 37 degrees showed slightly more recovered amount and the ones at 4 °C temperature showed the maximum recovered amount.

However, at each time point, the recovered amount was higher compared to respective recovery amounts in low-GLP-solution sample. For example, the recovery was 56%, 76% and 98 % respectively for high- GLP-solution sample at 37 °C with agitation, 37 °C without agitation and 4 °C without agitations (**Figure 6.6b**). For subsequent days also, the readings were higher than that of the low-GLP-1 samples. Unlike in the low-GLP-1 samples, there was a gradual drop in the recovered amount in the high-GLP-1 samples, eventually reaching 7%, 28% and 85% at day 13 respectively. This concentration dependent recovery of the GLP-1 hinted that the loss is due to the biomolecule sticking on the walls of the vials. GLP-1 is reported as a very sticky biomolecule and the issues of loss of proteins due to non-specific adsorption to the walls of the container when the concentration is low, has been frequently reported. As such, the release media had to be optimised to counteract this issue.

6.6 Optimization of the release medium

So far, it has been observed that the GLP-1 may be lost due to it sticking to the walls of containers at low working concentration. As our theoretical loading was set at 2% (w/w), our working concentration is quite low. Hence, it was important to counteract this issue by other means. BSA is often used as additional component in the media when working with proteins in lower concentration in cell culture. It is often reported that inclusion of BSA in

the media would competitively adsorb to the walls of the container, thereby effectively coating it, thus not allowing for the biomolecule of interest to stick on the walls of the containers. Thus, our release medium was optimised by adding BSA to form 0.1% BSA solution in PBS and the GLP-1 amount recovered for the first 4 days was monitored which was enough to conclude if the BSA incorporation in the release medium could be a solution to our problem. As before, 0.005 mg/mL GLP-1 solution was prepared in 0.1% BSA-PBS solution. This concentration of GLP-1 was chosen as our working concentration in the release study would be in the same level. The solution samples were then kept at three conditions, 37 °C with and without continuous agitation, and 4 °C without agitation. Interestingly, BSA greatly inhibited the loss of GLP-1 in all the samples. No loss was observed for the first two days for all the samples after which there was a slight loss in amount recovered for the samples at 37 °C under continuous agitation, while the other remaining samples still showed no loss by day 4 (**Figure 6.7**). Thus, BSA was chosen as an additional compound to be used at 0.1% in the release studies and the time points for the release study was set as every other day.

Figure 6.7 Graphical plot showing amount of the GLP-1 (0.005 mg/mL) recovered as a percentage of the initial amount at day 0 upon addition of BSA (0.1% w/v) in the release medium, data presented as mean $\pm$ SD (n=3)

6.7 Effects of intraparticle pH on release kinetics of GLP-1

Having observed the difference in the level of pH and the acidic microenvironment between the h-MPs and s-MPs, it is of interest to investigate if these attributes could be used to explain the release profiles of the MPs. Thus, a release study of GLP-1 from both the formulations was investigated, to understand how the particle morphology can influence release kinetics of the encapsulated biomolecules. A calibration curve and the equation were first generated by plotting the area under the absorption peak given by HPLC measurements versus the known concentration of the GLP-1 solution.

Figure 6.8 HPLC chromatogram of GLP-1 of known concentrations used for the preparation of the calibration curve (a) and a graphical plot showing the calibration curve and equation generated by plotting the absorbance area with respect to the known concentrations of the standard GLP-1 solutions

The cumulative release profiles of GLP-1 from the hollow and solid MPs over the 14 days release period is shown in **Figure 6.9**. While the cumulative release of GLP-1 from h-MPs showed a cumulative amount of $88 \pm 8\%$, the cumulative release from s-MPs plateaued at $33 \pm 6\%$, without any further release. In other words, a more complete and efficient release of GLP-1 was achieved from h-MPs. This result further supports our

observation that was presented in **Chapter 5** on the release of bovine insulin from MPs, whereby the release from the hollow MPs was significantly improved while the solid MPs suffered from incomplete release of insulin.

Figure 6.9 Comparative in vitro release profiles of GLP-1 peptide encapsulated in the hollow and solid MPs, data presented as mean $\pm$ SD (n = 3)

6.8 Study of polymer-biomolecule adsorption

A simple bulk concentration measurement experiment was conducted to elucidate the polymer-biomolecule adsorption. First, the amount of GLP-1 lost from the GLP-1 solution was investigated when exposed to: (a) same amount of blank polymer MPs in the medium at different pHs, and (b) different amounts of the polymer at a constant pH. **Figure 6.10a** shows that there was a significant decrease in the recovery of the GLP-1 amount in the solution when interacted with an equal amount of the MPs at acidic pH 2 compared to neutral pH 7. While the amount of GLP-1 in the solution remained almost unaffected at pH 7, $30 \pm 4\%$ of the initial amount of the GLP-1 couldn't be detected in the solution at pH 2.

Figure 6.10 Bar graph showing the influence of pH on the adsorption of the peptide GLP-1 to PLGA polymer (a) and the influence of increased amount of the polymer on the adsorption of the peptide GLP-1 (b), data presented as mean $\pm$ SD ($n = 3$)

Likewise, when the GLP-1 solution was mixed with 0, 1, 10, 50, and 100 mg of MPs in release medium adjusted at pH 2, it was observed that with an increasing amount of MPs, the recovery of GLP-1 in the solution also decreased. The peptide solution alone, however, was confirmed to be stable at pH 2 by comparing the HPLC chromatograms with that of peptide at pH 7 (**Figure 6.11**). While the control without polymer MPs in the solution showed no loss ($100 \pm 4\%$) in the recovery of GLP1, the recovery was $88 \pm 2\%$, $71 \pm 3\%$, $43 \pm 3\%$ and $28 \pm 1\%$ of the initial amount for 1 mg, 10 mg, 50 mg and 100 mg of MPs in the solution respectively. This strongly suggests that the disappearance of GLP-1 from the solution is likely due to polymer-biomolecule interaction, and thus polymer-biomolecule adsorption can be a significant factor affecting the release of the encapsulated biomolecules.

Figure 6.11 HPLC chromatogram of GLP-1 solution in PBS adjusted to pH 7 (red) and pH 2 (blue)

6.9 Assessment of structural integrity of GLP-1 by MALDI TOF MS

So far, solid MPs have been shown to have significantly low intraparticle pH in comparison to hollow MPs, as a result of greater accumulation of acidic degradation products. Furthermore, it was shown that there is a greater adsorption of the positively charged GLP-1 and negatively charged polymeric particles which results in the incomplete release of GLP-1 from solid MPs. However, this strong acidic environment inside the MPs and the polymer-biomolecule interactions may have serious implication on the structural integrity of the biomolecules. Thus, it is of interest to probe into the structural integrity of the biomolecule.

Circular Dichroism is preferably used for elucidation of the structural integrity of the proteins and peptides as it can directly provide information on their secondary structures that are considered crucial pointers of structural integrity. However, the low

concentrations of GLP-1 solution samples in our study made it extremely difficult to analyse the samples using Circular Dichroism. As such, we resorted to using MALDI TOF MS instead as it offers the possibility of analysing the sample of concentration as low as in the range of 1-100 pmol/ μ L. MALDI TOF MS is one of the techniques that can be used in analysis of the molecular mass of the biomolecules. Molecular mass analysis of the encapsulated GLP-1 can also provide insight into the fragments of the biomolecules that might have formed as a result of degradation of the biomolecules owing to the various stresses in the MPs. Thus, this could be one approach to elucidate the structural integrity of the encapsulated biomolecules.

For this study, GLP-1 that was still entrapped in the hollow and solid MPs were extracted at day 7 and day 14. Briefly, the particles were collected by centrifugation and washed twice with DI water. Then the MPs were dissolved in acetonitrile, dried at room temperature under vacuum and reconstituted in DI water. The concentration of the extracted solutions was measured by HPLC and the concentrations were normalised by dilution with DI water to match the lower concentration of GLP-1 solution obtained after extraction from hollow MPs. Standard reference GLP-1 solution and heat denatured GLP-1 solution (24 hours at 90 °C) of same concentration was also prepared in the same way as the controls for comparison. Finally, aliquots of 2 μ L of the prepared samples were then mixed with 6 μ L of α -cyano-4-hydroxycinnamic acid (α -CHCA) solution and 2 μ L of this mix was loaded on a clean sample plate and left to dry at room temperature. After the samples were dried, the plate was then inserted into the mass spectrometer.

MALDI-TOF MS analysis of the samples were conducted on a MALDI-TOF instrument (Shimadzu, Japan) using a 337 nm nitrogen laser. Spectra were acquired in linear mode, with power set at 110. Comparing the spectrum of standard reference solution, denatured solution with the ones extracted from the hollow and solid MPs, it became clear that there was some degree of fragmentation of the encapsulated GLP-1 in solid MPs. An additional peak was detected near 2960 m/z for the GLP-1 extracted from the solid MPs at day 7 and day 14 (even higher in intensity) which is also detected in the spectrum of the heat denatured GLP-1 solution (**Figure 6.12a**). However, this additional peak is not present in

the standard reference solution of GLP-1 and the one extracted from the hollow MPs (**Figure 6.12b**). Thus, the MALDI-TOF MS revealed that there is onset of degradation of encapsulated GLP-1 in the solid MPs while hollow MPs are shown to be unaffected from this issue.

Figure 6.12 MALADI TOF MS spectra showing peaks for molecular mass of the GLP- solution at day 0 (yellow line), day 7 (blue line), day 14 (red line) extracted from solid MPs (a) and hollow MPs (b). Spectra for standard GLP-1 solution (orange line) and heat denatured GLP-1 solution (green line) were obtained to serve as controls for comparison

6.10 Assessment of bioactivity of GLP-1

The fragmentation of biomolecules as observed in the MALDI-TOF MS spectroscopy may not always be the sole reason of loss of functionality of the biomolecules. The biomolecule may not be fragmented, yet there might be loss in the bioactivity owing to disruption in secondary structures etc. Thus, it is important to conduct an experiment that can be directly used to assess the bioactivity of the biomolecules.

Figure 6.13 The results showing bioactivity of the peptide extracted from hollow and solid MPs at day 7, normalized to 1000 pg/mL (a), data presented as mean $\pm$ SD ($n = 3$), and HPLC chromatogram of the peptide extracted from the MPs at day 7 showing the intact and degraded peaks of GLP-1(b)

As such, the bioactivity of GLP-1 was assessed using a HTRF active GLP-1 assay kit. This is a direct method of determining whether the GLP-1 is bioactive or not. This kit uses sandwich immunoassay involving two monoclonal antibodies, one labelled with Lumi4Tb-Cryptate (Donor) and the second with d2 (Acceptor).^[2] The principle of

detection is based on HTRF® technology. The two antibodies bind to the active GLP-1 and upon the excitation of the donor with a light source, Fluorescence Resonance Energy Transfer (FRET) towards the acceptor is triggered which in turn fluoresces at 665 nm wavelength. This fluorescence signal intensity is proportional to the antigen-antibody complexes formed and therefore to the active GLP-1. This kit was used to test the level of bioactivity of the GLP-1 extracted at day 7 from both the formulations suspended in PBS.

The results in **Figure 6.13a** showed that 93% of the extracted GLP-1 was still bioactive for the h-MPs. In contrast, a significant drop in the bioactivity was observed for s-MPs and only 58% of the biomolecule was observed to be bioactive. HPLC chromatograph analysis of the extracted GLP-1 at day 7 confirmed the acylation of GLP-1. HPLC chromatography of GLP-1 extracted from h-MPs showed a very small peak for intact biomolecule showing presence of very little amount of non-released intact biomolecule (**Figure 6.13b**). However, for s-MPs, not only a significantly large peak was observed for non-released intact peptide, a second peak was also observed which well indicates an acylation product of the GLP-1. Because of this degradation, the bioactivity of the encapsulated biomolecule is compromised in s-MPs. This limitation could thus be ameliorated through the use of h-MPs as a delivery vehicle considering the results above. Hence, h-MPs that can be easily fabricated through this one-step, osmogen-mediated technique is more efficient in delivery of biomolecules.

6.11 Discussions

The h-MPs loaded with GLP-1 were fabricated through the addition of an osmogen in the oil phase (polymer solution in organic solvent) before emulsifying with the water phase (water with surfactant).^[3] For the h-MPs, presence of the NaCl salt in the polymer solution droplet creates an osmotic pressure that drives water from the external continuous phase into the polymer droplets. With careful adjustments of the amount of NaCl as the osmogen, the amount of water that penetrates into the polymer solution droplet is controlled so as to facilitate the coalescing of the penetrating water droplets to be at the

core of the emulsion. By removing the water core through freeze drying, h-MPs are formed. In contrast, s-MPs were obtained, without the addition of salt during the fabrication process

When the particles were suspended in the release medium, even though both MPs were composed of the same PLGA polymer, with time, s-MPs experienced an earlier and more significant mass loss. The change in average molecular weight over time (**Figure 6.2b**) further corroborated above observations, whereby the s-MPs had an earlier onset and a relatively more drastic reduction of its average molecular weight. A decrease in average molecular weight is evidence of hydrolysis, and this leads to the formation of acidic degradation products, i.e. oligomers. These acidic oligomers dissolve in the aqueous environment, and dissociate into carboxylate anions.^[4] As the acidic degradation products accumulate within the particles, these anions increase the negative charge of the polymer matrix. At the same time, the accumulation of hydrophilic carboxylate anions further increases water diffusion into the MPs, leading to a faster hydrolysis of PLGA, i.e. an auto-catalytic effect. The results from the mass loss and average molecular weight change study, in totality, cohesively imply that both MP formulations undergo hydrolysis when exposed to the physiologically-relevant release medium. Although both formulations were composed of the same polymer, i.e. PLGA, they experienced different hydrolysis rates due to their difference in particle morphology. Because the s-MPs possess a higher polymer mass per particle, due to its dense solid morphology, more degradation by-products are expected to be formed within s-MPs. These acidic oligomers in s-MPs tend to be entrapped within these particles, leading to a lower intraparticle pH (**Figure 6.5a**). Because of the solid morphology of s-MPs, these oligomers could not diffuse efficiently out into the release medium,^[5] due to a slower diffusion rate through a dense polymer matrix and a longer diffusion distance. On the other hand, although acidic oligomers are also formed in h-MPs (**Figures 6.2** and **6.5**), this did not seem to affect its intraparticle pH as significantly. As the shell of h-MPs degrades, the acidic products have a shorter diffusion distance to the release environment. In addition, the water-filled hollow cavity also helps to dilute the concentration of the acidic oligomers, and further promotes diffusion. As such, the less acidic oligomers in h-MPs were better able to leach or diffuse

into the release medium. These results therefore consistently explain why a huge variation exists between the intraparticle pH of s-MPs and the pH of its release medium, and this is attributed to the dense particle morphology of s-MPs.

The release profiles from the two batches also showed a stark difference, which can be attributed to two main factors. Firstly, because of particle morphology, the rate of biomolecule release is different in each formulation. While a hollow morphology promotes release through diffusion, a solid morphology tends to restrict diffusion because of a greater diffusion distance and a denser polymer matrix. Secondly, the intraparticle pH of the degrading MPs can also influence the release kinetics of the biomolecule.^[4] When the intraparticle pH drops below the isoelectric point of the encapsulated biomolecule, the biomolecule attains a positive charge and will be electrostatically bound to the negatively-charged degrading PLGA matrix. This electrostatic interaction between positively-charged biomolecule and a negatively-charged polymer can significantly impede the release. To validate if the polymer-biomolecule interaction is a significant factor impeding the release, the net charge of GLP-1 as a function of pH was calculated. For this, the amino acid sequence of GLP-1 was analysed using the Bachem's peptide calculator with this formula:

$$Z = \sum_i N_i \frac{10^{pKa_i}}{10^{pH} + 10^{pKa_i}} - \sum_j N_j \frac{10^{pKa_j}}{10^{pH} + 10^{pKa_j}}$$

Z	Net charge of the peptide sequence
N _i	Number of arginine, lysine, and histidine residues and the N- terminus
pKa _i	pKa values of the N-terminus and the arginine, lysine, and histidine residues ^[4]
N _j	Number of aspartic acid, glutamic acid, cysteine, and tyrosine residues and the C-terminus
pKa _j	pKa values of the C-terminus and the aspartic acid, glutamic acid, cysteine, and tyrosine residues ^[6]
pH	pH value

The graph plot of the charge of biomolecule as a function of pH revealed that the isoelectric point of GLP-1 is at pH 7.7. With a decrease in the environmental pH, the positive net charge of this biomolecule increases to be as high as +5 net charge per kDa, when the pH is at or below 3 (**Figure 6.14**). Considering the results from our intraparticle pH measurements, it is evident that the encapsulated GLP-1 in s-MPs attains a more positive net charge per mass than in h-MPs. With an intraparticle pH of 2.64 (for s-MPs – **Figure 6.5a**), this would imply that the biomolecule would achieve a +5 net charge per kDa compared to +1.5 net charge per kDa for h-MPs. A stronger electrostatic interaction therefore exists between a more positively-charged GLP-1 and a more negatively-charged (i.e. acidic) polymer matrix. In a recent study by Balmert et al, it was further shown that there is an inverse correlation between the net positive charge of the biomolecule and its release rate.^[4] Hence, a simple, bulk concentration measurement experiment^[7] was carried out to confirm if the polymer-biomolecule interaction, as a result of a considerably stronger acidic environment, is the main reason for the incomplete release observed for s-MPs.

Figure 6.14 Graph showing net charge on the peptide GLP-1 at different pH (Graphical plot obtained from <http://www.bachem.com/service-support/peptide-calculator>)

Evidently, when the biomolecule was exposed to polymeric MPs suspended in the solution adjusted to pH 2, there was significant adsorption of the peptide to the polymer when compared to that of solution adjusted to pH 7.

Finally, upon analysing the bioactivity of the GLP-1 extracted from the particles suspended in the release medium, it was observed that the bioactivity of the GLP-1 in the s-MPs was significantly compromised in comparison to that in the h-MPs. These results are in good correlation with the intraparticle pH study whereby the pH of s-MPs was extremely low (pH 2.64) compared to h-MPs (pH 4.99) by day 7. This high level of acidity has been widely reported to be detrimental to the bioactivity of the encapsulated bioactive molecules such as peptides and proteins.^[8-10] Also, it has been reported that at such an acidic microenvironment, cationic peptides form new covalent bonds with the PLGA oligomers by acylation reactions resulting in PLGA-peptide adducts.^[4, 11] By HPLC chromatography, it was revealed that in fact there was acylation of the biomolecules in s-MPs (**Figure 6.13b**). Likewise, the results from MALDI TOF MS showed there also was degradation of GLP-1 into fragments of lower molecular weight in solid MPs (**Figure 6.12a**). Thus, this acylation of GLP-1 and also the degradation into fragments could have factored in the decreases of the bioactivity of GLP-1 encapsulated in s-MPs.

6.12 Summary

GLP-1 was successfully encapsulated into PLGA h-MPs using a one-step fabrication technique. Mass loss, changes in average molecular weight and intraparticle pH were measured and compared between h-MPs and s-MPs. The results in its entirety indicate that there is a significantly less accumulation of polymer degradation products, or acidic oligomers, in the h-MPs. In contrast, s-MPs suffered from a highly acidified microenvironment that drastically decreased the intraparticle pH. The acidic polymer matrix was found to impede biomolecule release through an electrostatic interaction between the negatively-charged polymer and positively-charged biomolecule. In addition,

the biomolecule from h-MPs maintained its structural integrity and bioactivity as shown by MALDI TOF MS and active GLP-1 HTRF assay. Therefore, h-MPs was shown to be a promising solution to the inherent problems of s-MPs as a delivery vehicle for biomolecules.

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Chapter 7

Conclusions and Future Recommendations

This chapter draws together the conclusions of the overall results obtained from the studies encompassed by this thesis that has been discussed in Chapter 4, 5 and 6, and reflects on the extent of fulfillment of the objectives that were set at the inception of the thesis. All the results discussed earlier are woven together to provide a conclusive gist of the findings of this thesis. Finally, the chapter ends by painting a brief picture of opportunities and strategies for future studies that can be further built upon the work presented in this thesis. The fabrication method we have developed and reported, can be easily modified to obtain double layered hollow microparticles. With the addition of additional layer of polymer, it can have great implications on the attributes of the MPs as carriers of biomolecules. Hence, the fabrication process is introduced in brief and the potential advantages of double layered hollow microparticles are highlighted leaving the reader with motivation of exploring the avenue of using osmogen mediated emulsion solvent evaporation technique to fabricate double layered hollow microparticles for encapsulation and delivery of biomolecules.

7.1 Conclusions

The work for this thesis was designed to answer some of the frequently reported, major limitations of using biodegradable polymer MPs for encapsulation and delivery of biomolecules. With extensive study of the literature, it became evident that despite the immense potential of biomolecules (proteins and peptides) in therapeutic industry, the limitations of biomolecules such as short half-life, low bioavailability due to rapid degradation and clearance from the body, paved the way for the development of varieties of delivery platforms to protect biomolecules, while making it possible to release them in sustained fashion. Probing further into this, it was clear that the MPs of biodegradable polymer such as PLGA was most widely used and accepted platform for encapsulation and delivery of biomolecules due to various properties (biocompatibility, biodegradability, suitable physical properties) allowing it to be easily designed to suitable shapes and size to cater to various applications. However, it further became obvious that this delivery system, although widely used by pharmaceutical industry in therapeutic formulation, is still lacking in certain aspects.

The major limitation of biodegradable polymer MPs as delivery platform stems from their inability to allow for the complete release of encapsulated biomolecules. As such, the efficiency of the particles is greatly affected. While the non-release of biomolecules would be a waste from economical point of view, the possibility of degradation and denaturation of non-released biomolecules and possible ensue of immunogenicity becomes additional concerns. As such, it becomes imperative to find a way to optimize the MPs delivery system to ameliorate these concerns.

Keeping this in mind, it was hypothesized that using MPs of hollow morphology instead of widely used solid morphology could be a possible answer to the problem. The rationale behind this was that, with the hollow morphology, the thinner shell because of hollow core instead of the solid, dense core, would greatly reduce the diffusion distance for the encapsulated biomolecules, thus facilitating their release. Also, the thinner shell would allow easy diffusion of the acidic degradation products of polymer out of the particle that

would otherwise accumulate and create extremely acidic microenvironment which has been frequently reported to be detrimental for encapsulated biomolecules' structural integrity and bioactivity. Thus, our first phase of work for this thesis was to develop an easy, one-pot single step method to fabricate hollow MPs that would be later used as an efficient platform for delivery of biomolecules.

The well-established emulsion solvent evaporation technique was modified to meet the goal of producing hollow MPs in a single step. Inclusion of osmogen in the oil phase of emulsion was a key aspect of modifying the technique to produce hollow MPs. The osmogen would withdraw water from the external aqueous phase into the polymer solution droplet which would then coalesce into the core of the MPs while the polymer precipitated and formed a shell around the water filled core upon evaporation of the solvent. Upon freeze drying these particles, the dry powder of hollow MPs was obtained. With a series of experiments, varying several process parameters such as initial polymer solution concentration, type and amount of osmogen, stirring speed etc., a correlation between these process parameters and the final morphology of the MPs was established which allowed us to obtain a comprehensive understanding of the method developed and equipped us with capability to control the final morphology of the MPs such as shell thickness, volume space of hollow cavity and size by simply adjusting the osmogen amount. Thus, our first objective was achieved.

In the second phase of this work, bovine insulin, a hydrophobic protein was encapsulated as a model biomolecule to test the suitability of the system as a delivery vehicle. It was encapsulated in solid microcrystals form and in solution form in the MPs of varying shell thickness and volume space of hollow cavity. While the encapsulation efficiency remained similar throughout all the formulations, interesting observation were made in regard to the release profiles obtained. The release study conducted revealed that changing the morphology of MPs from solid to hollow greatly improved the release profiles of the protein. While the MPs of solid morphology suffered from incomplete release, the release was subsequently improved upon decreasing the shell thickness and increasing the volume space of hollow cavity of the MPs. The method developed allowed us to have the ability

to control the shell thickness and volume space of the hollow cavity by simply changing the amount of osmogen introduced in the fabrication process, thus subsequently influencing the release of biomolecules. Experiments were further conducted to assess the structural integrity and bioactivity of the encapsulated biomolecules to ascertain if there was any significant effect due to the difference in morphology i.e. solid vs hollow. However, the assessment of structural integrity by MALDI TOF MS, Circular Dichroism spectroscopy and assessment of bioactivity by MCF-7 cell proliferation study revealed that there was not any significant loss of bioactivity of encapsulated biomolecule in MPs of both morphologies. Although the second objective to validate the hollow MPs as a system offering better release kinetics over solid MPs was achieved, the results could not substantiate the superiority of hollow MPs over solid MPs in terms of bioactivity of the encapsulated biomolecules. The hydrolytic degradation study provided a clue that for the chosen duration of release study, there was not any significant difference in the degradation of the MPs of both morphologies and thus the effect of accumulation of acidic bi-products of degradation could not manifest in loss in bioactivity. Thus, this motivated an extension to the study with the PLGA of lower molecular weight and lower intrinsic viscosity which would allow for substantial degradation of the polymer over a shorter duration, making it possible to investigate on the effects of accumulation of the polymer degradation products on the encapsulated biomolecules.

With this in mind, hollow and solid MPs were fabricated using PLGA of lower molecular weight and intrinsic viscosity. A hydrophilic biomolecule, GLP-1 was chosen as model peptide as it would elucidate the versatility of the platform as a carrier of both hydrophilic and hydrophobic biomolecules (bovine insulin). The release study conducted further corroborated the previously obtained results. As expected, solid MPs suffered from incomplete release, while the hollow MPs release was near complete. Upon investigation into the bioactivity of the encapsulated GLP-1 in these MPs, interestingly, it was found that there was a significant loss in the level of bioactivity of the biomolecule in solid MPs while hollow MPs suffered a minimum loss. Intraparticle pH measurement study, mass loss and molecular weight loss study and polymer-biomolecule interaction study were carried to explain the observation. The results obtained made it clear that there was

significant accumulation of negatively charged acidic degradation products in the solid MPs, thus the pH had dropped to extremely low values in solid MPs. Further, upon analyzing the peptide sequence, it was found that GLP-1 attains significantly high net positive charge at low pH. Thus, the possibility of strong electrostatic interaction between the negatively charged polymer and the positively charged biomolecules had to be given a thought. This was later observed to be true when experiments were carried out to quantify the polymer-biomolecule adsorption at acidic pH in comparison to neutral pH. This adsorption not only factored in incomplete release of the biomolecule, but also promoted acylation of the biomolecule which was observed in the HPLC chromatogram. As a result, the bioactivity of the biomolecules was significantly compromised in solid MPs while hollow MPs were observed to effectively prevent this detrimental effect. Thus, the hollow MPs were validated to be superior over solid MPs for effective encapsulation and delivery of biomolecules, achieving the final goal of this thesis.

Considering the results discussed earlier on the encapsulation and release of the biomolecules (bovine insulin and glucagon like peptide-1) from the hollow microparticles, the fabrication method and the microparticles fabricated thereof can be considered as a versatile platform allowing for effective encapsulation of biomolecules of different hydrophilicity. Successful encapsulation and release data together with the results pertaining to the preservation of bioactivity, obtained for the hydrophobic bovine insulin and hydrophilic glucagon-like peptide, are the testimony to the possibility of encapsulation and release of different biomolecules scattered within the spectrum of hydrophobic to hydrophilic characteristics. However, it is to be noted that the encapsulation efficiency of the hydrophilic biomolecule was comparatively lower than the hydrophobic biomolecule due to the natural tendency of the hydrophilic biomolecule to be easily solubilized and leached out into the external aqueous phase during the MPs fabrication. It may be possible that the encapsulation efficiency for hydrophilic biomolecule can be further increased by loading it into double layered hollow MPs which is discussed further in **Section 7.2** below.

7.2 Future recommendations

In this project, an easy one step technique to create hollow MPs was developed that were subsequently validated to be superior over solid MPs in terms of achieving better release and preservation of bioactivity. However, the full potential of the fabrication technique developed is yet to be explored. Preliminary investigation into the possibility of fabricating double layered hollow MPs showed that, with careful optimization of the fabrication parameters, double layered hollow MPs can be easily fabricated. The fabrication technique can easily be optimized to produce double layered and potentially multilayered hollow MPs. The process is briefly discussed below.

7.2.1 Fabrication of double layered PLGA/PLLA hollow MPs

The methodology involved is similar to the one that was developed to fabricate single layered hollow MPs. The only difference to note is that, two different polymers (PLGA and PLLA in this case) are dissolved in the organic solvent instead of one. **Figure 7.1** below shows the schematics of the fabrication process. Briefly, 0.1 g of PLGA and 0.1 g of PLLA (6.5% w/v) were dissolved in 3 mL of DCM to which 10 mg of NaCl (5 % w/w) were added. The content was put under magnetic stirring for 3.5 hours and vortexed for 20 minutes. Subsequently, the polymer/salt solution was poured into 100 mL of PVA aqueous solution (3% w/v) and emulsified under overhead stirring for 3.5 hours at 500 rpm to evaporate the volatile organic solvent resulting in double layered hollow MPs (**Figure 7.2**). During this solvent extraction and evaporation, the oil phase becomes more concentrated and reaches above the cloud point which triggers the phase separation of the two polymers. The cloud point is the polymer solution concentration at which the polymers begin to phase separate due to subsequent immiscibility while still remaining dissolved in the solvent^[1, 2]. If the polymer precipitation rate is slow enough, two polymers will configure themselves in their most thermodynamically stable configuration as dictated by the interfacial tensions of two polymers and the external aqueous phase, using the spreading coefficient theory which was developed by Harkin^[3] and further

extended by Torza and Manson.^[4] Likewise, when the precipitation rate is considerably fast, the polymer phases may be kinetically entrapped in a non-equilibrium configuration as a transient intermediate within the MPs. Therefore, there are two possibilities of forming the double-layered hollow structure. In the first case, where the complete engulfment is predicted by the spreading coefficient, the precipitation rate should be slow enough such that one polymer has sufficient time to spread onto the other. In the second case, the polymer precipitation rate should be carefully optimized so as to kinetically trap the polymers in the double-layered structure as a non-equilibrium configuration. As such, further study is necessary to fully investigate the double layered hollow microparticle formation process so that the polymers on each of these layers can be accurately determined while also a correlation can be established between the process parameters and the layer localization of each of the polymers. Having a thorough understanding of how the fabrication process parameter affects the polymer layer configuration could greatly help in optimizing the encapsulation and release of biomolecules.

Figure 7.1 Schematic illustration of the process of double layered hollow MPs fabrication

Figure 7. 2 SEM image showing the cross-sectional view of the double layered hollow MPs fabricated.

7.2.2 Significance of double layered hollow MPs

In this study, although the encapsulation efficiency for the hydrophobic biomolecule bovine insulin was high, it was significantly low for the hydrophilic biomolecule Glucagon like Pepide-1. Solid or hollow, the morphology couldn't improve the encapsulation efficiency. Thus, it seems to be an inherent problem of single-layered particle types, that they often suffer from low encapsulation efficiency for hydrophilic drugs.^[5] This could be because of easy diffusion of the hydrophilic biomolecules from the surface area of the MPs during fabrication while the particles are in the hardening process, due to their higher solubility and hydrophilicity. Thus, using double layered configuration with an outer polymer layer that has lower affinity with the biomolecules to be encapsulated, could possibly prevent the leaching of hydrophilic biomolecules during the fabrication process, thus increasing the encapsulation efficiency.

Likewise, it is also not possible to achieve layer localization of two different biomolecules in the single-layered MPs, giving rise to an inability of releasing two biomolecule drugs in a pulsatile or sequential manner from a single particle.^[6] Owing to the limitation on encapsulation efficiency and pulsatile/sequential release from the monolithic system, double layered hollow microparticle systems that is composed of an outer polymer shell surrounding inner second polymer layer is desirable. This double layered hollow morphology may provide improved control over drug release rates, through selective localization of each drug in the individual polymer phases^[5, 7-11], while also benefiting from reduction of accumulation of degradation products, biomolecule-polymer interaction and subsequent acylation of encapsulated biomolecules due to the hollow morphology. Thus, two different drugs could be effectively released at different doses with time through the layer localization of drugs in different layers^[6]

Although this thesis encompasses the study of release kinetics of individual drugs (GLP-1 and bovine insulin) from single layered hollow MPs, it is an interesting avenue to explore the release kinetic of these two therapeutics when they are loaded together into the double layered hollow MPs. It is reported that unlike a single layered system, the degradation of the top polymer layer would accelerate hydrolysis of the underlying layer in double layer systems.^[12] Therefore, the double layered hollow MPs may also possess unique hydrolytic degradation characteristics and morphological attributes that differ from single-layered hollow particles. As such, these double layered MPs may offer greater versatility in controlling release kinetics and profile, by manipulating the particles' layer thickness, configuration, size and the degradation rates.^[13, 14]

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