Studies of insulin transport across intestinal epithelium using nano-encapsulation for oral delivery

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**STUDIES OF INSULIN TRANSPORT ACROSS INTESTINAL
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NTU Institute for Health Technologies**

A thesis submitted to the Nanyang Technological University in partial
fulfillment of the requirement for the degree of

Doctor of Philosophy

2018

Statement of Originality

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

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Date

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8 Feb 2019

Date

Prof Subbu Venkatraman

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Abstract

Diabetes Mellitus is a chronic metabolic disease characterized by abnormally high blood glucose level. Conventional subcutaneous insulin injection has been the dominant treatment option for the diabetic patients due to its immediate glucose lowering effect. However, its long term application has generated a plethora of problems, relating to the complications and compliance issues. Great efforts have been devoted to the development of an effective alternative treatment but none of them has been turned into a solution to date. The discovery of insulin in the early 1920s brought promising hopes to pharmaceutical industries as increasing number of them have been attempting to load the drug in a capsule for the conventional oral route. However, the nature of the protein renders huge difficulties as it attempt to overcome the defensive barriers of the body. The bioavailability of the orally ingested protein drug is too low to achieve the desired glycemic control because of the degradation by proteolytic enzymes and low permeability through the small intestine. These shortcomings have limited the application of oral tablets and discouraged the diabetic patients to switch from the current dominant subcutaneous injection to oral treatment. However, this does not stop the research towards the oral route of delivery because of its promising future. An oral route not only minimizes the patient compliance issue by eliminating the use of invasive needles, more importantly, it offers hepatic glucose regulation as the orally administered insulin would potentially be carried by hepatic portal vein to reach the liver, where glucose homeostasis take place. This made the oral pathway extremely advantageous because it lowers the risk of hypoglycemia and hyperinsulinemia which are often associated with improper control of injected insulin dose. Hence, the aim of this study is to investigate the transport of insulin across intestinal epithelium using nano-encapsulation for oral delivery.

Liposomes has been the most translatable and extensively studied vesicles in the field of oral drug delivery. The studies of insulin transport across intestinal epithelial cells were investigated from three general areas; interaction of empty liposomes with intestinal epithelial Caco-2 cells, cellular interaction and transport of insulin loaded liposomes with appropriated surface modifications across Caco-2 cells, loading of

insulin onto surface of liposomes for enhanced transport, improved loading, and sustained release. Liposomes fabricated from lipids of different acyl chain length, degree of saturation, and, cholesterol content has been investigated for their capability to generate high cellular association. Rigid HSPC/DOTAP liposomes demonstrated higher cellular association compared to the softer ones. Eudragit S100 coating was performed on rigid HSPC/DOTAP liposomes with the help of Tween 80. In the presence of free Eudragit S100 and Tween 80, cellular uptake and transport was observed in Eudragit S100-coated insulin-loaded HSPC/DOTAP liposomes. With a modified rehydration protocol, insulin transport was further improved from 3.9ng/ml to 35.7ng/ml. Subsequently, a Layer-by-Layer approach was developed to load insulin onto the surface of HSPC/DPPG liposomes with the help of oppositely charged chitosan. The multilayer coated liposomes exhibited significant improvement in insulin loading, cellular uptake and transport, and sustained the release of insulin for up to 3 months. With only 1 layer of insulin loaded on the surface of liposomes, the transport of insulin across Caco-2 cells was enhanced by 2.6 fold from 5.7ng/ml to 15ng/ml. An enhancement factor of more than 8x was observed with just 2 layers of insulin coating. This thesis has demonstrated a novel approach to deliver oral insulin via the Layer-by-Layer approach. This approach based on simple electrostatic interaction generated particles with long sustained release window, therefore serve as a potential alternative to the current subcutaneous injection.

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List of Abbreviations

Active Pharmaceutical Ingredient	API
Gastrointestinal	GI
Gut associated lymphoid tissue	GALT
metabolizing enzyme cytochrome P-450 3A4	CYP3A4
P-glycoprotein	P-gp
Morpholine-urea-phenylalanine-homophenylalane-nine-vinylsulfone-phenyl	K02
Hydrochloric Acid	HCl
organic cation transporters	OCTs
Multi-drug resistance protein 1	MDR1
organic anionic transporting peptides	OATPs
ATP-binding cassette sub-family B member 1	ABCB1
low density lipoprotein	LDL
glycosylphosphatidylinositol	GPI
Receptor Tyrosine Kinase	RTK
poly(lactic-co-glycolic acid)	PLGA
poly(acrylic acid)	PAA
poly(methacrylic acid)	PMA
poly(ethylene glycol)	PEG
human papilloma virus	HPV
ovulatory cervical mucus	OCM
polyethylene glycol	PEG
cervicovaginal mucus	CVM
hydroxypropyl methyl cellulose phthalate	HP55
Blood Brain Barrier	BBB
Hanks' Balanced Salt Solution	HBSS
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	HEPES
Dichloromethane	DCM
trifluoroacetic acid	TFA
Isopropyl Alcohol	IPA
L- α -phosphatidylcholine	Egg PC

1,2-dimyristoyl-sn-glycero-3-phosphocholine	HEPC
Soybean phosphatidylcholine	SPC
1,2-dipalmitoyl-sn-glycero-3-phosphocholine	DPPC
1,2-Dimyristoyl-sn-glycero-3-phosphocholine	DMPC
soybean phosphatidylcholine	HSPC
1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol, sodium salt	DPPG
1 2-Dioleoyloxy-3-trimethylammonium propane chloride	DOTAP
Phase transition temperature	T _m
Human epithelial colorectal adenocarcinoma cells	Caco-2
Encapsulation efficiency (%)	EE (%)
Loading efficiency (%)	LE (%)
Enzyme-linked immunosorbent assay	ELISA
Transepithelial Electrical Resistance	TEER
Epithelial Voltometer	EVOM
Simulated Intestinal Fluid	SIF
Simulated Gastric Fluid	SGF

Chapter 1

Introduction

This chapter consists of the background, problem statement, objectives and scope, hypothesis, novelty of the research findings, and outline of the dissertation. Oral delivery has been the ultimate goal of insulin therapy for diabetic patients as it eliminates the reliance of needles, improve patient compliance, and minimize injection-associated complications by involving liver for insulin dose control. However, barriers in the GI tract has made the oral pathway extremely challenging as macromolecules such as protein will be degraded into its basic units which adversely affects the bioactivity and bioavailability of the drug. In this thesis, liposomes with appropriate surface modification were proposed to improve the transport of insulin. In addition, a Layer-by-Layer approach of coating insulin on the surface of liposomes was proposed to improve loading and release of insulin from liposomes. This thesis elucidates the insight into the mechanism of insulin transport with the help of Eudragit S100-coated liposomes; the coated liposomes demonstrated significant improvement of insulin transport compared to unmodified bare liposomes. The Layer-by-Layer approach of coating insulin on the surface of HSPC/DPPG liposomes was demonstrated to be effective for sustained release of insulin for up to 3 months. The Layer-by-Layer coated liposomes demonstrated superior stability in PBS and have a higher uptake and transport of insulin across Caco-2 cells compared to unmodified liposomes.

1.1 Background

Diabetes Mellitus is a chronic metabolic disease characterized by abnormally high blood glucose level. The number of people diagnosed with diabetes was 382 million in 2013 and it was estimated to increase to 592 million in the next 20 years largely due to aging population and urbanization-related change in lifestyle.^[1] A variety of medication choices for glycemic control have been discovered and it can be divided into two main categories; oral delivery of small molecular drugs and subcutaneous injection of protein and peptides.^[2] Subcutaneous injection has been the dominant treatment option for diabetic patient for decades since the discovery of insulin in the early 1920s.^[3] The subcutaneous route of insulin injection gives immediate glucose lowering effect and can relieve the diabetic symptoms temporarily; however, it is often associated with complications such as hyperinsulinemia and hypoglycemia due to improper dose control.^[4, 5] Moreover, the subcutaneous injection delivers insulin directly into the systemic circulation without passing through the liver, which is an important organ involved in homeostasis of glucose.^[4]

An oral route of delivery offers greater advantage as the administered insulin mimics the body's endogenous insulin secretion pathway. Furthermore, the oral route ensures insulin to be carried to the liver before entering systemic circulation, stimulating the role of liver in regulating blood glucose level, thereby reducing the possibility of hypoglycemia and improving patient compliance by eliminating the use of invasive needles.^[6, 7] However, oral delivery has only been made possible for small molecular drugs, peptides and protein drugs could not escape the fate of being degraded as it passing through the harsh environment in the GI tract.^[8] Insulin, being a peptide hormone, faces the same challenges as most of the orally administered protein drugs. Despite the advantages that oral insulin delivery could potentially bring about, and the efforts that have been devoted into the research of oral insulin formulation over the years, there are no oral insulin products available that can achieve the same potency as subcutaneously injected insulin, mainly due to the GI barriers that results in low oral bioavailability.^[9] Furthermore, the intestinal epithelium serves as another physical barrier that lowers the bioavailability of orally administered insulin by limiting the diffusion of intact insulin that reach the surface of the epithelial cells. The tight junctions strictly control the nature and size of molecules to be allowed for

diffusion via paracellular pathway. Only small hydrophilic drugs with molecular weight less than 200Da are able to cross the cell barrier via paracellular pathway.^[8] Hence, a nanocarrier would be important in protecting insulin while transiting through GI tract and enhancing its absorption by small intestine.

Liposomes have been widely used in the pharmaceutical industry as an efficient drug delivery vehicle due to its excellent biocompatibility and unique amphiphilic property.^[10] It is capable of encapsulating both hydrophobic and hydrophilic drugs in its bilayer and aqueous core. Although early attempts of using liposome to improve the oral bioavailability of insulin showed promising prospects in the 1970s,^[11] its application for oral delivery of protein and peptide drugs remains challenging due to the extreme conditions in the GI tract. Low pH and enzymatic degradation was the fate of most orally ingested protein drugs. Bile salts destabilize the lipid bilayer while lipases hydrolyze the phospholipids, leading to the loss of integrity of orally ingested liposomes.^[12-14] Even if the protein reaches small intestine with the protection of an acid resistance capsule, additional barriers such as the mucus layer^[15, 16] that constantly flushes away the adsorbed particulates and the brush border enzymes^[17] that break down any remaining macromolecules that reach the surface of enterocytes will adversely affect the permeability and bioavailability of drugs. Intestinal epithelium, or mainly the enterocytes, serves as another layer of protection against foreign particle invasion via the regulation of its tight junction.^[18] The tight junction closes the gap between adjacent cells, preventing the diffusion of large hydrophilic protein drugs. Orally administered protein and peptide drugs with high molecular weight (>3kDa) was unable to be absorbed without being degraded into amino acids.^[8] Large particles of size 50-500nm are able to be taken up by the enterocytes or M cells via endocytosis or phagocytosis.^[8] Intracellularly, the internalized particle undergoes a programmed degradation pathway by fusion with the acidic compartmentalized lysosome. This further reduces the chance of the protein being transported across the epithelial cell layer without losing its function. Therefore, a carrier that could protect the encapsulated protein drugs, and enhance its transport and uptake must be considered in order to overcome the barriers in the GI tract when designing a formulation for oral protein delivery.

To overcome these drawbacks, numerous studies have reported on the use of various types of nanocarriers including liposome, polymeric nanoparticle, and layer-by-layer

nanoparticle to protect the encapsulated protein drug. Strategies to improve the performance of the nanocarrier including surface modifications with targeting ligand to enhance the cellular uptake, use of pH responsive materials such as anionic polymers, which were shown to act as membrane destabilizing agent and assisted in endosomal escape of internalized particles for intracellular targeting.^[19, 20] In fact, most of the oral formulation failed during clinical trials and so far there is no product been developed for oral insulin delivery.^[9] Diasome Pharmaceuticals is the only company to-date that reach clinical trial phase III with its hepatic-directed nanoliposomes.^[21]

1.2 Problem statement

Oral delivery of proteins and peptides offers great advantages in terms of patient compliance and biomimicry; however, several challenges have to be considered before a formulation can be developed. As insulin is a peptide hormone, its bioavailability is adversely affected by (i) enzymatic and pH variation throughout the GI tract and (ii) intestinal epithelial cell layers as physical barriers in the small intestine.^[22-25] To overcome these barriers, there is a need to develop a carrier system capable of improving the uptake and transport of insulin, while having sufficient loading and sustained release of insulin with adequate protection during its transit in GI tract.

1.3 Hypothesis

It is hypothesized that insulin can be loaded both inside and on the surface of liposomes and transported across the intestinal epithelial cells with the help appropriate surface modification of liposomes. Three sub-hypothesis are described in the following:

- 1) Liposomes can assist the transport of insulin across intestinal epithelium with the help of appropriate surface modification using Tween 80 and Eudragit S100
- 2) Insulin can be coated onto the surface of negatively charged HSPC/DPPG liposomes with the help of positively charged chitosan
- 3) Loading and release of insulin can be improved by coating insulin on the surface of liposome via a Layer-by-Layer approach

Figure 1. The steps involved in nano-encapsulation of insulin for oral delivery. Enteric coated capsule break and release the liposomes in the intestinal lumen. Insulin-loaded liposomes can be surface modified with Eudragit S100 polymers to enhance uptake and transport across intestinal epithelium. Insulin-loaded liposomes can also be coated with multilayers of alternating chitosan/insulin to improve loading, release, stability, and transport.

1.4 Objectives and Scope

The objective of this project is to design a liposomal carrier system for loading insulin and study the uptake and transport of insulin-loaded liposomes across intestinal epithelial cell model (Caco-2). There are 3 sub-aims for this project.

- 1) To optimize the parameters for coating a gelatin capsule with pH-sensitive polymers such that the capsule will only release its contents in the intestine while protecting its cargo in acidic conditions of the stomach.
- 2) To investigate the physiochemical properties of liposomes in affecting the loading and transport of insulin across intestinal epithelial cells and enhance the transport via surface modification of liposomes using Eudragit S100 and Tween 80
- 3) To improve the loading and release of insulin by coating insulin on the surface of liposomes via a Layer-by-Layer approach

1.5 Novelty of the project

An attempt is made in order to study the transport of insulin with the help of liposome carriers for oral delivery. Several novel research findings were discovered while attempting to improve the transport of insulin across Caco-2 cells:

- 1) The coating of Eudragit S100 onto liposomes with the help of Tween 80 in aqueous solution ensured a final size within the nanometer sized range (<500nm) and has eliminated the use of organic solvent which was previously used to coat Eudragit S100 onto multi-laminar vesicles (MLVs).
- 2) A novel insight into the pathway of insulin transport with the help of Tween 80 stabilized Eudragit S100-coated liposomes was proposed; the coated liposomes demonstrated improved the cumulative transport of insulin from 0.139ng/ml to 3.89ng/ml via both paracellular and transcellular pathway compared to unmodified insulin loaded liposomes. A further increase of insulin transport from 3.89ng/ml to 35.7ng/ml was achieved by increasing liposome loading via a modified rehydration protocol.
- 3) A Layer-by-Layer approach to coat insulin on the surface of HSPC/DPPG liposomes was developed which significantly prolonged the release of insulin for up to 3 months. The Layer-by-Layer coated liposomes demonstrated stability for up to 1 month in PBS. With only 1 layer of insulin loaded on the surface of liposomes, the transport of insulin across Caco-2 cells was enhanced by 2.6 fold from 5.7ng/ml to 15ng/ml.

1.6 Outline

The thesis is divided into seven chapters.

In *chapter 1*, background information about the current state of the problem which drives the motivation of this project is summarized. Objectives and scope are outlined. Hypothesis of the project is discussed.

In *chapter 2*, literatures about the current achievements and limitations, barriers of oral drug delivery, type of carriers used to overcome the barriers in the GIT,

mechanism of transport across intestinal epithelial cells, application of liposome surface modification in the field of oral insulin delivery are reviewed.

In *chapter 3*, experimental methods performed to achieve the specific aims are described.

In *chapter 4*, experimental results from coating of gelatin capsules for enteric targeted release are discussed and analyzed.

In *chapter 5*, experimental results including the physicochemical properties of liposome in affecting its interaction with caco-2 cells and liposomal surface modification with Tween 80 and Eudragit S100 for enhanced transport across intestinal epithelial cells are discussed and analyzed.

In *chapter 6*, experimental results including loading, release, and stability of a multilayered system consisting of alternating layers of chitosan and insulin on the surface of HSPC/DPPG liposome are discussed and analyzed.

In *chapter 7*, conclusions and future recommendations of the thesis are proposed.

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Chapter 2

Literature review

This chapter reviews the current status of oral insulin technology developments. Literatures related to the disease state of diabetes mellitus, current treatment options, advantages and limitations of current treatments are described. The reasons why most oral formulations do not reach clinical development stage will then be reviewed followed by the obstacles that oral peptide drugs face while transiting through GIT with measures to overcome these barriers. Following on, the understanding of the mechanisms and pathways of nanoparticle transport across intestinal epithelial cells will be looked into. With that, different aspects of oral drug delivery vehicle will be discussed. This includes, the type of polymeric materials and nanoparticle that has been extensively used for oral drug delivery, the use of liposomes and the advantages of its surface modification, and the applications and implications of Layer-by-Layer coated nanoparticles in drug delivery.

2.1 Disease state

Diabetes Mellitus is a chronic metabolic disease characterized by abnormally high blood glucose level. The number of people diagnosed with diabetes was 382 million in 2013 and it was estimated to increase to 592 million in the next 20 years largely due to aging population and urbanization related change in lifestyle.^[1] The economic cost incurred by diabetes and its associated complications including cardiovascular disease, renal failure, microvascular disease and macrovascular disease was \$245 billion in the US in 2012.^[2]

Insulin is produced by the Islet of Langerhans in the pancreas, stored in secretory granules inside the cytoplasm of pancreatic beta cells, and released by exocytosis upon sensing of a minute change (~ 4.5 to 8 mM in Man) in blood glucose level by the beta cells.^[3] The 51 amino acids peptide hormone plays a key role in modulating blood glucose by assisting the storage and uptake of excess glucose into the peripheral tissue. It is a consisting of two polypeptide chains A and B covalently linked by two disulfide bonds. The deficiency of this hormone observed in diabetic patient can be due to either autoimmune destruction of the insulin secreting pancreatic beta cells, Type 1 diabetes,^[4] or a progressive development of resistance to insulin due to environmental and genetic factors, Type 2 diabetes.^[5]

A variety of medication choices for glycemic control have been discovered and it can be divided into two main categories; oral delivery of small molecular drugs and subcutaneous injection of protein and peptides.^[6] The oral delivery pathway offers greater advantage as the administered insulin mimics the body's endogenous insulin secretion pathway. Furthermore, the oral route ensures insulin to be carried to the liver before entering systemic circulation, stimulating the role of liver in regulating blood glucose level, thereby reducing the possibility of hypoglycemia and improving patient compliance by eliminating the use of invasive needles. However, oral delivery has only been made possible only for small molecular drugs. Peptides and protein drugs could not escape the fate of being degraded due to the harsh environment in the GI tract.

2.2 Insulin

Insulin is a 5808 Dalton small protein secreted by pancreatic islet β cells and is stored in high concentrations in the Golgi apparatus. The protein has 51 amino acids arranged in two polypeptide chains (21 amino acids in acidic chain A and 30 amino acids in basic chain B) connected by two disulphide bonds. In chain A, the acidic amino acid residues Glutamic acid (pI 3.22) dominates the charge making the chain slightly acidic, while in B chain there are more basic amino acid residues Histidine (pI 7.59), Arginine (pI 10.76), and Lysine (pI 9.74), than acidic amino acids Glutamic acid (pI 3.22) and Aspartic acid (pI 2.77) making it slightly basic. Insulin contains with an overall Isoelectric Point (pI) of pH 5.39, where the protein is at its minimal solubility due to the zero net charge. The charge of insulin can be easily tuned by adjusting the pH of the solution; the protein will be negatively charged when dissolved in a solution with $\text{pH} > \text{PI}$, while positively charged when dissolved in a solution with $\text{pH} < \text{PI}$.

Upon secretion by the β cells in pancreases, insulin is concentrated in storage vesicles and stabilized by zinc in hexamers forms. The hexameric structure dissociates into dimers and monomers when released into the circulation due to a drop in the insulin concentration. Interestingly, only monomeric and dimeric forms of insulin are able to stimulate the receptor, therefore the rate of dissociation of hexameric insulin to a large extent determines the pharmacokinetics and pharmacodynamics of subcutaneously administered insulin.^[7] The amino acid residue that assist in the formation of hexamer include B10 His, which binds to zinc ions and stabilized by close-packed hydrophobic interactions due to B14 Ala, B17 Leu, B20 Gly and A 13 Leu residues. In physiological conditions, pancreatic β cells secrete insulin at rate of 0.25-1.5 units/hr to ensure sufficient glucose entry into cells. ^[8] In fasting state, 180 pmol/L (1 ng/ml) insulin produced by pancreatic β cells reach the portal vein where first pass metabolism occurs and removes up to 70% of insulin, as a result only 54 pmol/L (313 pg/ml) reach the peripheral circulation. On the other hand, 800 pmol/L (4.6 ng/ml) insulin is produced during fed state of which 55% are degraded by the liver, resulting in 360 pmol/L (2 ng/ml) insulin reaching the peripheral circulation. ^[9]

Figure 2. Insulin clearance rates and insulin fluxes in a normal man (70kg, body surface area 1.73m²) in the fasting condition (upper panel) and in the fed state (lower panel). The rate of insulin clearance is expressed in L min⁻¹ below each tissue. The amount of insulin released from pancreas and the amount of insulin reaches the peripheral circulation is represented by the values expressed in “U day⁻¹” or in “U per 2h” units. The amount of insulin cleared by the near tissue is indicated by the values in the gray boxes. The numbers in the light gray arrows indicate the insulin concentration into the portal vein and in the post-hepatic veins. Adapted from Ref^[9]

Source of Insulin has evolved from early animal extraction to the current biosynthetic human insulin and its analogue since its discovery. Recent therapies of diabetes involved the use of insulin analogues whose amino acid sequences have been altered to achieve enhanced molecular properties such as fast or prolonged duration of action. Generally, insulin analogues can be classified into two categories, rapid acting insulin analogues such as insulin lispro, insulin aspart, and insulin glulisine and long-acting insulin analogues including insulin glargine and insulin detemir. Rapid-acting insulin analogues normally is injected at meals for correcting high blood glucose as it gives peak insulin concentration soon after injection and gets metabolized and eliminated within 3 to 5 hours, whereas long-acting insulin analogues provides basal insulin and helps to maintain a constant and stable insulin concentration for 12-24 hours after administration. ^[10]

2.3 Current clinical application of oral insulin delivery

Despite extensive research in the field of oral protein delivery, no oral insulin product has been approved and commercialized in the global market. Various industries have attempted different strategies of oral insulin delivery using polyelectrolytes nanoparticles, liposomes, and enzyme inhibitor and permeation enhancer. Some of them have made it to the clinical phase while others were eliminated due to the limitations or side effects. The failure and success of these technologies that entered clinical trials and the challenges they faced has been described in various reviews, [11-15] and summarized in table 2.

Table 1 Table summarizes current oral insulin delivery technologies under clinical trials. Adapted from Table summarizes current oral insulin delivery technologies under clinical trials. Adapted from Ref [12]

Company	Product	System	Clinical trial status	Ref.
Diasome	HDV-I	hepatic targeting nanoLiposomes	Completed Phase II in T1DM and T2DM; approved by FDA for Phase III	[16] [17]
Oshadi drug administration	Oshadi Icp	Enteric capsules containing particle matrix of polysaccharides, insulin, silica and oil	Completed Phase II in T1DM	[18]
Oramed	ORMD-0801	Enteric capsules, enzyme inhibitor, and absorption enhancers	Completed Phase IIa in T1DM; completed Phase IIb in T2DM; approved by US FDA for Phase Iib	[19, 20]
Diabetology	Capsulin	Enteric capsules, absorption enhancers and a solubiliser	Completed Phase IIa in T1DM and Phase II in T2DM	[21]
Biocon	IN-105	Chemical modification of insulin with a small PEG and penetration enhancers	Phase III in T2DM failed to clear the primary end point; planned Phase I and II studies in collaboration with Bristol–Myers Squibb	[22, 23]
NovoNordisk – Merrion	NN1953 NN1954 NN1956	PE – sodium caprate activates micelle formation	Completed Phase I; planned Phase Iia	[24]
NovoNordisk – Emisphere	Eligen	PE – salcaprozate sodium (SNAC)	Phase II in T2DM	[25]

Oral insulin technologies generally involve an enteric coated capsule that ensures intestinal targeted release of therapeutic formulation. According to the active pharmaceutical ingredient (API), it can be broadly divided into nanotechnology and permeation enhancer based delivery.

Oramed's technology involved the use of enteric coating protected capsules for delivering insulin together with permeation enhancers. The product named ORMD-0801 was adversely affected by meal when the trial was conducted on fed state volunteers. The duration of effect lasted longer between 5-6 hours.^[19, 20] Biocon developed the technology of chemically modified insulin to improve its hydrophobicity, transcellular permeation and resistance to enzyme degradation. In this technology, Lysine 29 on the insulin β chain was conjugated to PEG and permeation enhancer and the final product was named IN-105, an advancement of HIM2 (hexyl-insulin mono-conjugate 2). The new molecule's activity lasted for 1.5-2 hours onset of oral administration via hard gelatin capsules during clinical trials.^[22, 23] However, IN-105 failed to lower the plasma HbA1C level by 0.7% in T2DM patients in clinical phase III study. Capsulin developed by Diabetology, Ltd., consisting of an enteric coated capsule encapsulating insulin, PE, and other excipients. Phase II trial reveals that glycemic control was demonstrated with an oral insulin dose of 150U and 300U in 16 T2D patients over 6 hours.^[21] Despite the promising phase II results, further development of the product was not disclosed to the public.

Oshadi Drug Administration Ltd., has developed oral carrier Oshadi Icp for encapsulating insulin, proinsulin and C-peptide (Patent US8936786B2). The formulation involves an enteric coated capsule encapsulating a particle matrix consists of ~100nm silica nanoparticles embedded in polysaccharides, suspending peptide in an oil layer.^[26] Safety and efficacy studies for oral insulin were demonstrated in phase I and II trial, however, the mechanism or the rationale behind such a system still remain unknown to the public.

Diasome Pharmaceuticals's hepatic-directed vesicles-insulin (HDV-I) uses insulin loaded nanoliposomes (<150nm) decorated with a liver targeting molecule to facilitate hepatocyte uptake following intestinal absorption (Patent US8962015B2). These surface modified nanoliposomes demonstrated significant glycemic control during oral glucose tolerance test with a low dose of 5 IU insulin, a 100 fold higher potency

in hepatic glucose uptake than injected porcine or human insulin of the same dose.^[16] It was the first liposome based oral insulin technology entering clinical phase III approved by FDA, however metabolic control was poor after long duration of exposure to the drug which could be due to resistance to the drug.^[17]

Permeation enhancers are primarily medium chain fatty acids salts (i.e. Sodium caprate) or MW N-acylated alpha-amino acids (i.e. salcaprozate sodium), that are capable of reversibly opening tight junctions between adjacent enterocytes in the small intestine, leading to enhanced absorption of therapeutics. Most permeation enhancer based technologies have made it to the furthest in clinical phase II or III with FDA approval because of the promising safety and efficacy results during short term trials. However, unknown side effects associated with chronically increasing intestinal permeability still needs to be assessed.

In a collaboration between NovoNordisk and Merion, gastrointestinal permeation enhancement technology (GIPET®) was used to deliver a variety of insulin and GLP-1 analogs. Sodium caprate, a medium chain fatty acid salt (C₁₀), the main composition of GIPET was found to act as mild surfactant at high concentration >150mM, increasing mucosal membrane fluidity for improving both para and transcellular transport *in vivo*. At low concentration, paracellular transport was facilitated by cytoskeletal actin filament contraction, mediating the opening of tight junction by reducing the claudin-5 expression for paracellular transport.^[24] GIPET was able to form mixed micelles above their critical micellar concentration (CMC) when interacting with bile salt at upper jejunum, causing increased permeation by triggering surface attachment and subsequent endocytic uptake of peptides. Emisphere partnered with NovoNordisk in the development of Eligen, low MW N-acylated alpha-amino acids known as Sodium N-[8-(2-hydroxybenzoyl) aminocaprylate (SNAC or salcaprozate sodium), which weakly associates with poorly permeable drugs to allow transcellular absorption by an unknown mechanism. Nevertheless, Eligen produced promising results for delivering a long acting GLP-1 analog (semaglutide) in a phase II trial 2015. According to the company report, plasma Hb1AC level reduced by 1.9% among 600 T2DM patients over 26 weeks.^[25]

2.4 Why most oral insulin formulations do not reach clinical trial

Oral route of insulin delivery offers many promising advantages over subcutaneous injection. Firstly, an oral route of administration improves patient compliance by avoiding the use of needles and injections which could be a life-long treatment for chronic diabetes. Secondly, orally administered drugs mimic the physiological route of insulin secretion whereby the drugs would be absorbed by the small intestine, enters the portal vein and reaches the liver, where glucose homeostasis takes place.^[27] With the regulation by the liver, hyperinsulinemia-related complications such as neuropathy and retinopathy could be reduced.

Despite the promising advantages of oral insulin delivery, challenges from the GIT hinder the development of oral insulin technologies. Firstly, low pH condition in the stomach inactivates the function of protein based drugs by causing a conformational change in its structure. This low pH activates enzymes such as peptidase to break down any protein that arrives in the stomach. Even though some of the proteins managed to reach small intestine, mucus layer prevents the diffusion of these macromolecular drugs; as a result, majority was trapped and lost due to the continuous shedding of mucus layer. Additionally, the presence of brush border enzymes would break down any remaining proteins into amino acids.^[28] Intestinal epithelial lining cells enterocytes further prevents the absorption of macromolecular protein drugs because of the size and hydrophilic nature of insulin.

The demands from diabetic patients drives the development of an oral insulin formulation, however, there is no oral insulin product to date since its discovery in the early 1920s. Many pharmaceutical companies have been investing in the research and development of oral insulin products but few entered the clinical trials. Looking from a statistical perspective, only one-quarter of the *in vivo* studies investigated *in vitro* cell models beforehand.^[12] Most of the companies failed in clinical phase II indicating the lack of metabolic control in the diabetic patient. From the patient perspective, no clear clinical advantage over subcutaneous injection has been demonstrated in the clinical trial because of low bioavailability and lack of sufficient evidence to support the long term safety and efficacy of these formulations.

There is a need for pharmaceutical companies to have more detailed study on meal-related absorption and get reproducible *in vivo* results for better disease management.

As most of the pharmaceutical products entering clinical trials are permeation enhancer based technologies, it is necessary to evaluate the long term effect on safety, efficacy, and tolerability of these formulations because of the potential side effects of prolonged opening of tight junctions in the small intestine. It is important for them to disclose the causes of their failure during early clinical trials. Furthermore, companies need to overcome the barriers during translation and scale-up, sufficient amount of insulin need to be manufactured in a cost-effective way.

2.5 Obstacles for oral route

The main obstacle of oral drug delivery is the barriers of the gastrointestinal (GI) tract. GI tract is not only responsible for digestion, absorption of the nutrients and water homeostasis. Furthermore it is the first line of defense to against harmful microorganisms in the gut. It regulates the paracellular transport of macromolecules such as intestinal antigens from bacteria by adjusting intestinal permeability via the tight junctions. Gut associated lymphoid tissue (GALT) consist of peyer's patches and lamina propria, which are the primary location of immune cells to safeguard the body, prevent the entry of any invasion of foreign antigens or particles.^[29] Mucus adds an additional barrier to slow down the diffusion of the antigens and trap harmful microorganisms.

Figure 3. Structure of intestinal epithelium. Adapted from Ref^[30]

2.5.1 Cell types (M cell, enterocyte, goblet cell)

The intestinal epithelial lining consists of a variety of cell types each carrying its own unique function. The main cell types of the intestinal lining include enterocytes, goblet cells, Paneth cells, M cells, and crypt cells. Each cell type has their specific role in regulating the intestinal permeability and safeguarding the body from foreign particle invasion. Enterocytes are the main absorptive cells and it regulates the permeability of molecules via the tight junctions. Enterocytes are the most abundant cells in the intestinal epithelial lining with average life span 3-6 days. It is characterized by dense, well-ordered brush border microvilli at the apical surface in the intestinal lumen. The enterocytes are mainly responsible for absorbing nutrients; amino acids and monosaccharides are absorbed via cell surface receptors while fatty acids are absorbed via simple diffusion.^[31-33] The main secretory cells are goblet cells and Paneth cells. Goblet cells constantly secrete mucus that act as both lubricant for passage of chyme as well as a barrier for the attachment of microorganisms. Paneth cells are the mucosal defense cells which secrete antimicrobial peptides into the lumen of the small intestine when exposed to bacteria.^[34] M cells are characterized by flattened apical surface without mucus coverage, and with high numbers of mitochondria and low numbers cytoplasmic lysosomes. It is located in the gut associated lymphoid tissue or the Peyer's patches which has been considered the primary site of particle uptake by transcytosis.^[35] The main function of M cells is to sample the surrounding environment for antigens from microorganisms and facilitate antigen presentation to the T lymphocytes inside the Peyer's patches in the underlying mucosal immune system.^[36, 37] Crypt cells are the undifferentiated basal stem cells that have the potential to differentiate into all cell types.^[38-40]

2.5.2 Unstirred aqueous boundary layer and GI mucosal barrier

The rate of nutrient diffusion to reach the surface of brush border microvilli for absorption is controlled by a layer of 30–100 μm thick unstirred water layer which consist of water, mucus and glycocalyx.^[40] Specialized secretory goblet cells secrete mucus which serves as a barrier between the commensal microorganisms in the gut and the host. The main composition of mucus is mucin glycoproteins which are large homo-oligomers giving rise to the viscous properties for trapping any foreign particles. Other antimicrobial molecules such as immunoglobulins are also found in

the mucus to safeguard the host from any opportunistic microorganisms.^[41] The extracellular mucus barrier consists of a sterile inner firmly adherent layer and a non-sterile outer loosely adherent layer. The thickness increases from 200 μm in stomach, 200-500 μm in the small intestine to 800 μm in the large intestine.^[42] The apical surface of intestinal epithelium cells are densely packed with transmembrane mucin glycoproteins. These filamentous structures offer steric hindrance that prevents bacteria that reach the inner adherent layer from getting in contact with the cell surface.^[41]

2.5.3 Intestinal cytochrome P450 and p-glycoprotein

The enterocytes of the small intestine express intracellular drug metabolizing enzyme cytochrome P-450 3A4 (CYP3A4) and the cell surface P-glycoprotein (P-gp) to prevent the passage of big molecular drugs. CYP3A4 is bound within the Endoplasmic Reticulum of enterocytes that functions to metabolize the drug substrates in the small bowel. P-gp is a 170-kDa membrane transporter protein found in the liver, small intestine, and predominantly expressed in cancer cells, pumping chemotherapeutics out of the cells rendering the cells multidrug resistant.^[43] It works synergistically with CYP3A4 by pumping the metabolites of a drug catalyzed by CYP3A4 back into the intestinal lumen, limiting the bioavailability of orally administered drugs.^[44] A cysteine protease inhibitor Morpholine-urea-phenylalanine-homophenylalanine-vinylsulfone-phenyl (K02) has been studied for oral bioavailability with the co-administration of ketoconazole, an inhibitor of CYP3A family. Surprisingly, bioavailability of K02 increased from 2.9% to 31%, the 11 fold enhancement suggested that inhibition of metabolism in the small intestine could improve the bioavailability of orally administered drugs.^[45] Gan *et al.* studied the *in vitro* transport of cyclosporin A, a substrate for both CYP3A4 and P-gp, across Caco-2 cell monolayer. They observed that metabolites of cyclosporin A were transported back from within the cells to the apical medium containing micromolar concentration of the parent drug. Furthermore, the rate of metabolism of cyclosporine A was greater when the drug is added to the apical side than the basal side of the transwell, suggesting that P-gp present in the apical membrane assisted in determination of the residence time of drug inside the cells.^[46]

2.5.4 Chemical and Enzymatic barrier

The chemical, enzymatic, and diffusional barriers of the GI tract functions to maximize the absorption of nutrients, electrolytes, fluid, and at the same time defend against the invasion of harmful microorganisms. Bioavailability of orally administered protein drugs are constantly challenged by the mucosal enzymatic degradation, diffusion across the densely glycosylated mucin layers, and permeation across the intestinal epithelial cell layer. Stomach lining secrete highly acidic Hydrochloric Acid (HCl) that lowers the pH of the environment to pH 1.2-3. Proteases such as pepsin are activated at such pH and will break down any large protein into fragments polypeptides. In the small intestine, a cocktail of enzymes and emulsifying agents carried by bile and pancreatic juice further assist in the breaking down of any remaining macromolecules of fats, protein, and carbohydrates into their basic units.^[47, 48] Other than the luminal enzymes (trypsin, chymotrypsin, elastase and carboxypeptidase), brush border cells secrete aminopeptidase that further aids in the digestion of any protein that is approaching the enterocyte cell surface. About 94-98% of orally ingested protein drugs are lost via oxidation, deamination or hydrolysis.^[49] With the additional first pass metabolism by hepatic enzymes including Type I (oxidation, reduction, hydrolysis), Type II (Glucuronidation, Sulfation, Methylation, Acetylation) and P450 which Metabolize 75% drugs, the amount of drug that eventually reach systemic circulation while remain bioactive was extremely low.^[50]

2.5.4.1 Mucus as a diffusional barrier

Small intestine is the major digestive and absorptive organ in the GI tract, the 6 meters long lumen has an enormous surface area of 200-500m² due to the presence of brush-like projections known as microvilli.^[50] The diffusion of nutrient to the surface of brush-border cells is controlled by an extracellular mucus layer, which consists of water, mucin, and glycocalyx. The extracellular mucus layer is divided into a non-sterile outer loosely adherent layer and a sterile inner firmly adherent layer. The thickness of the unstirred water layer is 30–100 µm thick while the sterile inner firmly adherent mucus layer increases from 200 µm in stomach, 200-500 µm in the small intestine to 800 µm in the large intestine.^[40, 42] Mucus is constantly secreted by specialized goblet cells, and renewed every 4-5 hours to protect against attachment of gut pathogens. Mucin glycoproteins are large homo-oligomers that accounts for the

viscosity of the mucus for trapping foreign particles. Other antimicrobial molecules such as immunoglobulins are also found in the mucus to safeguard the host from any opportunistic microorganisms.^[41] The apical surface of intestinal epithelium cells are densely packed with transmembrane mucin glycoproteins. These filamentous structures offer steric hindrance that prevents bacteria that reach the inner adherent layer from getting in contact with the cell surface.^[41] This mucus layer permits the diffusion of nutrients, water and small molecules while limit the movement of bacteria. Electrostatic repulsion due to negatively charged mucin slows down the travel of drug to reach the underlying epithelial cell lining.

2.5.4.2 Permeability barrier

a. Transcellular and paracellular transport

The permeability of the drugs in the small intestine is mainly regulated by the intestinal epithelial cells or enterocytes via transcellular and paracellular pathways. The epithelial cells of the small intestine are joined by various junctional proteins. Tight junctions consist of structural proteins (occluding and claudins) and scaffold protein (ZO-1, ZO-2, fodrin, cingulin, symplekin, 7H6, p130) that regulates the passage of molecules and fluids across the paracellular space between adjacent cells.^[50] Hydrophilic drugs are transported based on their size and charge, small hydrophilic drugs MW<200Da are transported via paracellular passive diffusion.^[51] Due to the hydrophobicity nature of phospholipid cell membrane, small lipophilic drugs molecular weight (MW)<700 Da are transported across the enterocytes via the transcellular passive diffusion.^[47] Most clinical drugs are absorbed by the transcellular passive diffusion, with increasing absorption as the size reduces. Transport of most protein drugs is limited because of their hydrophilic nature with a high MW>3000 Da.^[47] Without a permeation enhancer that opens up the tight junction, the transport of proteins is virtually impossible.^[52]

b. Enterocyte surface transporters

Other than simple diffusion, hydrophilic drugs can be transported by enterocyte surface transporters which can be classified into uptake and efflux transporters based on adsorption and elimination of drugs. Small positively charged organic cationic drugs MW<500Da are transported with the help of organic cation transporters (OCTs)

on the enterocyte surface.^[53] Transport of amphiphatic compounds MW>350Da are facilitated by organic anionic transporting peptides (OATPs).^[53] Multi-drug resistance protein 1 (MDR1), or P-glycoprotein (P-gp; ABCB1) is localized in both enterocytes of the small intestine and hepatocytes of the liver and was found to be the efflux pump of a variety of drugs including amphipathic, hydrophobic, cationic drugs with planar ring structure and MW between 200-2000Da.^[53]

2.6 Mechanism of transport through the mucosal

Oral delivery of protein drugs is constantly challenged by the barriers in the GI tract. One of the ways to improve the stability and bioavailability of the orally administered protein drugs is to encapsulate them in a nano-sized carrier, which offers greater surface area to volume ratio, controlled release, separation from the luminal environment, and enhanced absorption. Owing to its large size, transport of nanoparticles via simple diffusion is limited. Transcellular transport of nanoparticles follows an energy dependent endocytosis process, which can be broadly classified as phagocytosis and pinocytosis.

Phagocytosis occurs primarily in phagocytes for ingesting antibody or complement opsonized particles. Fc receptors expressed on these immune cells are responsible for binding and taking up the antibody opsonized particles. Particles of size greater than 20µm have been shown to be taken up by murine bone marrow derived macrophages.^[54] In contrast, pinocytosis occurs in most of the cell types as a process to sample the surrounding environment for fluid and nutrients. It can be further classified into clathrin-dependent, caveolin-dependent, clathrin- and caveolin-independent, and macropinocytosis pathways depending on the protein associated with the endocytosis.^[54, 55]

Clathrin-dependent endocytosis is found in all mammalian cell types as the dominant pathway for cell entry. Most nutrients such as cholesterol and iron are taken up by the cells upon binding of surface low density lipoprotein (LDL) and transferrin receptors, respectively. Clathrin-1 is the major protein involved in the internalization and formation of coated-pits, enclosing the receptor and its bound ligand. The clathrin coats then dissociate intracellularly and the internalized vesicle fuse with lysosome for degradation or sorted to endosomes for recycling of receptors.^[54, 55] **Caveolin-**

dependent endocytosis is mediated by Caveolin-1 and -2 in endothelial cells, fibroblast, adipocytes and Caveolin-3 in muscle cell to participate in membrane invagination initiated by nanoparticles or pathogens binding to the surface receptor on the membrane, while absent in leucocytes and neurons. The membrane bound calveosome with a neutral pH is trafficked along the intracellular microtubule network to the Endoplasmic Reticulum and nucleus, bypassing the degradation from lysosome.^[55] This behavior is seldom seen in other endocytic pathway whose fate is usually lysosomal degradation, it is therefore crucial for the design of nanomedicine to overcome the epithelial barrier because transcytosis is possible with the incorporation of ligand that targets caveolin-mediated endocytosis. Previous studies have shown that albumin could be transcytosed with the help of caveolin by the epithelial cells upon binding to its receptor gp60.^[56] Paclitaxel loaded nanoparticles decorated with albumin demonstrated enhanced antitumor activity following transcytosis.^[57] **Clathrin- and caveolin-independent endocytosis** is facilitated by cholesterol rich regions on the plasma membrane known as lipid raft. It can be further classified into Arf6-dependent, Cdc42-dependent, and Rho-dependent based on the type of effector proteins involved in the internalization process. Lipid raft can be found in membrane invagination area rich in caveolin and planar area rich in flotillin, which mediate the endocytic process in the absence of clathrin-1. Folate decorated nanoparticles was found to be binding to glycosylphosphatidylinositol (GPI)-anchored folate receptor, initiating the lipid raft facilitated, clathrin- and caveolin- independent endocytosis.^[55, 58] **Macropinocytosis** is a process of non-specific taking in surrounding fluids and large particles driven by the transient activation of Receptor Tyrosine Kinase (RTK). Growth factors bind to the RTK, initiating the rearrangement of cytoskeleton for the formation of membrane ruffles that protrudes and encloses the fluids and nutrients in the surrounding environment. The micropinosome of size 0.5-10 μ m then pinches off from the plasma membrane and translocated to fuse with lysosome in macrophages.^[54]

Figure 4. Schematic diagrams representing different pathways of nanoparticle uptake in the intestinal epithelium. Insets are the mechanisms of phagocytosis, macropinocytosis, clathrin-mediated endocytosis and caveolae-mediated endocytosis. Adapted from Ref^[47]

Physicochemical properties of nanoparticles including size, charge, shape, hydrophobicity, and surface roughness played an important role in modulating the surface attractive forces for cellular uptake. The uptake of nanoparticles depends on the bio-physicochemical interactions at the nano-bio interface. The initiation of membrane wrapping requires a localized reduction in Gibbs free energy to overcome the resistive forces from elastic recoil of the plasma membrane, which can be achieved by non-specific or ligand specific interaction at the cell surface.^[59, 60]

Size of the nanoparticle could have a profound influence on the cellular uptake. The cell surface has a limited number of receptors for nanoparticle to associate with. Larger particles have bigger surface area to cover more receptor-ligand binding sites thus are thermodynamically more favorable condition for initiating endocytosis than smaller particles, however, they may interfere and limit the interaction of additional nanoparticles.^[60] Particle size of 30-50 nm ensures a fully occupied receptor on the

localized cell membrane and ligand on the nanoparticle, thus is the optimum condition for endocytosis.^[60] Unfortunately, the effect of particle size on cellular uptake has been primarily focused on immortalized cell lines which may behave differently as primary cells in terms of phenotype and level of expression of surface receptors. Effect of nanoparticle size on the mechanisms of cellular uptake has been studied by various groups. However, the exact pathway by which the nanoparticle is undertaken remains controversial due to the poor particle characterization and selection of non-specific endocytic inhibitors. For example, one study suggested that clathrin-mediated endocytosis dominates the uptake when nanoparticle size is less than 200 nm, while the uptake mechanism changed to caveolin-mediated endocytosis when size increase above 200 nm.^[61] This was challenge by another study which suggested that particle size of 50-80 nm favors the calveolin-dependent pathway while 120 nm favors clathrin-dependent pathway.^[59] Desai *et al.* compared the intestinal uptake of poly(lactic-co-glycolic acid) (PLGA) particle between size 100nm, 500nm, 1um, and 10um *in situ* in a rat model. Their study suggested that 100 nm sized PLGA particle exhibited 15-250 fold higher uptake comparted to the larger sized particles.^[62] Other studies suggested that transcellular transport of nanoparticles via non-specific uptake is size dependent, with the optimum size range of 50-500 nm by the enterocytes while M cells tend to transcytoses larger ones.^[47]

Charge of the particle affects its binding or attachment on the transmembrane glycoproteins and phospholipid head groups. Cell membrane is slightly negatively charged because of the phospholipid head groups and the presence of glycans on the transmembrane proteins. Cationic particles are therefore preferred over anionic particles for interacting with the cell surface via electrostatic attraction.^[63] One study found that small cationic particle of size 2 nm could induce a transient Ca^{2+} influx.^[64] Particle of size 4-20 nm was shown to cause a localized membrane reconstruction.^[65] Interestingly, negatively charged particles were found to induce gelation of localized lipid membrane while positively charged particles would make the membrane more fluidic.^[60] Furthermore, charged particles are more prone for serum protein attachment, forming protein corona. Some studies suggested that the opsonized particle encourage cellular uptake.^[66]

Particle of same size but different in geometry has been found have different levels of cellular uptake. Shape of the particle for particle size larger than 100nm demonstrated

decreasing uptake with the following shapes: rod, sphere, cylinder, cubes.^[67] However, sphere was better than rod when particle size is less than 100nm.^[68] Chithrani *et al.* has examined cellular uptake capability of HeLa cells for 14, 30, 50, 74, and 100 nm gold nanospheres 40x14 nm and 74x14 nm gold nanorods. Their results have shown that cellular uptake was highest for 50nm AuNP and significantly higher for 14nm and 74nm spheres than 74x14 nm rods.^[69] Increasing the aspect ratio seemed to decrease cellular uptake. This could be due to the larger surface area of the longitudinal side of rods which occupied more receptor binding sites, limiting the particle binding to the cell surface.

Particle material type, hydrophobicity, and surface roughness could also affect its interaction with cell surface. Studies has compared cellular uptake between 50nm spherical particles of different materials including gold nanoparticles, silica nanoparticles, single-walled carbon nanotubes, and quantum dots. A 1000 fold enhancement in endocytosis rate was observed for single-walled carbon nanotubes compared to gold nanoparticle of the same size and shape.^[70-72] Nanoparticles with hydrophobic surfaces were found to be more efficient of being endocytosed because of the hydrophobic nature of the cell membrane. Increased surface roughness also enhances the adhesion by minimizing the repulsive forces between the particle and cell surface.^[59]

2.7 Nanomedicine for oral insulin delivery

Application of nanomedicine in the field of oral insulin has been extensive studied over the past few decades. Encapsulating insulin in nanocarriers in the size range 10-1000 nm and microcarriers 1-1000 μm could protect insulin from harsh environments in the GI tract, improving its absorption. Many different types of materials has been investigated for the synthesis of nano- or micro-sized carriers, including, muco-adhesive or muco-penetrating, cell-penetrating, polymeric nanoparticles, and liposomes. Each of these will be reviewed in the following sections.

2.7.1 Nanoparticles and Microparticles

Both nano- and micro-particles are able to protect insulin from degradation in the GI tract; however, nanoparticles have been shown to be more efficiently absorbed via transcellular pathway due to its smaller size. Tight junctions having an average gap of

70-90 Angstroms limits the diffusion of solutes no larger than 1 nm.^[47, 73] While the narrow junctional space between adjacent enterocytes limits the diffusion of nanoparticles and microparticles via paracellular pathway, which can be achieved through reversible opening of the tight junction with permeation enhancers. A great advantage of nanocarrier used in oral delivery of insulin is that these nanosized carriers after absorption tend to accumulate in the liver irrespective of their composition,^[12] allowing improved glycemic control in the liver mimicking the physiological insulin secretion.

Microparticle has difficulty transverse through the densely glycosylated mucus layer which has an average pore size of 340 ± 70 nm which limits its diffusion.^[74] Most of the microparticles ended up being trapped at the loosely adherent outer mucus layer, which could be cleared within 4-5 hr due to mucus shedding. Enzyme inhibitors and permeation enhancers and are often co-administered to protect insulin from degradation by brush border enzymes while making its way to reach enterocyte surface and open the tight junction to allow the permeation to blood stream. Microparticles can be broadly divided to microspheres (drug-polymer matrix) and microcapsules (drug-polymer core shell). Various materials has been used in the formation of microparticles including poly(methacrylic acid), chitosan, alginate, and the Eudragit family for oral delivery of insulin.^[75] N.A. Peppas developed a pH sensitive microsphere consists of copolymer networks of poly(methacrylic acid) grafted with poly(ethylene glycol), which gives targeted and pH triggered release of insulin at small intestine in rats. The microspheres PEG moiety confers the particle excellent mucoadhesive properties, resulting in a pH dependent swollen and release of insulin from polymer matrix.^[76] In another study, chitosan microspheres containing insulin, protease inhibitor (bacitracin) and absorption enhancer (sodium taurocholate) protected insulin from enzymatic degradation and exhibited extended release up to 12 h.^[77] Sun *et al.* prepared pH-sensitive hydroxypropyl methyl cellulose phthalate (HP55) microcapsules, encapsulating PLGA nanoparticles of insulin-sodium deoxycholate complex using organic spray-drying method. The insulin-sodium deoxycholate complex enables a 94.2% encapsulation and the microcapsule results in a pH dependent release of insulin and an improved bioavailability in diabetic rats.^[78]

2.7.2 Muco-adhesive and muco-penetrating

Before nanoparticles could reach the surface of intestinal epithelial cells and play its role in transcellular transport, it must be able to cross the dense layer of mucus which slows down diffusion due to the 1000-10000 fold increase in viscosity compared to in water. ^[79] Most orally administered nanoparticles therefore suffer from short transit time through the GI tract because of the rapid clearance from the GI lumen. Nanoparticles prepared using Mucoadhesive materials offers great advantage in enhancing absorption via muco-adhesion which prolongs residence time, leading to sustained release of the encapsulated therapeutic drug for generating a high drug concentration at localized site. Mucoadhesive materials can be characterized as high molecular weight hydrophilic polymers with hydroxyl, carboxyl, amine, and amide side groups that is able to interact with the mucus via H-bonding, electrostatic interaction or Van Der Waals forces. Examples of mucoadhesive polymers are Carbopol, ^[80-82] alginate, ^[83] chitosan, ^[84, 85] poly(acrylic acid) (PAA), ^[86] thiomers, and pectin ^[87].

Chitosan being a cationic polymer that not only interacts strongly with the negatively charged mucin molecules, but also played a role in modulating the transient opening of tight junctions, thus are among the popular choices of muco-adhesive polymers. Takeuchi *et al.* have compared the mucoadhesiveness between positively charged non-coated, carbopol-coated, and chitosan-coated liposomes in an ex vivo experiment using isolated intestines from Wistar rats. Results have shown that both chitosan-coated positively charged liposomes and carbopol-coated negatively charged liposomes exhibited higher mucoadhesiveness, than positively charged liposomes. This suggests that other than electrostatic interaction, physical entanglement between polymer and mucins played an important role in enhancing mucoadhesion of liposomes.^[80-82] Ionotropic gelation of alginate and chitosan using divalent calcium ions produced nanoparticles of 750nm that was able to encapsulate insulin at loading capacity of 9.9% and lower the serum glucose level by 59% in streptozotocin-induced diabetic rats. The mucoadhesive nanoparticles ensured a bioavailability of 6.8% and were able to be retained on the intestinal tissue for a sustained hypoglycemic effect of 18 hours.^[83] Crosslinking of alginate with di- or poly-valent ions creates a porous gel matrix that could encapsulate and stabilize hydrophilic protein drugs. Addition of

chitosan or methycellulose reinforces the encapsulation and release by electrostatic interaction with the amino acid residue of the protein.^[84] Similar study on ionic gelation of chitosan and Tripolyphosphate (TPP) was shown to generate positively charged 300-400nm particles capable of encapsulating insulin at 55% loading efficiency which greatly improved nasal absorption.^[85] Other permeation enhancers like Spermine has similar polyamine groups as chitosan, has been formulated with PAA based on ionic interactions. Temporary reduction of TEER values was observed when these particles were applied on intestinal epithelial cell model (Caco-2) *in vitro*. FITC-dextran showed a 5.56 fold increase in transport when encapsulated in SPM-PAA nanoparticles compared to its free solution.^[86] Thiomers are thiolated polymers are designed to improve the muco-adhesiveness by interacting with cysteine rich regions of mucins via strong disulfide bond. Metformin loaded thiolated pectin beads were prepared by ionic gelation with calcium chloride as cross linking agent. The mucoadhesiveness was prolonged by 2.5h compared to the unthiolated pectin beads in an ex vivo study.^[87]

Majority of the muco-adhesive nanoparticles attach only at the loosely adherent mucus layer and are rapidly cleared by the constant renewal of mucus before reaching the underlying firmly adherent mucus layer. Since the intestinal mucin turn over time is about 47-270min, most mucoadhesive nanoparticles would be removed within 4-5 hours before being able to traverse across the loose adherent layer and reach the cell surface.^[88] As a result, the promising *in vivo* results of mucoadhesive nanoparticles are more likely to be due to sustained release and improved protection of protein drug at the loosely adherent layer mucus layer. The design of mucus penetrating nanoparticles should be targeted at reducing the interaction between mucin proteins while being relatively small to diffuse in between the porous space in the mucin gel matrix.

Mucus is both negatively charged due to the glycosylated areas and hydrophobic due to lipid-coated regions of mucin. The hydrophobicity nature of the mucus gel traps hydrophobic molecules, reducing the diffusion of even small molecular weight lipophilic molecules such as testosterone.^[89] Nanoparticle surface modified with positive and negative charges, generating net neutral charge and small in size, was found to be the ideal muco-penetrating characteristics.^[79] Capsid viruses such as Norwalk virus (38 nm) and human papilloma virus (HPV; 55 nm) consist of equal

number of positive and negative surface charge, were able to diffuse at the same speed in human ovulatory cervical mucus (OCM) and water.^[90] Wang *et al.* decorated the 200nm polystyrene nanoparticles with high density of low molecular weight (2kDa) polyethylene glycol (PEG), converting the mucoadhesive particle to mucopenetrating particle with neutral surface charge that minimize the interaction with mucin via hydrophobic interaction and improves diffusion in human cervicovaginal mucus (CVM) (pore size $340\pm 70\text{nm}$, range 50-1800nm).^[91] They reported an 8000 fold higher in diffusion of 200nm and 500nm PEG-polystyrene in CVM than smaller sized Herpes simplex virus (HSV; 180 nm) of enveloped hydrophobic surfaces.^[92-94]

2.7.3 Cell-penetrating

Transport of drug loaded particles can be improved by incorporating a targeting ligand that recognizes the specific receptor on the cell surface to initiate receptor mediated endocytosis and subsequent transcytosis of the drug loaded particle. Fc receptor is commonly expressed in the vascular endothelium, Blood Brain Barrier (BBB), kidney, liver, lungs, and hematopoietic system, discovered in neonatal intestine for absorption of IgG from mother to offspring.^[95, 96] Fc part of IgG protein binds to FcR at $\text{pH} < 6.5$, initiating a sequence of events from endocytosis of Fc-FcR to intracellular trafficking. Finally, IgG would be exocytosed to the basal part and released from the receptor at $\text{pH} 7.4$. The transcytosis of intact IgG across intestinal epithelial cells was used to design nanoparticles with specific targeting of the intestinal cell receptor to improve cellular uptake and transport. Pridgen *et al.* showed that PLA polymeric nanoparticles decorated with Fc ligands were efficiently transported across intestinal epithelial cells *in vitro* and *in vivo*. The Fc nanoparticles improved mean absorption efficiency from 1.2% to 13.7% in mice.^[97] A similar targeting ligand folic acid also initiate cellular uptake via receptor mediated endocytosis. Folic acid is an essential nutrient required by all dividing cells for nucleotide synthesis. Uptake of folic acid results in the deposition in cytosol rather than lysosome, making folic acid a popular choice for improving oral absorption of drugs.^[98] Ling *et al.* decorated liposome surface with folic acid, resulting in a 1.4–2 fold increased bioavailability of poorly absorbable cefotaxime.^[99]

2.7.4 Polymeric nanoparticle

Nanoparticles made from natural polysaccharides such as chitosan (CS), alginate, pectin and dextran are hydrophilic in nature, biodegradable and biocompatible for oral drug delivery. Among these, CS is most extensively studied due to its capability to enhance permeation by transiently loosening tight conjunctions between adjacent intestinal epithelial cells and to electrostatically adhere to the negatively charged mucus layer.^[100-103] Several groups have demonstrated promising results for oral delivery of insulin and peptides using CS-based nanostructures such as CS-coated gold nanoparticles^[104], CS-coated liposomes^[105-108], CS-coated nanocapsule^[109], CS-coated lipid nanoparticles^[110]. However, the physicochemical properties of chitosan depend on the pH of the environment. CS is a weak base having D-glucosamine residue pK_a of 6.2-7, would lose its charge and precipitate at neutral to basic pH in the intestine^[101, 103]. Modification of CS has been done by many groups to improve its intrinsic solubility, mucoadhesiveness as well as paracellular permeability for oral delivery of insulin. One of the CS derivatives Trimethylchitosan–cysteine conjugate (TMC–Cys) has shown to exhibit increased mucoadhesion of 2.1– 4.7-fold over TMC/insulin NPs due to the disulfide bond between cysteine and mucin.^[100] Poly(lactic acid-co-glycolic acid) (PLGA) is a hydrophobic copolymer consisting of repeating units of PLA and PGA. It is widely studied for its application as drug carrier due to its excellent biodegradability and biocompatibility. However, its hydrophobic nature renders difficulty in encapsulating hydrophilic proteins such as insulin. Various approaches has been attempted to increase the loading efficiency of insulin. Conjugating insulin with soybean phosphatidylcholine improved its lipophilicity and therefore loading efficiency up to 90%.^[111] In addition, it is frequently used together with hydrophilic PEG as diblock PLGA-PEG which forms a micellar structure as PEG self-assembles to orient itself towards the aqueous phase, encapsulating hydrophilic drugs in its core.^[112]

2.7.5 Liposome

Oral insulin delivery using liposome as a carrier has been considered back in 1975. Patel and Ryman reported that insulin entrapped in liposomes showed significant drop in blood glucose level when administered orally in diabetic rats.^[113] The ability of liposome as a delivery vesicle for insulin was later on challenged by Kawada, *et al* in

1981 as they reported that no reduction of blood glucose was observed after oral administration of insulin in diabetic rats.^[114] In 1997, Iwanaga, K., *et al.* studied the stability as well as hypoglycemic effect of positively and negatively charged DPPC DSPE liposomes conjugated with PEG and mucin as surface coating. Results showed that coated liposomes had increased stability in acidic pH with the presence of bile salts and GI enzymes as compared to uncoated liposome. Positively charged and surface coated liposomes demonstrated significant reduction of plasma glucose as compared to neutral liposomes when administered *in vivo* in a rat model.^[115]

The most common cause of low bioavailability was a result from low drug permeability and solubility. By encapsulating the drug using liposome carrier, improved bioavailability could be expected as it improves the solubility of the drug by preventing the drug degradation and precipitation in acidic pH. Liposomal drug carriers consist of a bilayer of amphiphilic phospholipids that self-assemble naturally into spherical vesicles which are capable of encapsulating both hydrophobic and hydrophilic drugs in its lipid bilayer and aqueous core. It offers a platform to allow surface modification with various targeting ligands such as antibody, small molecules, carbohydrate for specific recognition and targeted delivery at the site of action. Polyethylene Glycol (PEG) can be directly conjugated to the lipid molecule for assembly of PEGylated liposomes which prevent vesicle opsonisation (attachment of serum protein) and subsequent clearance by Reticuloendothelial System (RES) and help to prolong the circulation time in the body. The first FDA approved PEGylated liposome encapsulating doxorubicin.

Figure 5. Schematic drawing of various types of liposomal drug delivery system. Adapted from Ref^[116]

Liposomes possess excellent physio-chemical properties such as biomimetic, biodegradable, and biocompatible, however, their hydrophobic nature limits the application as an efficient carrier for delivery of oral drugs because of the difficulty in crossing the hydrophilic mucus barrier in the small intestine. The instability of liposomes during the GI transit when administered via the oral route further reduces its effectiveness in delivering intestine or colon targeted drugs.^[117, 118] Low pH condition in the stomach causes influx of H⁺ ions inside the aqueous core, destabilizing the integrity of liposome. Bile salts that function to emulsify fats would act on liposome's lipid bilayer and disrupt the integrity of vesicular membrane.^[119, 120] Liposomes are excellent carriers for loading small hydrophobic drugs as the drug could be incorporated in its similarly hydrophobic lipid bilayer during thin film formation, achieving high loading and encapsulation efficiency easily. However, in the case of insulin, a small hydrophilic protein, it is difficult to achieve high loading of the drug inside the liposome aqueous core because of the low storage volume of the aqueous core and solubility of the protein.

To improve the effectiveness of liposome, various surface modifications and formulations has been explored. Incorporation of cholesterol and saturated lipids greatly improves the rigidity of liposome and protect them from enzymatic degradation under the harsh environment in the GI tract.^[121] Surface coating of liposome with PEG or glycans from mucin stabilizes the liposome under low pH condition and increases the encapsulation efficiency of nystatin.^[115, 122] Lecithin liposomes encapsulating Cyclosporin A, a cyclic peptide consisting of 9 amino acids, have been administered orally for a comparison study with its free drug form Sandimmun Neoral®. Equal absorption was observed in the rat intestinal tissue despite the presence of mucus barrier for big sized liposomes.^[123] In another study, cyclosporine A together with lecithin were dissolved in organic solvent and then sprayed onto lactose to form a free flowing powder. The rehydrated powder formed liposomes exhibited a 9 times higher bioavailability compared to cyclosporine free drug.^[124]

2.7.5.1 Surface modification: To improve stability, solubility, sustained release, increased adherence,

Despite the numerous advantages that liposomes possess, their application in oral administration has been limited due to the instability in GI tract, causing rapid burst release of the encapsulated payload within hours after administration. Surface modification emerged as the recent trend of liposomal formulation, as it has been demonstrated to have superior stability, residence time, and sustained release as compared to unmodified liposomes. Liposomes have been modified with a variety of surfactants such as bile salt and Tween 80, or polymers such as chitosan and Pluronic F127 to alter their physiochemical properties and improve their effectiveness in oral application.

Figure 6. Emerging trends of liposomes surface modification and its advantages in the field of oral drug delivery. Adapted from Ref^[125]

2.7.5.2 Surfactant

Niu *et al.* reported that insulin-loaded liposomes incorporated with bile salts such as sodium glycocholate sodium taurocholate or sodium deoxycholate exhibited improved stability of liposome and bioavailability of insulin when administered orally to diabetic rats.^[126] Non-ionic surfactant Tween 80 was found to increase the vesicular flexibility for forming ultra-deformable liposomes.^[127-129] Huang *et al.* incorporated

tween 80 in the liposomes and demonstrated that the modified liposomes have improved stability in simulated SIF.^[130] Other than improving the stability of liposomes, the role of tween 80 could be extended to enhancing absorption of liposomes by intestinal epithelial cells^[131] and crossing the blood brain barrier (BBB).^[132] In fact, numerous drugs have been shown to be transported across the BBB with only in the presence of Tween 80 coated poly(n-butylcyano-acrylate) nanoparticles, including dipeptide kyotorphin^[133], loperamide^[134], tubocurarine^[135], and doxorubicin^[136]. The possible mechanism was that Tween 80 acts as an anchor for binding of nanoparticles to brain endothelial capillary cells and inducing cellular uptake via lipoprotein receptor-mediated endocytosis.^[137-139] Furthermore, Tween 80 was reported as an inhibitor of p-glycoprotein^[140], which could potentially help to enhance the absorption of peptide drugs by Caco-2 cells.^[141]

2.7.5.3 Chitosan

Surface modification of liposomes with polymers provides a shield for the liposomes, protecting it from the harsh environments in the GI tract. The polymer coating on liposome surface could improve its stability, control drug diffusion giving rise to sustained release, and improve mucoadhesiveness by introducing charged moieties that could interact with mucus, improving bioavailability for oral drug delivery. Chitosan is a natural polymer commonly used in the surface modification of liposomes. Chitosan coated liposomes was found to not only improve the stability but also the muco-adhesiveness of liposome. Sugihara *et al.* demonstrated prolonged residence time and sustained release of chitosan-coated indomethacin encapsulated liposomes in the small intestine in both fasted and fed state rats *in vivo*. Encapsulation of indomethacin prevented the precipitation of the drug at acidic pH and improved the solubility of the drug by releasing the drug at neutral pH where the solubility is higher, increasing the chance of drug reaching intestinal cell surface and improving the bioavailability.^[142, 143] Han *et al.* reported an 8 times mucin adsorption on chitosan coated liposome leading to high retention and enhanced drug permeation compared to uncoated liposomes. The coated liposomes exhibited 24 hour stability in SGF and SIF, improved uptake in Caco-2 cells, and 2.6 fold improved bioavailability of alendronate in rats.^[144] Furthermore, Manconi *et al.* showed that by coating liposomes with chitosan cross-linked with biocompatible β -glycerolphosphate, increased

mucoadhesion was observed than chitosan coated liposomes in ileum and colon.^[145] Chitosan-aprotinin coated calcitonin loaded MLV liposomes prepared by coating of chitosan-aprotinin on negatively charged liposomes via electrostatic interaction, inhibited the action of trypsin and achieved a 15 fold increase in the area above blood calcium concentration time curve in rats.

Figure 7. Chemical structure of chitosan comprising of N-acetyl-D-glucosamine (acetylated units) and glucosamine (deacetylated) units. Adapted from Ref^[146]

2.7.5.4 Chitosan degradation

Chitosan dissolves in organic or inorganic acids where pH is lower than its D-glucosamine residue pK_a of 6.2-7, at which the amino groups starts to protonate, resulting in a viscos non-Newtonian fluid.^[103] Despite the wide range of applications in the pharmaceutical and biomedical field, few chitosan-based products reached the market. The primary reason lies in the inconsistency of its properties including molecular weight, polydispersity, degree of deacetylation, and purity level, which varies between different sources of extraction and played an important role in determining the degradation mechanism of the polymer. Chitosan degradation arise from chain depolymerization caused by oxidation, chemical or enzymatic hydrolysis reactions.^[147] Depolymerization starts with random splitting of β-1,4-glycosidic bonds followed by subsequent breaking of -acetyl linkage, known as deacetylation. The degree of deacetylation determines the speed of degradation, the higher the degree of deacetylation, the greater the porosity between the polymer chains because more protonated amino groups would be available to repel each other, as a result, the speed of acid hydrolysis would be faster. Depolymerization results in the production of free radicals which induce subsequent oxidation^[148]. Decrease in average molecular weight begins with polymer chain scission, accompanied by cleavage of functional groups such as amino, carbonyl, hydroxyl. Covalent crosslinks between fragments of chitosan irreversibly alters the physicochemical properties of the polymer.

Figure 8. Schematic drawing representing the degradation processes of chitosan. Adapted from Ref^[149]

2.7.5.5 Pluronic F127

Despite the promising application of chitosan as mucoadhesive material to improve the residence time of liposomes as shown by various groups, Chen *et al.* claimed that neutrally charged Pluronic F127- modified liposomes has better mucus penetrating ability due to the minimal interaction with negatively charged mucin moiety compared to positively charged chitosan, which tends to being trapped inside the dense mucus layer instead of traversing through to reach the surface of enterocyte.^[150, 151] These trapped liposomes could be releasing drugs locally inside the mucus and be cleared due to mucus shedding. Peptides released in the mucus could still unable to escape the fate of degradation by brush border enzymes. In another study, Pluronic F127-modified liposome-containing cyclodextrin (CD) inclusion complex was shown to improve the solubility, cellular uptake, and penetration of tacrolimus *in vitro* and *in vivo*.^[152]

2.7.5.6 Eudragit

The human GI tract is a harsh environment for orally administered drugs. It is important to protect insulin from degradation due to proteases and low pH environment in stomach. To achieve this protection, pH responsive polymers that are unaffected in low pH but swell and open up in high pH environment have been frequently used in the design of oral drug delivery vehicles. Eudragit family polymers are block copolymers consisting of repeating units of methacrylic acid and methyl methacrylate. Owing to its well-established medicinal use as enteric coating material, various groups have attempted fabricating drug loaded Eudragit microparticles for oral delivery.^[153, 154] In one study, insulin-loaded Eudragit S100 microspheres demonstrated a 24% reduction in blood glucose level effect continuing up to 6 hours.^[154] Barea *et al.* developed a pH responsive microsphere of Eudragit S100 encapsulating drug loaded liposomes for colon targeted release. The microspheres demonstrated minimal release in SGF and SIF condition while subsequent release in colon-mimicking conditions.^[155, 156] The carboxylic acid groups from methacrylic acid are responsible for the pH responsive property of the Eudragit polymer, which gets deprotonated and dissolved at pH above its pK_a . The pK_a of Eudragit L100 and S100 are estimated to be 6.45 ± 0.03 and 6.66 ± 0.05 . The slightly lower pK_a value of Eudragit L100 is due to the higher carboxylic acid content which causes its dissolution pH to be lower than Eudragit L100.^[157]

Table 2. Percentage carboxylic content, pK_a and dissolution pH of the pH-sensitive polymers.

Polymer	Carboxylic acid content (%)	pK_a	Dissolution pH	Molecular weight (g/mol)	Polydispersity index	Reference
Eudragit S100	27–30	6.66 ± 0.05	7	125,000	1.7-2.3	[157-159]
Eudragit L100	46–50	6.45 ± 0.03	6	125,000	1.7-2.3	[157-159]
HP55	21–27	4.83 ± 0.04	5.5	45000	2.67	[157, 160]

Figure 9. Molecular structure of anionic copolymers (Eudragit) based on methacrylic acid and methyl methacrylate. Eudragit L having x:y equals 1:1. Eudragit S100 having x:y equals 2:1. Adapted from Ref^[161]

Figure 10. Molecular structure of Hydroxypropylmethylcellulose phthalate. Adapted from Ref^[160]

2.7.5.7 To improve liposome loading

In general, hydrophilic drugs are loaded into liposomes through either passive or active methods. Passive loading is achieved by introducing water soluble drugs to the dried lipid film during thin film rehydration which allows encapsulation to occur during vesicle formation. However, the efficiency of loading is often low and the drug tends to stay in the bulk of rehydration solution instead of being loaded inside the liposome aqueous core because of its small volume compared to the surrounding aqueous environment. To improve the loading of liposomes for hydrophilic proteins and peptides, many groups have been studying on methods such as active loading and repeated freeze thawing. ^[162, 163]

2.7.5.8 Active loading

In active loading, drugs are loaded into the pre-formed liposomes using to a transmembrane pH gradient. The pH of the buffer outside liposome makes the drug unionized and able to cross the lipid bilayer to reach the inner aqueous core. Once inside, the difference in pH in the aqueous core causes the drug to be ionized and trapped inside the liposome core. The pH of the liposome aqueous core is normally acidic that set the requirement of the drugs to have weakly basic groups for active loading. Anticancer drugs such as Doxorubicin ^[164, 165] and Vincristine ^[166] are weak bases that have been successfully loaded into preformed liposomes via the pH gradient method. Remote loading of insulin into neutral or positively charged liposomes was attempted by Hwang et al. A two-fold improvement of loading was reported for insulin using the transmembrane pH gradient method compared to conventional reverse-phase evaporation vesicle method. However, the distribution of insulin was primarily on the surface of the positively charged liposomes ^[167].

Figure 11. Active loading of ionizable hydrophilic drugs into liposomes with the help of a transmembrane pH gradient. Adapted from Ref^[168]

2.7.5.9 Repeated freeze thawing

Repeated freeze thawing the liposomes reduce the lamellarity and results in physical disruption of the bilayer due to ice crystal formation, allowing diffusion of drug molecules into the liposome for higher encapsulation. Multiple cycles of repeated freeze and thawing were often required to achieve equilibrium drug concentration inside and outside the aqueous core.^[169] Xu *et al.* studied the lipid protein interaction using superoxide dismutase (32500Da), 6 times the size of insulin, and achieved 50% encapsulation with 150nm DPPC liposome containing cholesterol using repeated freeze and thawing technique.^[170] However, the reason this technique is not widely used in the encapsulation of protein drugs is that most protein drugs are sensitive to temperature changes, its function or conformation changes when experiencing

extreme temperature change. The stability of the protein would be adversely affected by the extreme temperature introduced during repeated freeze thawing at -196°C and 65°C.

2.7.5.10 LbL approach

A Layer-by-Layer (LbL) approach of building up drug or polymer on the surface of nanoparticles or bulk materials has gained growing interest in the field of drug delivery research over recent years. The method of layering drugs on the surface of nanoparticle is based on simple electrostatic interaction, hydrogen bonding, or other secondary interaction, which are able to generate highly stable drug delivery vehicle. One of the unique advantages of such a system is modular sustained release with high drug loading, which could overcome the limitations of liposome to hydrophilic drugs when modified on the liposomes surface. More recently, liposomes has been explored as the substrate for coating polymers layer by layer on the surface. The application of LbL on the surface of liposomes, which are already FDA approved for several cancer drugs, would be of great clinical translational value. The ability to load drugs in both core and the layers enables an independently tunable multidrug delivery device. Paula et al. has demonstrated that LbL modification significantly improves the *in vivo* stability and pharmacokinetics of nanoparticles for systemic delivery.^[171] The same group has demonstrated extended stability of LbL-liposomal formulation in tissue culture medium with less than 20% release over 24 hours.^[171] Loading of the LbL nanoparticles can be achieved at high drug density range from 10-40 wt % which is significantly higher than the 2 to 5 wt% obtained by traditional polymer drug blended matrices.^[172] Furthermore, the coating layers are generated in aqueous solution at room temperature, which are mild conditions for preserving the activity of proteins, nucleic acids, and other fragile biomacromolecules.

2.8 References

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Chapter 3

Methods and Materials

This chapter describes the experimental methods and materials involved in achieving the objectives of the thesis. The experimental design revolves around the synthesis of particles for testing the uptake and transport across Caco-2 monolayer. Experiments designed for testing the first hypothesis of an enteric targeted capsule release include capsule dip-coating in pH responsive polymers, SEM characterization, and release in simulated bodily fluids. Experiments designed for testing the second hypothesis of enhanced transport of liposomes via surface modification include synthesis of Eudragit S100 coated liposomes, NanoDSC for characterizing the T_m of liposomes, TEM and DLS characterization of coating, HPLC and micro BCA for determining encapsulation and loading, flow cytometry and fluorescence imaging for characterizing cellular uptake, ELISA for quantifying transport and release. Experiments designed for testing the third hypothesis that improved loading and release can be achieved through Layer-by-Layer coating insulin onto the surface of liposomes involve the DLS optimization and characterization of the multilayers, NanoITC for studying the thermodynamics of binding, flow cytometry and fluorescence imaging for characterizing cellular uptake, ELISA for quantifying transport and release.

3.1 Materials

Capsule coating. Eudragit L100, S100, and Hydroxypropylmethylcellulose phthalate (HP-55) were gifts from Jebsen & Jessen (SEA) and Shin-etsu Chemical Co., Ltd (Toyama, Japan). Hard gelatin size 0 capsules were purchased from Bractaco Chemicals. Dip coating mandrel was purchased from UFO Labglass (S) Pte Ltd. Dichloromethane (DCM), Trehalose, Triethyl Citrate, Human recombinant insulin (Sigma Aldrich, ≥ 27.5 IU/mg), Triton X-100, Tween 80 (Polysorbate 80), Coumarin-6, sodium phosphate dibasic (SPD, $\geq 99.5\%$ purity), trifluoroacetic acid (TFA, $\geq 99\%$, HPLC grade) and HPLC grade acetonitrile (ACN, $\geq 99.93\%$), Hanks' Balanced Salt Solution (HBSS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), NaHCO_3 , medium molecular weight chitosan, simulated gastric fluid without enzyme (SGF, pH 1.1-1.3), simulated intestinal fluid without enzyme (SIF, pH 6.5-6.6) were purchased from Sigma-Aldrich. Phosphate buffered saline (PBS) was prepared by the addition of potassium chloride (KCl), sodium chloride (NaCl), potassium dihydrogen phosphate (KH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4), all of which were purchased from Sigma-Aldrich. Chloroform (Amylene Stabilized), Isopropyl Alcohol (IPA) and Methanol (MeOH) were obtained from Tedia Company Inc. **Lipids.** 18:1 PE CF polar head group fluorescently labelled carboxyfluorescein lipid, L- α -phosphatidylcholine (Egg PC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine, HEPC, L- α -phosphatidylcholine (Egg PC), Soybean phosphatidylcholine (SPC) were purchased from Avanti® Polar Lipids, Inc. Hydrogenated soybean phosphatidylcholine (HSPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol, sodium salt (DPPG), and 1 2-Dioleoyloxy-3-trimethylammonium propane chloride (DOTAP) were obtained from NOF America Corporation, Coatsome®. **Cell culture.** Caco-2 (passage 17) cell line was purchased from ATCC. 12-well Corning(R) Transwell (R) Polyester Membrane (Sigma-Aldrich), DMEM High Glucose with 4.0 mM L-Glutamine and Sodium Pyruvate, 1X Phosphate Buffered Saline (PBS) 0.0067M PO_4 without Calcium, Magnesium, and Phenol Red were purchased from GE Healthcare. Chopstick Electrode Set for EVOM2, 4mm (World Precision Instruments). Insulin ELISA kit (Mercodia). Vectashield® with DAPI was purchased from Vector Laboratories (USA). Rabbit polyclonal to Claudin 1, Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488), AF488 anti insulin and secondary

antibodies. **Others.** Hydrochloric acid (HCl) was purchased from Emsure and sodium hydroxide pellets (NaOH, grade AR) were purchased from Schedelco. Biotech cellulose ester dialysis membrane 300KD packed in 0.05% sodium azide was purchased from Sciencescan Consultancy & Services Pte Ltd.

Figure 12. Diagram summarizing the experimental procedures designed for achieving the specific aims of this thesis.

3.2 Capsule coating

Gelatin capsules were dip-coated in a sequential of enteric coating solutions following the procedures in section 3.2.2. Capsules would be left to dry at room temperature for a minimum of 2 hours before being placed into a 37°C vacuum oven overnight to ensure complete drying. The capsules were tested in a release study thereafter. Fluorescently tagged liposomes were fabricated following the traditional thin film rehydration method in section 3.2.3 and lyophilization was described in section 3.2.4. Finally, lyophilized liposomes or Rhodamin 6G dye were loaded into the gelatin capsule before coating with enteric polymers and studied for release in SGF and SIF as described in section 3.2.5. Coated capsules were characterized by SEM for measuring the thickness of the coating layers as described in section 3.2.6.

3.2.1 Solution preparation for capsule coating

Eudragit polymers (S100/L100) were prepared by dissolving in isopropyl alcohol (IPA) containing triethyl citrate. HP55 was prepared by dissolving in dichloromethane (DCM) containing triethyl citrate and methanol. Both solutions were stirred at 350rpm for at least 4 hours to ensure complete dissolution of the polymers and achieve a homogenous solution. Detailed composition of the coating solution is summarized in **Table 3**.

Table 3. Solvent composition used for enteric polymer coating

Organic solvent	Eudragit polymer (600mg)	HP55 (550mg)
IPA	8.54ml	-
Triethyl citrate	60μl	50μl
DCM	-	8ml
Methanol	-	2ml

3.2.2 Enteric coating of gelatin capsules

Manual dip-coating. Four sets of gelatin capsules coated with different polymers of varying thickness were subjected to release study in simulated gastric and intestinal fluids to determine the optimum coating parameters to achieve intestinal targeted release. Each capsule was loaded with 1mg Rhodamin 6G for quantifying the release by fluorescence. Two sets of capsules were coated with 4, 6, 8 layers of Eudragit L100, the other two sets were coated with 2, 4, 6 or 8 layers of Eudragit S100 solution. One of these two sets was pre-coated with 2 layers of HP55 before coating with Eudragit solution. The capsules were first dipped into the coating solution and left to dry with the help of a mini USB fan. The 2nd layer was coated after the 1st layer has been fully dried. The coated capsules were stored at room temperature overnight before release study.

Automated dipcoating. Stainless steel capsule holder mandrel from UFO Labglass for the NIMA Technology Micro-Processor Interface IU4 dip-coater was used to coat the capsules half by half. Gelatin capsules was filled with 20mg of 2mM lyophilized HSPC liposomes tagged with 0.5 mol% Coumarin-6 dye, dip coated using the dip-coater machine at a speed of 240mm/second. The holder was designed to hold the

capsule near its bottom to ensure complete submerge of the capsule connecting part in the coating polymer solution. Once lifted out from the coating solution, the capsule was overturned to prevent concentrating of solution at the domed end of the capsule, and to allow the coating to be uniformly developed. Coated capsules were stored at room temperature for at least 2 hours before being transferred to a 37°C vacuum oven and kept overnight for complete removal of any remaining organic solvents.

The number of coating layers was optimized to protect capsule from releasing in SGF while giving targeted release in SIF. Capsules were pre-coated with HP55 which was served to enhance Eudragit polymer adhesion. Two types of Eudragit polymers S100 and L100 were compared in this study to determine the suitability as enteric coatings for intestinal targeted release. Gelatin capsules were first coated with Eudragit S/L100 polymer with and without 2 layers of HP55 pre-coatings, and tested for release in SGF and SIF to select the suitable polymer that gives the desired release window for intestinal targeted release. From the release study, Eudragit L100 was selected and used for subsequent optimization of coating thickness. Next, both HP55 and Eudragit L100 coatings were varied between 2 - 3 layers to determine the minimum coating thickness required to achieve the intended release.

3.2.3 Liposome fabrication

Liposomes were prepared by thin film hydration method as previously described.[1] Briefly, known amount of phospholipids dissolved in chloroform and methanol solvents in a 2:1 ratio in a round bottom flask. The different types of lipids used in this study are summarized in Table 4. Cationic lipid DOTAP or anionic lipid DPPG were added to the organic solvent mixture at 10mol% for modifying the surface to be positively or negatively charged, respectively. Cholesterol was added at 10 mol%, 30 mol%, and 60 mol% during liposome fabrication to alter the rigidity of the liposomes. Fluorescent dye coumarin-6 was added at a 0.5mol% for transport study of empty liposomes. Solvents were allowed to evaporate under a stepwise reduction in pressure and finally stabilized at 20 mbar at constant temperature at 60°C for an hour in a rotary evaporator (BUCHI Rotavapor® R-100). After the solvents were completely removed, 5 ml of PBS 7.4 was added into the round bottom flask to rehydrate the lipid film, reaching a final liposome concentration of 20 mM. The liposome suspension

was extruded 5 times through a 0.2 μm pore size filter followed by 10 times through 0.08 μm pore size filter to obtain final size around 100nm.

Table 4. List of phospholipids and fluorescent dyes used for liposome fabrication. Adapted from Ref [2-8]

Lipid	Carbon atoms: C=C bonds	Structure
Egg phosphatidylcholine (EPC)	16 : 0 18 : 1	
Soy phosphatidylcholine (SPC)	18 : 2	
Hydrogenated egg phosphatidylcholine (HEPC)	16 : 0 18 : 0	
Hydrogenated soy phosphatidylcholine (HSPC)	18 : 0	
Dimyristoyl-sn-glycero-3- phosphocholine (DMPC)	14 : 0	
1,2-Dipalmitoyl-sn-glycero- 3-phosphocholine (DPPC)	16 : 0	
1,2-dioleoyl-3- trimethylammonium-propane (DOTAP)	18 : 1	
1,2-Dipalmitoyl-sn-glycero- 3-phosphatidic acid, sodium salt (DPPG)	16 : 0	
1,2-dioleoyl-sn-glycero-3- phosphoethanolamine-N- (carboxyfluorescein) (ammonium salt)	18:1 PE CF	
Coumarin-6	-	

3.2.4 Lyophilization of liposomes

Lyophilization conditions were optimized by adding 100mg of cryoprotectants (sucrose or trehalose) into 5ml 2mM, 200 μ M and 20 μ M liposomes in PBS. The samples were frozen at -80°C overnight before being transferred to freeze-dryer to allow the sublimation of water. Samples were collected after 24 hours and reconstituted in DI water to study the effect of cryoprotectant in shielding the liposome during lyophilization. Reconstituted liposomes were characterized by DLS to measure the change in size, zeta potential, and PDI.

3.2.5 Release study of enteric coated capsules

Simulated Gastric Fluid (SGF), pH 1.2 was prepared by dissolving 2g NaCl in a mixture of 7.0ml of HCl (37% v/v, 12.06M) and 1000ml of DI water.^[9] Simulated Intestinal Fluid (SIF), pH 6.8 was prepared by dissolving 6.8g KH₂PO₄ and 0.9g NaOH in 1000ml DI water.^[10] To study the release of coated capsules, transparent hard gelatin size 0 capsules were loaded with Rhodamin 6G dye or lyophilized HSPC liposomes containing 0.5% coumarin-6. The coated capsules were immersed in 10ml of SGF with magnetic stirring at 350RPM. Sampling was collected hourly for analysis of percentage of dye released by fluorescence. Following 4 hours of immersion in SGF, capsules were transferred to glass bottles containing 10ml of SIF and continued with a magnetic stirring until completely released. The collected samples were transferred to Corning 96-well black plate at 100 μ l/well and analyzed for fluorescence readings using Tecan i-Control Fluorescence Microplate Reader with excitation and emission wavelengths of 526nm and 555nm for Rhodamin 6G samples. Similarly, excitation and emission wavelengths of 480nm and 530nm were used to detect the fluorescence of Coumarin-6 tagged liposome. Calibrations of the dyes were performed to allow the conversion of data from the fluorescence to concentration of dye/liposomes released.

Figure 13. Release setup. Capsules coated with Eudragit L/S100 with and without HP55 precoat after 4 hours in SGF (0 hour in SIF)

3.2.6 Scanning Electron Microscope (SEM) Analysis

To characterize the surface morphology and coating thickness of the capsules, scanning electron microscopy (JEOL JSM-5410-LV, Germany) was performed to provide direct visualization of the coated capsule samples. Empty coated and uncoated capsules were manually sectioned into halves to allow the cross section to be exposed and the sectioned capsules were mounted onto the SEM stage both vertically and horizontally to reveal cross sections and surface morphology. Thickness of the coating was directly measured from the SEM image of cross sections using Image J software and normalized against uncoated capsules. Surface morphologies such as porosity were observed from the image of the capsule main body. The mounted samples were gold sputtered for 70 seconds to allow the sample to be conductive of electrons.

3.3 Insulin loaded liposomes with Eudragit coating

Insulin was loaded into liposomes based on a progressive rehydration method described in section 3.3.1. Circular dichroism (CD) was used to characterize the conformation of insulin in different buffered conditions, as described in section 3.3.2. HPLC was used for quantification of insulin for loading and encapsulation determination, as described in section 3.3.3-3.3.4. Liposomes were fabricated using lipids of different carbon chain length, degree of saturation, and charge and

characterized by NanoDSC (section 3.3.6.3) for phase transition temperature. Eudragit S100 coated liposomes were characterized by DLS and TEM to study for the change in size, zeta potential, and PDI (section 3.3.6.1-3.3.6.2). Caco-2 cells were cultured on transwell inserts and glass coverslips for studying transport and uptake of liposomes based on the protocol described in section 3.3.7-3.3.8 and 3.3.11, respectively. ELISA was used to quantify the amount of transported (section 3.3.9) and released insulin (section 3.3.10). Flow cytometry and fluorescence imaging was used to study cellular uptake of liposomes (section 3.3.11).

3.3.1 Insulin solution preparation and liposome loading

Insulin solution (1mg/ml) containing sodium phosphate dibasic was adjusted to pH 7.5 using 1M NaOH and 1M HCl measured by a pH meter (Mettler Toledo). A gradient dilution aimed to improve the loading was achieved by rehydrate the thin film following a gradient dilution. Generally, insulin solution of 500 μ l 1mg/ml, 500 μ l 0.5mg/ml, 500 μ l 0.25mg/ml, 1ml 0.125mg/ml was first added to the thin film in a step wise manner with 5 mins interval in between each addition. Subsequently, 2.5ml of insulin buffer was added to dilute the liposome solution so that the lipid concentration of the final solution remains at 20mM.

3.3.2 Insulin characterization by Circular dichroism (CD)

CD scan was performed to detect changes in the secondary structure of insulin. Samples were prepared by loading 50 – 100 μ l of 0.3 mg/ml of insulin samples onto a sandwiched cuvette. The CD spectrum was attained using a spectrometer (Chirascan, Applied Photophysics) in the far UV region (190 – 260 nm), with a 0.5 nm bandwidth and a step size of 1 nm. The CD spectra of the background (SPD buffer) was measured and subtracted from the insulin spectra.

3.3.3 Quantification of insulin by HPLC and nanodrop

Insulin concentration was estimated using UV visible spectrophotometer (Thermo scientific NanoDrop 1000) according to the direct A280 method. The measurement pedestal was cleansed with DI water and blanked with SPD buffer before pipetting 2 μ l of insulin sample onto the pedestal for measurement. For more precise measurement of insulin concentration, High performance Liquid Chromatography

(HPLC) was used. HPLC is a quantitative method of measuring the concentration of analytes based on its interaction with stationary and mobile phase to effect a separation. For non-polar or weakly polar analytes, Reverse-phased high-performance liquid chromatography (RP-HPLC) with a non-polar stationary phase is commonly used. The stationary phase is either chemically bonded to a surface or coated onto a support for retention of analytes. In a gradient elution method, the polarity of the mobile phase is gradually decreased, increasing the interaction between analyte and mobile phase until sufficiently high which allows the release of analyte from the stationary phase and elution of analyte as it is carried by the mobile phase to the detector. RP-HPLC (Agilent Technologies 1200 series) method for insulin was developed for accurate measurement of insulin concentration. A C18 column (Zorbax) of pore size 300 Å, 250 mm length, and 4.6 mm wide internal diameter was used. The mobile phase consisted of 0.1% TFA in ACN and 0.1% TFA in DI water which was set at a flow rate of 1 ml/min. A gradient elution technique was executed whereby composition of the non-polar mobile phase – 0.1% TFA in ACN, was increased from 20% to 80% in 10 min. Optimization of elution time and calibration of insulin concentration will be further discussed in section 5.4.4. Assays for each sample were conducted in triplicate with an injection volume of 100 µl each and at an UV absorbance at 235 nm.

3.3.4 Quantification of Encapsulation Efficiency and Loading Efficiency

Fabricated liposomes were ultracentrifuged (Thermofisher, SORVALL WX ultra) at 50,000 rpm, 4 °C for 30 min. The concentration of free insulin in the supernatant, $C_{rhINS,SN}$ (mg/ml), was determined using a reverse-phase (RF)-HPLC assay against a standard curve obtained by serial dilution of insulin of known concentration. The ratio of $C_{rhINS,SN}$ (mg/ml) to $C_{rhINS,SN}$ (mg/ml) can be used to calculate the percentage encapsulation efficiency (EE %) using the following formula:

$$EE (\%) = \left(1 - \frac{\text{insulin concentration in supernatant}}{\text{original insulin concentration in hydration solution}} \right) \times 100 (\%)$$

Following the ultracentrifugation step, the pellets were separated from the supernatant and re-suspended in DI water. Complete resuspension was achieved by vortex mixing. Thereafter, 300 µl of this liposomal suspension was transferred to a pre-weighed 1.5 ml microtube which was then frozen for at least 4 h in a -80 °C freezer. In its frozen

state, the liposomal suspension was placed in a manifold freeze-dryer (lyophilizer) for 12 h. Duration of freezing, transfer and lyophilisation were carefully planned to ensure that the eventual powdered liposomes were completely dried. The mass of the insulin-loaded liposomes were determined from the difference between the mass of microtubes containing powdered liposomes and that of empty microtubes. Ultimately, the percentage loading efficiency (LE %) was calculated from the following steps:

$$\text{Mass of encapsulated insulin (mg)} = \text{EE} * C_{rhINS,0} \text{ (mg/ml)} * 300 \text{ (}\mu\text{l)}$$

$$\text{LE (\%)} = \left(\frac{\text{Mass of encapsulated insulin}}{\text{Mass of insulin loaded liposomes}} \right) \times 100 \text{ (\%)}$$

3.3.5 Eudragit S100 coating of liposomes

Eudragit S100 coated liposomes were prepared by electrostatic interaction between the positive charged DOTAP lipids in the liposome and the negatively charged Eudragit S100 polymer solution. 0.0125% (w/v) Eudragit S100 was first prepared by dissolving Eudragit S100 powder in alkaline water. Briefly, 1 mM Liposome solution was first mixed with 3.5 μ l Tween 80 and vortexed for 5 mins to achieve a sterically stabilized surface. Subsequently, 15 μ l 0.0125% (w/v) Eudragit S100 polymer solution was added to the Tween 80 stabilized liposome solution and vortexed for another 5 mins. The detailed formulation used for coating the liposome is listed in **Table 5**.

Table 5. Volume of liposome, PBS, Tween 80 and Eudragit S100 used for coating of liposomes

Type of coating	Liposome volume (μ l)	PBS volume (μ l)	Tween 80 volume (μ l)	0.0125% (w/v) Eudragit S100 volume (μ l)
Uncoated	10	203.5	0	0
Tween 80	10	200	3.5	0
E15	10	185	3.5	15
E20	10	180	3.5	20
E30	10	170	3.5	30
E40	10	160	3.5	40

3.3.6 Characterization of liposome

Eudragit S100 coated liposomes were characterized by Transmission Electron Microscopy (TEM) for direct visualization of size, Dynamic light scattering (DLS) for hydrodynamic radius in solution, and Nano differential scanning calorimetry (Nano DSC) for transition temperature.

3.3.6.1 Transmission Electron Microscopy

Morphology of Eudragit S100 coating on the liposome vesicle was characterized by TEM (JEOL JEM-2010). Typically, 6 μ l of 160 μ M Eudragit S100 coated liposomal suspension was dropped casted onto a copper grid (Lacey Formvar/Carbon, 300 mesh, Copper) and air-dried completely overnight prior to TEM observation.

3.3.6.2 Dynamic Light Scattering

The liposome suspension was diluted 100 times with Deionized (DI) water for analysing its size and zeta potential using a Zetasizer Nano (Malvern Instruments, UK). Disposable polystyrene cuvettes were used for measuring size, while folded capillary cell DTS1070 was used for measuring the charge of liposomes. The characterization of the size and zeta potential of liposomes are analyzed using dynamic light scattering (DLS). DLS predicts the particle size in suspension by measuring their Brownian motions, and the signal was converted into hydrodynamic diameter using Stokes-Einstein equation.^[11] Zeta potential measures the electrophoretic mobility of particles in suspension under the influence of an applied electric field. Charged particles in solution are surrounded by oppositely charged ions, forming a tightly bound stern layer which stays with the particle as the particle moves. Additional ions surrounding the stern layer is known as the diffusive electric double layer which does not stay with the particle as the particle move. The boundary between stern layer and the diffuse layer is called the slipping plane, where zeta potential is measured. Zeta potential is calculated based on Henry Equation.^[12]

Figure 14. Schematic representation of electrical double layer surrounding a particle in aqueous solution.^[12]

Stokes-Einstein equation:

$$d(H) = \frac{kT}{3\pi\eta D}$$

$d(H)$ = hydrodynamic diameter

D = translational diffusion coefficient

k = Boltzmann's constant

T = absolute temperature

η = viscosity

Henry's equation:

$$U_E = \frac{2\varepsilon z f(Ka)}{3\eta}$$

U_E = electrophoretic mobility

ε = Dielectric constant

$f(Ka)$ = Henry's function

3.3.6.3 Nano differential scanning calorimetry (Nano DSC)

Thermal properties of liposomes and insulin were characterized by Nano DSC (TA Instrument). PBS and samples were first degassed using a degasser and subsequently loaded into the reference and sample channel. The samples were heated up at 1 °C/min up to 90 °C. Sample curves were normalized with PBS blank during analysis. The concentration of lipids and insulin used were 15.7mg/ml and 1mg/ml, respectively. NanoAnalyze software was used to analyze the thermogram and fit the integrated area according to nonlinear least squares regression using two state scaled model to obtain melting temperature (T_m) and change in enthalpy (ΔH).

3.3.7 Cell culture for transport study

Caco-2 cells were cultured in T75 flasks with Dulbecco's Modified Eagle Medium (DMEM) high glucose medium containing 20% FBS and 1% Penicillin/streptomycin and 1% non-essential amino acids and harvested when the cells were 80-90% confluent. To establish an *in vitro* cell model for transport study, Caco-2 cells were grown on 12-well transwell inserts with 0.4µm pore size for 30 days with TEER values monitored every second day for the development of a monolayer. Cells were seeded at 300,000 cells/well on a 12 mm permeable membrane support for 30 days. Medium was changed every other day post seeding and Transepithelial Electrical Resistance (TEER) values were recorded using Epithelial Voltmeter (EVOM) equipped with “chopstick” electrodes (World Precision Instruments, Sarasota, FL). Transport experiment was performed 30 days post-seeding when the normalized TEER values reached above 300 Ω.cm². The normalized TEER values were calculated using the following equation:

$$\text{TEER} [\text{Ohm} \cdot \text{cm}^2] = (\text{TEER}_a - \text{TEER}_b) \cdot A$$

TEER_a = TEER value across transwells seeded with cells

TEER_b = TEER value across cell free transwells

A = surface area of filter membrane

3.3.8 *In vitro* Transport study

Transport experiment was performed 21 days post-seeding when the TEER values reached above 300 $\Omega\cdot\text{cm}^2$. HEPC, EPC, HSPC, SPC, DMPC, DPPC liposomes were fabricated from lipids containing different carbon chain length and degree of saturation. HBSS-HEPES buffer was prepared by dissolving 9.7 g HBSS, 5.95 g HEPES, and 0.35 g NaHCO_3 in 1 liter DI water. NaOH (1 M) was used to adjust the pH of the solution to pH 7.4 and the buffer solution was sterile-filtered using 0.22 μm pore size membrane. Transport experiment was carried out following an established protocol.^[13] Briefly, liposomes solution was diluted to a final lipid concentration of 1 mM using HBSS-HEPES buffer. 500 μl of the 1mM liposome solution was added in the apical compartment and 1.5 ml of buffer was added into the basal compartment of the transwell insert. Subsequently, sampling was done at 0, 1, 2, 3, and 4 hour and buffer from the basal compartment was replaced with fresh HBSS-HEPES. Both apical and basal compartment fluid were collected, and cells were washed twice with PBS before lysing with 0.1% Triton-X. Cell debris was removed by centrifugation for 10 mins. Supernatant, apical and basal fluids were collected for fluorescence reading of coumarin-6 from the liposomes by fluorescence plate reader at excitation 488 nm emission 523 nm.

PC lipid assay (BioVision) was performed according manufacturer's protocol. Briefly, samples collected from basal compartment of transwell inserts during transport study were mixed with 50ul of reaction mix containing PC probe, PC hydrolysis enzyme, development mix, and assay buffer. The reaction mixture was incubated for 30 min at room temperature in dark before measuring absorbance at 570 nm.

3.3.9 ELISA quantification of insulin

Transport buffer in the basal compartment was collected hourly for quantification of transported insulin. ELISA (Mercodia) was performed according to manufacturer's protocol using known amount of human recombinant insulin as standard. Enzyme-linked immunosorbent assay (ELISA) is an immunoassay based on antibody sandwiched detection of antigen in a sample. Typically, anti-insulin monoclonal antibodies were coated onto the bottom of the microtiter wells which is capable of

capturing the antigenic sites of insulin. For the detection of insulin, peroxidase-conjugated anti-insulin antibodies were added to the wells containing the bound insulin to initiate a colorimetric reaction when peroxidase converts the substrate into a colored product which can be measured by absorbance at 450nm.

3.3.10 Release of insulin from liposomes

Release study was performed on coated liposomes in PBS pH 7.4 at 37°C under constant stirring. 50ul of coated or uncoated liposomes were placed in 300kDa MWCO dialysis bag sealed and immersed in 15ml of release buffer and incubated for 89 hours. Sampling was performed at 0, 1, 2, 3, 4, 19.2, 45.8, 66.8, and 90.3 hours and medium was completely replaced with fresh PBS following each sampling. Amount of insulin released was quantified by ELISA as described in section 3.3.8.

3.3.11 *In vitro* cellular uptake study

Caco-2 cellular uptake was studied by both flow cytometry and fluorescence microscopy. For **flow cytometry**, Caco-2 cells were seeded in 12-well plates and were allowed to grow for at least 14 days before treating with AF546 tagged insulin loaded liposomes. Cells were treated with liposomes with and without Eudragit S100 and incubated for 4 hours. After treatment, cells were trypsinized and washed with PBS and stored in 0.2% FBS in PBS for flow cytometry analysis using BD FACS Aria.

Fluorescence microscopy was used to study cellular uptake of empty and insulin loaded liposomes with and without Eudragit S100 coating. 22x22 mm glass cover slides were cleaned using 70% ethanol, washed with PBS before adding 2 ml DMEM. Cells were seeded at 1×10^5 cells/well in 6 well plates and incubated for 14 days at 37°C in CO₂ incubator. Cells were washed twice with PBS, fixed with ice cold methanol and perforated with 0.1% Triton-X, before staining immunostaining. For studying the cellular interaction of empty liposomes, confluent cells were treated with coumarin-6 tagged liposomes at 1 μ M for 4 hours. For studying the uptake of insulin loaded liposomes, insulin was first manually tagged with AF546 fluorescent dye before being loaded into the liposomes. Cells were treated with insulin loaded liposomes with and without Eudragit S100 coating and immunostained with primary rabbit anti-claudin-1 antibodies, followed by secondary AF488-tagged mouse anti-rabbit IgG. For studying the uptake of LbL-liposomes, AF488 tagged anti-insulin

antibody was used to stain insulin. Claudin-1 was stained by primary rabbit anti-claudin-1 antibodies, followed by secondary AF568-tagged goat anti-rabbit IgG. VectaShield mounting media which stains cell nucleus with 4',6-diamidino-2-phenylindole (DAPI) was dropped onto the coverslips which were inverted and mounted onto a microscopy slide for imaging by ZEISS Slide Scanner.

3.4 Insulin loaded liposomes with multilayers of chitosan/insulin on the surface

Negatively charged HSPC/DPPG liposomes were used as a core for coating multilayers of alternating chitosan and insulin on the surface. Optimization of chitosan and insulin coating layers on HSPC/DPPG liposomes surface was described in section 3.4.1-3.4.2. Nano ITC was performed to study the binding stoichiometry between each coating layers (section 3.4.3). Following the optimized protocols, LbL coated liposomes were fabricated after repeated cycles of coating and purification, collected after 1, 3, and 5 layers of coating with chitosan/insulin for release (section 3.4.4), loading (section 3.4.5), and stability studies (section 3.4.6). Caco-2 cells were cultured on transwell inserts and glass coverslips based on the protocol described in section 3.3.7-3.3.8 and 3.3.11, respectively. ELISA was used to quantitate the amount of transported (section 3.3.9) and released insulin (section 3.3.10). Flow cytometry and fluorescence imaging was used to study cellular uptake of LbL-liposomes (section 3.3.11).

3.4.1 Optimization of chitosan and insulin coating layers

Insulin solution was prepared as described in section 3.3. Medium molecular weight chitosan solution 1% (w/v) was prepared by dissolving 1g of chitosan in 100ml 1% (v/v) acetic acid. For optimizing the chitosan layer on HSPC/DPPG liposomes, 10ul of 20mM HSPC/DPPG (10%mol) was diluted with 185ul of DI before mixing with chitosan solution. Increasing amount of chitosan solution 0.1% (w/v) was added to a fixed amount of HSPC/DPPG liposomes and characterized by DLS to find out the optimum condition for obtaining homogeneously coated liposomes. Liposomes with smallest changes in size and PDI but with a reversed charge were selected for optimizing the next layer of insulin coating. For insulin coating optimization, the same procedure was applied on chitosan-coated HSPC/DPPG liposomes. Amount of

insulin was increased from 0 to 250 μ l while keeping the volume of chitosan-coated HSPC/DPPG liposomes fixed at 10 μ l. DLS was used to measure the size, zeta potential and PDI change during the optimization to find out the optimum condition to achieve stable coating.

3.4.2 Fabrication of Layer-by-Layer (LbL) coatings onto liposome core

Layer by layer coating of nanoliposomes was achieved by a stepwise mixing and centrifuge-based purification method. All the odd layers and even layers were coated following the same formulation. Briefly, chitosan solution (0.1% w/v) containing 1% v/v acetic acid was mixed with insulin loaded HSPC/DPPG liposomes at a ratio of 7:1 and vortex for 5 mins for thorough mixing. Due to opposite charges between chitosan and liposome surface, chitosan adsorbs on the surface of negatively charged HSPC/DPPG liposomes giving rise to an electrostatically stable chitosan coated liposome. The chitosan coated liposomes were purified by ultra-centrifuging the liposomes at 50000 rpm 4°C for 30 minutes. Supernatant was decanted, and pellet was re-suspended before coating the next layer. The same procedure was performed on even layers with insulin to chi-HSPC/DPPG liposome ratio of 16:1.

3.4.3 Nano Isothermal Titration Calorimetry for PG/chitosan/insulin binding study

Nano Isothermal Titration Calorimetry (NanoITC, TA Instruments) was carried out to characterize the interaction and binding between different layers. Protein concentration was determined by NanoDrop™ 2000 according to UV absorption at 280 nm. All samples were degassed prior to the experiments. Data was collected while stirring at 300 rpm. NanoAnalyze software was used to analyze the binding isotherms and fit the integrated area according to nonlinear least squares regression using independent model to obtain Michaelis-Menten kinetic parameters. Briefly, nanoITC measures the thermal property of reactions involved in binding. The heat released or absorbed following each injection is reflected by the heat flow during binding. To study the binding of chitosan onto HSPC/DPPG liposomes, sample cell was filled with HSPC/DPPG liposomes (2mM PG content) in 0.1% acetic acid and syringe was filled with 0.1% chitosan (average MW 250k, average monomer units 1472) in 0.1% acetic acid. During analysis, syringe injects chitosan solution 2 μ l at a

time with 5mins intervals for 25 injects at 25°C. Heat of dilution was performed by injecting the 0.1% acetic acid into 20mM HSPC/DPPG liposomes in 0.1% acetic acid and was used as blank for creating the Blank Constant model. The thermogram of chitosan-HSPC/DPPG titration was normalized with the heat of dilution and fitted using independent model. To study the binding of insulin onto chitosan- HSPC/DPPG liposomes, sample cells was filled with chitosan- HSPC/DPPG liposomes in DI water and syringe injects 1mg/ml (0.172mM) insulin solution 2ul at a time with 5mins intervals for 25 injects. Heat of chitosan dilution was performed by injecting the DI water into chitosan- HSPC/DPPG liposomes in DI water.

3.4.4 LbL release

The release study of 3- and 5- layers coated liposomes was carried out at 37°C with constant stirring at 100rpm. The 3- and 5- layers coated liposomes were purified by ultracentrifuge to remove the free uncoated polymers, subsequently 50ul of each coated liposome were pipetted into a piece of Biotech cellulose ester dialysis membrane with 300kDa pore size, and the two ends were sealed with closure to allow only diffusion of free insulin. The sealed membranes were placed in 60ml jar containing 15 ml of PBS pH 7.4. Sampling was collected every 3-4 days with complete buffer replacement was at each time point for 89 days. Amount of drug released was quantified by ELISA and used for the calculation of loading efficiency.

3.4.5 LbL loading

Loading of the LbL-liposomes was determined by the ratio between weight of encapsulated insulin and total particle weight. The weight of encapsulated insulin was calculated from cumulative amount of insulin released in PBS pH 7.4 over the entire release period. The total particle weight was the weight of lyophilized LbL-liposome, which has been ultracentrifuged to remove free chitosan. 3- and 5- layers coated LbL-liposome was normalized according to the fluorescence intensity difference reference to the uncoated HSPC/DPPG liposome to account for the loss of particle during centrifugation and sample transfer.

$$LE (\%) = \left(\frac{\text{Mass of encapsulated insulin}}{\text{Mass of insulin loaded liposomes}} \right) \times 100 (\%)$$

3.4.6 Stability study

Stability of the LbL coated liposomes in SGF and PBS were assessed by DLS for both sizes, PDI, and zeta potential. Briefly, uncoated HSPC/DPPG liposomes and 1-, 3-, 5-layers coated liposomes were diluted 50x with SGF and PBS and incubated at 37°C for 4 weeks. Sampling was done weekly by withdrawing 250ul and further diluted 4 times with DI before subjecting to DLS measurement for size and charge measurement.

3.4.7 Statistical Analysis

Student's t-test was used to analyze the difference between means of two populations, while one-way analysis of variance (ANOVA) was used to compare the means between more than two populations. Assumption was based on a normal distribution of population data and the sample populations are independent and random. Measurement data was represented as mean $\pm$ standard deviation (SD). Significant difference was considered when $p < 0.05$.

3.5 References

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Chapter 4

Capsule coating for intestinal targeted release

This chapter describes the experimental results from coating of gelatin capsules for enteric targeted release. Gelatin capsules loaded with fluorescent dye or lyophilized liposomes were dip-coated with pH sensitive polymers. The pH responsive polymers HP55, Eudragit S100, and Eudragit L100 were evaluated for their effectiveness as enteric coatings on gelatin capsules via in vitro release studies in SGF and SIF. Areas including capsule dip-coating, parameter optimization, capsule release, liposome lyophilization were investigated and will be discussed in this chapter. Capsules having a coating thickness of at least 114 μm of Eudragit L100 has demonstrated intended release in simulated intestinal conditions.

4.1 Introduction

The demand by diabetic patients over the past decades accelerated the research towards an oral formulation which offers the advantage of non-invasiveness and delivers insulin to the liver where glucose homeostasis takes place. Harsh pH and enzymatic conditions in the gastrointestinal (GI) tract drive the development of a nanocarrier that offers protection of insulin, which is commonly degraded before absorption. An oral route of delivering protein therapeutics faces constant challenges from the harsh environments of the GI tract. The natural defensive mechanisms and digestive system of our body against harmful micro-organisms invasion and for nutrient absorption are the underlining reasons for the difficulties of oral drug delivery. The low pH in the stomach serve as the first line of defense against any ingested foreign body. The pepsin activated by the acidic pH virtually degrades any protein or peptide from the food, microbes, or drugs ingested before they are able to reach the intestine for absorption.

The most convenient way of overcoming this barrier is by using a pH sensitive capsule that is able to resist the low pH in the stomach and give the intended release at intestinal pH. In fact, most of the pharmaceutical companies developed technologies along this line; details were reviewed in chapter 2.2. Oramed Pharmaceuticals Inc. for example, developed an oral insulin formulation by loading bare insulin together with permeation enhancers into enteric coated capsules. Their technology has successfully completed clinical phase II trials. In this study, different pH responsive polymers Hydroxy-propylmethylcellulose phthalate (HP55), Eudragit S100 and Eudragit L100 will be investigated for its ability to protect the gelatin capsules in simulated gastric conditions and give intended release in simulated intestinal conditions. Hydroxypropyl Methylcellulose phthalate (HP55) has been widely used as an enteric coating material by the pharmaceutical industry due to its pH resistant property. Moreover, the polymer has been frequently adopted as pre-coating material to prevent moisture from getting into the capsule core as well as to enhance adhesion between the Eudragit polymer and the underlying capsule surface as the pre-coating increases the roughness of the capsule surface.^[1, 2]

4.2 Objective

The current study focuses on developing a pH responsive enteric coated capsule, which remains intact at simulated gastric condition but break and release the contents when the pH change to alkaline, for oral delivery of insulin. Such a capsule when loaded with lyophilized nanocarriers would be able to resist gastric degradation, protect insulin integrity, and achieve targeted release in small intestine. The objectives of this study are as follows:

1. To optimize coating parameters such as number of coating layers for different polymers, and the coating techniques for intestinal targeted release
2. To optimize lyophilization conditions by adjusting the ratio between cryoprotectant and lipid for extended stability

Figure 15. Schematic diagram illustrating the objective of chapter 4. Enteric coating of gelatin capsules for intestinal targeted release.

4.3 Optimization of capsule coating parameters for intestinal release

Gelatin capsules were coated with varying layers of enteric coating materials HP55 and Eudragit polymers for optimizing the coating formulation for intestinal targeted release. Optimization was performed by coating 4, 6 or 8 layers of Eudragit L100 or

Eudragit S100 onto Rhodamine 6G loaded gelatin capsules with and without 2 layers of HP55 pre-coating. Release of Rhodamine 6G from the enteric polymer-coated gelatin capsules in SGF and SIF was studied, and results were shown in **Figure 15**, **Figure 16**, and **Table 6**.

Figure 16. Photos for release samples of Rhodamine 6G-loaded gelatin capsules coated with 4, 6, and 8 layers of Eudragit S/L 100 in the presence and absence of HP55 pre-coating. (a) Release of Rhodamine 6G from gelatin capsules coated with 4, 6, 8 layers of Eudragit L100 in the presence and absence of HP55. Release studies performed for 4 hours in SGF, followed by 4 hours in SIF. (b) Release of Rhodamine 6G from gelatin capsules coated with 4, 6, 8 layers of Eudragit S100 in the presence and absence of HP55. Release studies performed for 4 hours in SGF, followed by 36 hours in SIF.

Figure 17. Release study 4, 6 or 8 layers of Eudragit L100 coated on gelatin capsules with (a) and without (b) HP55 pre-coatings. (c) Release profile of HP55 protected Eudragit L100 coated capsules in 6 hour SGF followed by 1 hour SIF. Release study 4, 6 or 8 layers of Eudragit S100 coated on gelatin capsules with (d) and without (e) HP55 pre-coatings.

Table 6. Summary of repeated study on the release of enteric polymer coated gelatin capsule in SGF and SIF

	Time (hour)	Eudragit		HP55-precoated followed by Eudragit	
		L100	S100	L100	S100
SGF	1				
	2				
	3				
	4				
SIF	1				
	2				
	3				
	4				
	Over 12				

No release

2 coats dissolve

4 coats dissolve

6 coats dissolve

8 coats dissolve

desired dissolution window

Results of capsule release were tabulated in **Table 6**, rows enclosed by red box were considered as effective coating for targeted release in the small intestine. Both Eudragit S100 and L100 coated capsules were intact after 4 hours immersion in SGF but released after transferring to Simulated Intestinal Fluid (SIF) solution, demonstrating excellent pH responsive property.

All the 4, 6, and 8 layers of Eudragit S100 coated capsules remained intact for at least the first 2 hours after being transferred to SIF. With or without pre-coating of HP55, the 6 and 8 layers of Eudragit S100 coated capsules remained intact even after 12 hours upon being transferred to SIF. This suggests that Eudragit S100 might not be suitable for intestinal targeted release as the release timeframe is beyond the absorption window, however it could be considered for colon targeted release.

The Eudragit L100 coated capsule dissolved within the first hour as the pH changed from 1.2 to pH 6.8; which would allow more time for absorption of the capsule content in the small intestine. All the 4, 6, and 8 layers of Eudragit L100 coated capsules released within 1st hour upon being transferred to SIF. With pre-coating of HP55, the release of 6 and 8 layers of Eudragit L100 coated capsules appeared to be

delayed by 1 and 2 hours, respectively. This suggests that with pre-coating of HP55 on Eudragit L100, it presents an optimal release timeframe of Rhodamine 6G loaded gelatin capsules.

Eudragit polymers belong to the family of poly(methyl methacrylate-acrylic acid) copolymers, with L100 bearing higher acrylic acid content than S100 (**Figure 9**). From **Table 2**, Eudragit L100 has a pK_a value of 6.45 ± 0.03 while Eudragit S100 has a slightly higher pK_a value of 6.66 ± 0.05 .^[3] As the pH increases above the pK_a , the polymers start to dissolve. It has been found that the dissolution pH increases as the molar percentage of acrylic acid in Eudragit polymer decreases.^[4] Barba, A.A., et al., reported a linear relationship between dissolution pH and volumetric percentage of methyl methacrylate in poly(methyl methacrylate-acrylic acid) copolymers as shown in **Figure 18**.^[5] This relationship could be possibility due to the decreased number of protonation sites as the acrylic acid content decreased, causing the polymer to dissolve at a higher pH.

Figure 18. Swelling and dissolution pH versus volumetric percentage of MMA in the copolymer. Adapted from ^[5]

As the HP55 protected Eudragit L100 coated capsules exhibited consistent results of releasing within the first hour in SIF, the immersion time in SGF was increased from 4 hours to 6 hours to investigate if the release was time or pH dependent. In **Figure 17(c)**, HP55 and Eudragit L100 coated capsule remained intact within the 6 hours in

SGF, and break release within the first hour upon being transferred to SIF. This confirmed that the release of coated capsule was due to the change in pH instead of immersion time.

4.4 Optimization of lyophilization conditions for liposomes

The 0.5% fluorescently tagged HSPC liposomes (HSPC (0.5% FL)) were fabricated by thin film hydration method, DLS measurement results for size, PDI and zeta potential were summarized in **Table 7** and **8**. The fabricated liposomes were homogeneously distributed with average PDI 0.136, average size 93 nm, and carrying a neutral charge. Liposomes in solution form have limited shelf-life and are prone to degradation by hydrolysis and oxidation. Lyophilization is a common way to preserve the shelf-life in the pharmaceutical and food industry. Samples are first allowed to freeze and subsequently transferred at its frozen state to a vacuum environment to allow sublimation of water to take place. During lyophilization, liposomes tend to be affected by the buildup of ice crystals which adversely affects the size when reconstituted in aqueous solution. Cryoprotectants such as sucrose and trehalose are often added to prevent water crystallization and protects the liposomes from fracture and rupture instability.^[6]

HSPC liposomes (20mM) were diluted to 2mM, 200 μ M and 20 μ M concentrations containing 0%, 1%, and 3% sucrose or trehalose. Liposomes were immediately lyophilized after freezing at -80°C overnight. After rehydrating in DI water, DLS characterization of the reconstituted liposomes showed that 2mM HSPC containing 2% sucrose recovered closest to the size before lyophilization, while a large variation in size and PDI was observed for other concentrations of liposome and cryoprotectants, indicating the insufficient protection of the liposomes during lyophilization.^[7] Zeta potential of the liposomes in the presence of cryoprotectants has also shown to be similar to the zeta potential before lyophilization (**Table 8**). The 2mM HSPC liposomes lyophilized with 2% sucrose produced reconstituted liposomes size of 104.1 ± 35.66 nm and PDI of 0.081, suggesting that the liposome is relatively stable during the lyophilization and is uniformly distributed upon reconstitution as shown in **Table 7**. Hence, 2mM lyophilized HSPC liposomes were loaded into the enteric coated capsules for subsequent release study.

Table 7. Effect of varying lipid concentration and cryo-protectant on the size of liposomes before and after lyophilization

Liposomes		Size (nm)				
		Sucrose			Trehalose	
		0%	1%	2%	1%	2%
HSPC Lipid concentration	2mM	-	151.9±47.48 PDI 0.404	104.1±35.66 PDI 0.081	-	-
	200µM	-	143.3±43.76 PDI 0.428	117.5±36.00 PDI 0.304	-	-
	20µM	190.5±93.90 PDI 0.442	206.9±36.36 PDI 0.684	-	298.4±103.3 PDI 0.702	338.1±197.0 PDI 0.492
HSPC (0.5% coumarin-6)		93.08 ± 34.31 PDI 0.136				

Table 8. Effect of varying lipid concentration and cryo-protectant on the zeta potential of liposomes before and after lyophilization

Liposomes		Zeta potential (mV)				
		Sucrose			Trehalose	
		0%	1%	2%	1%	2%
HSPC Lipid concentration	2mM	-	-7.41±3.98	-5.52±5.37	-	-
	200µM	-	-6.25±3.80	-3.22±3.55	-	-
	20µM	-8.89±15.8	-3.31±10.6		-4.22±13.2	-4.42±20.3
HSPC (0.5% coumarin-6)		-5.99 ± 6.71				

4.5 Release study of coated capsules loaded with lyophilized liposomes

Liposomes fabricated using the conventional thin film hydration method generated uniformly distributed large unilaminar vesicles with an average size of about 100nm. Based on the optimized lyophilization conditions, lyophilized 2mM liposomes recovered were closest to the original size before lyophilization thus were loaded into the enteric coated capsules and subjected to 4 hours release in SGF followed by SIF. Based on the rhodamine 6G loaded capsule release studies in **Figure 17**, which suggest that both Eudragit L100 and Eudragit S100 were able to protect the coated capsule from releasing in SGF, but Eudragit L100 had a more optimal dissolution time than Eudragit S100 upon being transferred to SIF, therefore further coating

optimization was focused on Eudragit L100 polymer. Gelatin capsules were first coated with 2 (H2) or 3 (H3) layers of HP55 before subsequent coating with 2 (L2) or 3 (L3) layers of Eudragit L100, generating H2L2, H2L3, H3L2, H3L3 enteric coated capsules. Coumarin-6 fluorescence calibration was used to estimate the concentration of released HSPC liposomes at various time points from the release study. Release study of the liposome loaded H2L2, H2L3, H3L2, and H3L3 capsules showed that all the coated capsules were able to resist degradation in SGF and break release within 20 mins upon being transferred to SIF, except for H2L2 coated capsules which released after immersing in SGF for 2 hrs, as shown in **Figure 19-20**.

Figure 19. Released capsules after 20mins in SIF. Close up of H2L2 and H3L3. Released capsules.

Figure 20. Release of lyophilized powder of coumarin-6 tagged HSPC liposomes from HP55 and Eudragit L100 coated gelatin capsules in SGF for 240 mins followed by SIF for 20 mins.

4.6 Characterization of capsule surface morphology and coating thickness by Scanning Electron Microscopy

The JEOL JSM-5410-LV scanning electron microscope (SEM) was performed to characterize the morphology and thickness of the coated capsules. Thickness of the coatings of H2L2, H2L3, H3L2, H3L3 enteric coated capsules was measured using ImageJ. In the SEM images shown in Figure, there is a distinct difference between the thicknesses of different layers of coatings observed in each of the cross-sections compared to the uncoated capsule. ImageJ software was used to measure the thickness of coatings and each measurement was an average of 3 random points from 3 different images summarized in **Table 9**. The body of gelatin capsule and the coating has been labelled in each SEM image. In **Figure 21B**, air bubbles were observed at the interface between capsule and coating. The coating also shows a different texture as compared to the bulk of capsule.

Figure 21. Scanning Electron Microscopic images of capsules with and without coating. **A.** Uncoated gelatin capsule, cross-section under different magnifications (1) x50 (2) x150 (3) x500. **B.** H2L2 coated capsules, cross-section under different magnifications (1) x30 (2) x160 (3) x270. **C.** H2L3 coated capsules, cross-section under different magnifications (1) x50 area 1 (2) x150 (3) x50 area 2. **D.** H3L2 coated capsules, cross-section under different magnifications (1) x50 (2) x150 (3) x270. **E.** H3L3 coated capsules, cross-section under different magnifications (1) x30 area 1 (2) x30 area 2 (3) x160.

Table 9. Total capsule coating thickness measured from SEM images

Capsule	Coating Thickness (μm)	$\pm$ SD
H2L2	90.73	4.79
H2L3	114.42	31.48
H3L2	116.52	3.4
H3L3	169.05	20.15

Figure 22. Coating thickness, measurements obtained from SEM images using ImageJ software * $P < 0.001$

The coating thickness measured from SEM was compared between capsules with different layers of coating. Data expressed were tabulated with one-way ANOVA and Student's t-test. As seen in **Figure 22**, H3L2 (5 coats) and H3L3 (6 coats) show significant difference (* $p < 0.001$) when compared with H2L2 (4 coats) coating. H2L2 (4 coats) and H2L3 (5 coats) coated capsules did not show significant difference in coating thickness.

Table 10. Correlating coating thickness with percentage liposome release from HP55 and Eudragit L100 coated gelatin Capsules

Cumulative percentage release (%)								
Capsule	Coating Thickness (μm)	SGF (pH 1.2)					SIF (pH 6.8)	
		0 min	60 min	120 min	180 min	240 min	250 min	260 min
H2L2	90.73 $\pm$	4.23 $\pm$	4.28 $\pm$	4.28 $\pm$	40.0 $\pm$	100 $\pm$	-	-
	4.79	3.79	3.78	3.79	37.5	31.4	-	-
H2L3	114.42 $\pm$	4.70 $\pm$	5.10 $\pm$	5.28 $\pm$	5.33 $\pm$	5.49 $\pm$	12.2 $\pm$	18.9 $\pm$
	31.48	3.82	3.81	3.86	3.82	3.90	1.56	6.47
H3L2	116.52 $\pm$	6.24 $\pm$	7.19 $\pm$	7.31 $\pm$	7.43 $\pm$	7.69 $\pm$	7.46 $\pm$	33.5 $\pm$
	3.40	3.84	3.95	3.91	3.92	3.85	1.25	4.07
H3L3	169.05 $\pm$	4.84 $\pm$	4.76 $\pm$	4.78 $\pm$	4.78 $\pm$	4.65 $\pm$	2.61 $\pm$	99.6 $\pm$
	20.15	4.04	3.79	3.81	3.83	3.84	1.00	35.1

The coating of 2-3 layers of HP55 followed by 2-3 layers of Eudragit L100 resulted in H2L2, H2L3, H3L2, H3L3 enteric coated capsules with coating thicknesses ranged from 90 μm to 169 μm . The enteric coated capsules were immersed in pH 1.2 SGF for 4 hours before being transferred to SIF pH 6.8. As shown in **Table 10**, a coating thickness of 90 μm possessed by H2L2 was insufficient to protect the capsules in SGF as the capsule released its contents within 3 hours in SGF. Increasing the coating thickness of either Eudragit L100 or HP55 as represented by H2L3 and H3L2, respectively, protected the capsules during the 4 hours of study in SGF and released within 20 mins after being transferred to SIF as compared to H2L2 capsules. A further increase in the coating thickness to 280 μm as represented by H3L3 coated capsules did not show any further delay in release compared to H2L3 and H3L2 capsules, indicating that 5 layers of coating is sufficient to protect the capsules and achieve the intended release in SIF. Hence, 114 μm coating thickness of H2L3 is the minimum required thickness for protection. H3L3 coating, which is about 169 μm , was also sufficiently strong for resistance to SGF during the release study. Therefore, minimal coating thickness is 114-169 μm . From this study, we can conclude that the optimal number of coating layers would be at least 5 coats, in order to minimize the number of coats for intestinal release.

From the percentage release table as shown above, the H2L2 coated capsules completely released its contents by 240 minutes in SGF and a spike in concentration was detected at 180 minutes, this shows that the coating is insufficient for gastric protection. However, H2L3, H3L2 and H3L3 coatings all showed concentration spikes only after the change to pH 6.8 SIF immersion. Furthermore, H3L3 coated capsules show nearly complete release of contents after 20 minutes of immersion, showing delivery to absorption site whilst resisting acid environment. ^[8]

4.7 Discussion

The dip-coating parameters were optimized to obtain the minimum coating thickness required for intestinal targeted release in SIF. Lyophilized liposome powder was loaded in the capsules for the simulated release study. Liposomes made from saturated lipid HSPC was selected for the study because of its higher transition temperature (55°C) and rigidity in its lipid structure, thus offering better stability at physiological temperature (37°C). Sucrose and trehalose were studied for their applications as cryoprotectants to prevent damage of liposomes during the lyophilization process and to ensure recovery of size upon reconstitution in water.^[6] Sucrose appeared to be more effective in protecting HSPC liposomes and maintaining its structure during lyophilization since the reconstituted size was closer to the original liposome size before lyophilization as compared to trehalose (**Table 7**). The optimum condition for lyophilizing HSPC liposomes were shown to be occurring at 2mM HSPC containing 2% sucrose, which demonstrated the best size recovery with only less than 7nm increase in size after being reconstituted. The lyophilization could prolong the storage shelf life of liposomes for its application in the pharmaceutical industry. From the release study of enteric polymer coated capsules, Eudragit L100 with 2 layers of pre-coated HP55 displayed the desirable results of protecting the capsule in SGF and releasing its contents in the proximal part of the intestine, the duodenum or jejunum where the pH gradually increases to pH 6-7 due to the pancreatic juice. The capsules coated with 4, 6 or 8 layers of Eudragit L100 as well as the capsules coated with 2 layers of HP55 and 4 layers Eudragit L100 coated released within the first hour in SIF which would give the maximum time for the nanocarrier to be absorbed along the intestinal wall. On the other hand, capsules coated with same layers of Eudragit S100 released over 12 hours, which far exceeded the intestinal

transit time, hence would be more suitable for colon targeted delivery. From the SEM images in **Figure 21B**, air bubbles were observed at the interface between gelatin capsule surface and coating. The air bubbles could have originated during the dip-coating process and trapped by the coating layers as the air escape from inside the capsule during drying. This defect could be minimized by first applying a seal on the connecting part of the capsule before dip-coating.

4.8 Conclusion

The pH responsive polymers HP55, Eudragit S100, and Eudragit L100 were evaluated for their effectiveness as enteric coatings on gelatin capsules via *in vitro* release studies in SGF and SIF. The polymers were coated onto the capsule surface through repeated cycles of dipping and drying. Gelatin capsules coated with varying number of Eudragit S/L coating layers in the presence and absence of HP55 pre-coating were loaded with Rhodamin 6G dyes and subjected to release study for selecting the between Eudragit S100 and Eudragit L100 for intestinal targeted release. Generally, Eudragit S100 coated capsules had longer dissolution times as compared to Eudragit L100 coated capsules. It was also noted that HP55 polymer delayed the dissolution time for Eudragit L100 coated capsules. The Eudragit L100 polymer having a lower dissolution pH ^[9, 10] was more effective for the intended application compared to Eudragit S100, which took longer time to break and release the content in SIF. By correlating the thickness measured from SEM images and the *in vitro* release study, a total coating thickness (HP55 and Eudragit L100) of at least 114 μ m was required to prevent capsule dissolution in acidic pH and to break release within 20 minutes in SIF conditions.

4.9 References

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Chapter 5

Liposome assisted insulin transport across Caco-2 monolayer

This chapter describes the experimental results from investigation of liposome and cellular interaction. A panel of liposomes fabricated from different lipids was compared for their ability to associate/retain on the cell surface. Areas including particle fabrication, parameter optimization, loading and encapsulation, cellular uptake were investigated and will be discussed in this chapter. Rigid and charged HSPC/DOTAP liposomes demonstrated the highest cellular association compared to other liposomes of different lipids thus was selected for subsequent studies in this thesis. Surface modification of liposomes using Tween 80 and Eudragit S100 was also investigated for enhancing the transport across intestinal epithelial cells. Human epithelial colorectal adenocarcinoma cells (Caco-2) was selected as a model cell line for testing the transport of surface modified liposomes. Eudragit S100 was layered on the surface of HSPC/DOTAP liposomes and sterically stabilized with the help of Tween 80.

5.1 Introduction

Liposomes are biocompatible and biodegradable vesicles self-assembled by amphiphilic phospholipids to enclose an aqueous core within the phospholipid bilayer. In addition to its ability to compartmentalize both hydrophilic and hydrophobic drugs to improve their solubility and stability, the size is tunable and the surface served as a base for diverse modification.^[1] Early attempt to encapsulate insulin in liposome for oral application achieved promising results in 1976, the encapsulated insulin was able to lower blood glucose level when administered orally in a diabetic rat.^[2] Although liposomes were shown by various studies to be capable of protecting the encapsulated insulin, the *in vivo* bioavailability still remained low due to limited permeation across the intestinal epithelial cells. In this study, liposomes of different lipids having different properties will be compared for their ability to interact with intestinal epithelial Caco-2 cells. Insulin loading capability will be studied for the identification of potential liposomal carrier as candidate for oral insulin delivery.

Various ways have been developed to enhance absorption including the incorporation of targeting ligands (eg. cell penetrating peptide)^[3] to initiate uptake, modifying the surface charge for better mucoadhesion, or the use of permeation enhancers such as sodium caprylate and bile salts. However, ligand functionalization is often time-consuming and requires multistep synthesis. Mucoadhesive carriers generally release the insulin locally in the mucus, which can be aided by co-delivery of permeation enhancers to reversibly open intercellular tight junctions to promote paracellular transport of insulin.^[4] Although residence time of the particle could be improved, there is still high chance of drug degradation as the protein makes its way to the cell surface, where brush border enzymes are found. Furthermore, prolonged exposure to conventional permeation enhancers could potentially damage the intestinal lining, resulting in local inflammation.^[5] There is a need to design a simple, effective and safe carrier that can achieve enhanced absorption.

One of the simplest ways to improve the performance of liposome is through surface modification with biocompatible polymers via electrostatic interaction. Takeuchi et al. (1996) reported a 12 hours sustained glucose lowering effect with insulin loaded chitosan coated liposome in rats.^[6] In another study, oral administration of chitosan-

aprotinin coated multilamellar vesicles (MLVs) significantly improved the pharmacological effect of the loaded calcitonin compared to calcitonin in solution.^[7] pH sensitive anionic carboxylated polymers such as acrylic and methacrylic acid (MAA) copolymers are potentially membrane-destabilizing agents which undergo transition from coil to globule conformation when experiencing a decrease in environmental pH.^[8] This property enables the protection of endocytosed cargo from lysosomal degradation, making them good candidates as excipients and coating material for intracellular delivery.^[9] With the help of anionic polymers, endocytosed liposomes could potentially be transcytosed across the intestinal epithelial cells, overcoming the permeability barrier.

Although the use of anionic polymers to overcome permeability barrier is promising, the majority of the studies have been focused on its application for intracellular delivery of fragile biomolecular drugs such as DNA,^[10] RNA, proteins and peptides,^[11] and there is lack of concrete evidence to show its effect in enhancing the uptake and transcytosis of drug loaded liposomes. Poly(ethyl acrylic acid) (PEAA) and poly(propylacrylic acid) (PPAA) have been previously shown to disrupt cell membrane and hemolyzes red blood cells at acidic pH, while no haemolytic activity was found at physiological pH.^[12, 13] Covalent conjugation of PEAA onto liposome surface caused membrane fusion at physiological pH and subsequent release of encapsulated contents into the cytosol under acidic conditions.^[14] Eudragit S100 is a commercially available copolymer of methyl methacrylate and methacrylic acid (PMAA-co-MAA) that is insoluble at acidic pH, and is therefore widely utilized as a protective capsule coating for bypassing stomach acidity, allowing it to release its drug load in the intestines.^[15, 16] Applying this concept to nanocarriers, M Bareaa et al. (2010) have coated liposomes with pH responsive polymer Eudragit S100 for enteric targeting.^[17] Although the coating prevented initial burst release of liposomes, the micron sized MLVs were too large to be taken up by the epithelial cells and transported across the intestinal epithelium via the transcellular pathway, which is the main pathway for most nanocarriers.^[18] Furthermore, the fabrication method involved the use of organic solvent (acetone) which may degrade protein and peptide drugs and complete removal is often difficult. Nevertheless, there was no comprehensive study on whether Eudragit S100 can enhance the transport and uptake of nanoliposomes in Caco-2 cells.

The objective of the current study is to investigate the ability of Eudragit S100 to enhance uptake and transport of insulin loaded liposomes across intestinal epithelial cells (Caco-2). Eudragit S100 was coated onto 6 different types of fluorescently labelled positively charged liposomes, percentage lipid transported and accumulated with cells were fluorescently quantitated and compared with uncoated liposomes. The amount of Eudragit S100 was varied to generate liposomes with different coating thickness, and liposome size was precisely controlled to be below 500nm for optimal uptake.^[19] The transport of insulin loaded Eudragit S100 coated HSPC liposomes in the presence and absence of free Eudragit S100 was measured using enzyme-linked immunosorbent assay (ELISA). The effects of free Eudragit S100 in the solution and as a coating on the liposome surface on transport across Caco-2 were assessed.

5.2 Objectives

This study focuses on improving insulin encapsulation and surface modification of the liposomes which have been identified as a promising candidate for oral insulin delivery. The objectives of the project are as follow:

1. To investigate the insulin encapsulation and loading efficiencies using different rehydration methods and different lipid structures
2. To study the effects of Eudragit S100 coating on the uptake and transport of insulin-loaded liposomes across Caco-2 cells *in vitro*

Figure 23. Schematic diagram illustrating the objective of chapter 5. Coating Eudragit S100 polymer onto liposomes for enhancing its transport across intestinal epithelium.

5.3 Transport study of fluorescently tagged empty liposome

The brightfield microscopic images in **Figure 24A-C** represent the morphological changes of Caco-2 cells during the development on the glass coverslip. The cells changed from rounded and floating (day 0), to attached and spread out (day 9), and to the fully developed monolayer with obvious borders (day 30) as seen in **Figure 24A-C**. The tight junctional protein claudin-1 was immunostained with anti-claudin-1 antibodies after 14 days of culturing on glass cover slip **Figure 24D**.

Figure 24. Characterization of Caco-2 monolayer development by bright field microscopy at (A) day 0, (B) day 9, and (C) day 30 post seeding. (D) Immunostaining of Caco-2 cells cultured on glass coverslip. Green represents Claudin-1, Blue represents DAPI staining.

5.3.1 Association of liposome with caco-2 cells

To identify a potential liposomal carrier for oral delivery, the effectiveness of liposomal interaction with intestinal epithelial Caco-2 cells was studied between lipids of different charge, acyl chain length, and cholesterol content *in vitro*. DMPC, DPPC, HSPC has 14, 16, and 18 carbon in the saturated hydrocarbon lipid tail. EPC has the same lipid chain length as HSPC but with a degree of unsaturation in the hydrocarbon lipid tail. Effect of cholesterol content and charge on cellular interaction was studied

on HSPC liposomes incorporated with 10 mol %, 30 mol %, 60 mol % cholesterol and 10 mol% DOTAP and 10 mol% DPPG lipids. All liposomes were incorporated with 0.5 mol % Coumarin-6 during fabrication to allow visualization under fluorescence microscopy. The fabricated liposomes were characterized by DLS for its size, PDI, and zeta potential before the study and the results were summarized in **Table 11**. The fabricated liposomes after extruding with 0.08 μ m membrane ranged in size from 89 nm to 108 nm. The zeta potential were neutral for all the liposomes made from pure phosphatidylcholine (PC) while positively charged when incorporated with 10mol% DOTAP and negatively charged when incorporated with 10mol% DPPG. PDI were below 0.126 indicating that the synthesized liposomes were homogeneously distributed.

The difference between the two lipids lies in the alkyl chain length and double bond content. Liposomes fabricated with EPC are more flexible because the two lipid tails are of different length, one having 18 carbon, the other having 16. Furthermore, the double bond in the 18 carbon chain causes a kink in the lipid tail that makes the bilayer more fluidic in nature. On the other hand, HSPC has two 18 carbon alkyl chains attached to the polar head group. This means that the liposomes fabricated with this type of lipid are more rigid and denser than EPC liposomes at the same temperature. In a normal somatic body cell membrane, the saturated and unsaturated phospholipids are present as a mixture and the ratio between these two determines the fluidity of the membrane.

Table 11. Size, zeta potential and PDI of different types of liposomes used for investigation of cellular interaction

	PDI	Size (nm)	Zeta potential (mV)
DMPC	0.101	89 $\pm$ 33.11	-2 $\pm$ 6.54
DPPC	0.099	89.3 $\pm$ 37.17	-0.9 $\pm$ 5.44
EPC	0.067	102.4 $\pm$ 30.68	0.1 $\pm$ 4.87
HSPC	0.056	113.6 $\pm$ 33.11	-0.7 $\pm$ 5.7
HSPC 10% cho	0.073	108.4 $\pm$ 28.7	-1.9 $\pm$ 4.8
HSPC 30% cho	0.034	105.9 $\pm$ 27.52	-1 $\pm$ 6.34
HSPC 60% cho	0.049	108.4 $\pm$ 26.88	-5.7 $\pm$ 7.86
HSPC DOTAP	0.126	107.4 $\pm$ 38.23	36.3 $\pm$ 4.66
HSPC DPPG	0.074	108.1 $\pm$ 33.54	-40.9 $\pm$ 8.29

Nano DSC was performed on all the fabricated liposomes to study the phase transition temperature (T_m) and thermodynamic behaviors. Comparing the thermogram between HSPC incorporated with DOTAP and DPPG, it can be seen that both T_m and ΔH of the HSPC liposomes decreased when either was incorporated **Figure 25a**. This could be due to the repulsion between the charged lipid head groups that disrupted the packing of the lipid membrane, introducing freedom and flexibility which in turn lowered the T_m of liposomes. Cellular interactions studied by fluorescence microscopy showed that this change in membrane fluidity was not significant as compared to the surface property of the liposomes. Positively charged HSPC/DOTAP liposomes were able to have a stronger interaction with Caco-2 cells compared to neutral and negatively charged liposomes. This is because the cell surface is negatively charged due to the abundance of sugar and glycoprotein moieties which could attract the oppositely charged HSPC/DOTAP better than neutral HSPC and could possibly repel the negatively charged HSPC/DPPG liposomes. Flow cytometry analyzed the liposomes association and reflected as a peak shift in the fluorescence intensity measured. Cells treated with HSPC/DOTAP and neutral HSPC showed a bigger peak shift in the flow cytometry data and stronger fluorescence signal compared to HSPC/DPPG as shown in **Figure 25b-c**.

Figure 25. (a) Nano Differential scanning calorimetry (NanoDSC) analysis of the effect of charge on liposome T_m . (b)-(c) Study of effect of charge cellular uptake by (b) flow cytometry and (c) fluorescence microscopy

Table 12. Thermodynamic parameters of HSPC/DPPG, HSPC/DOTAP, and HSPC liposomes characterized by nanoDSC

Models			
	Model	Variable	Value
HSPC/DPPG	TwoStateScaled	Aw	1.0001
		T _m (°C)	51.9049
		dH (kJ/mol)	1351.01
HSPC/DOTAP	TwoStateScaled	Aw	0.99993
		T _m (°C)	50.0239
		dH (kJ/mol)	795.612
HSPC	TwoStateScaled	Aw	0.9999
		T _m (°C)	52.7519
		dH (kJ/mol)	1466.13

The thermographs between lipids of different carbon chain lengths were compared between HSPC, DMPC, and DPPC liposomes in **Figure 24a**. The T_m increased as the carbon chain length increased from DMPC (C14), to DPPC (C16), to HSPC (C18). As the lipid chain length increased, the hydrophobic interaction between the lipid chain increased, and this means that more energy is required to break the molecular interactions and make the lipids mobile as they transform from gel to liquid phase, thus an increased T_m was observed. EPC (C18) on the other hand, has a negative T_m of −15 °C to −7 °C which is below the lower measurement limit of nano DSC, due to the presence of double bonds in its lipid tail.^[20] Comparing the cellular interaction studied by fluorescence microscopy and flow cytometry, liposomes with increased T_m showed increased cellular interaction (**Figure 24b-c**). Notably, the fluorescence intensity was much lower as observed in EPC compared to HSPC. Despite that both lipids have the same chain length; the presence of double bonds increased the flexibility of the membrane, which could be the possible reason for the decreased cellular association. Between DMPC, DPPC, and HSPC, enhanced cellular association was observed as the lipid chain length increased, this could be due to the increasing rigidity of the liposome. This phenomenon has been reported by various groups^[21-23], Shi and Jiang et al. investigated the cellular uptake of lipid-coated poly(lactic-co-glycolic) acid (PLGA) nanoparticles, and they reported that rigid lipid-coated PLGA NPs enter cell more efficiently than soft ones.^[24] The reason that rigid particles enhance endocytosis more efficiently than softer ones is because lower adhesion energy was required to achieve full membrane wrapping around the

nanoparticle upon binding onto cell surface, resulting in a higher binding rate and lower dissociation rate compared to the softer nanoparticles.^[22, 23]

Figure 26. (a) Nano Differential scanning calorimetry (NanoDSC) analysis of the effect of lipid chain length on liposome T_m . (b)-(c) Study of effect of lipid chain length on cellular uptake by (b) flow cytometry and (c) fluorescence microscopy

Table 13. Thermodynamic parameters of DPPC, DMPC, and HSPC liposomes characterized by nanoDSC

Models			
	Model	Variable	Value
DMPC	TwoStateScaled	Aw	0.9959
		T _m (°C)	24.3406
		dH (kJ/mol)	2000
DPPC	TwoStateScaled	Aw	1.00305
		T _m (°C)	40.8872
		dH (kJ/mol)	1471.69
HSPC	TwoStateScaled	Aw	1.00377
		T _m (°C)	52.7632
		dH (kJ/mol)	1490.68

Given that HSPC showed the highest fluorescence signal among the groups of lipids with different carbon chain length and charge, further study was conducted to determine the effect of cholesterol on HSPC liposome cellular interaction. The thermograms of HSPC liposomes incorporated with 10mol%, 30mol%, and 60mol% cholesterol is shown in **Figure 26a**. It is clearly seen that the increasing cholesterol content from 0 to 60% broadened the phase transition peak and lowered the amount of heat required to change the phase from gel to liquid from 1443.3kJ/mol to 184.6kJ/mol. Cholesterol is known to inhibit phase transition by creating an additional liquid ordered phase (L_o) at which shares the characteristics of both solid ordered (S_o) and liquid disordered (L_d) phase (**Figure 25**).^[25] In this phase (L_o), the lipid membrane has more lateral packing order than L_d phase but more rotational freedom than in the S_o phase. The addition of cholesterol into a lipid membrane at S_o phase would disturb the lateral triangular lattice and increase its permeability. On the other hand, the rigidity would be increased when cholesterol is intercalated between flexible and mobile lipids at L_d phase, favoring a more closely packed trans-isomer form of the lipid tail.^[26] As a result, the lipids at L_o phase is capable of both lateral and rotational diffusion like in the L_d phase but conformational similar to S_o phase.^[27, 28] Given that the T_m of HSPC liposome is at 52.7 C° and the Caco-2 cells study was conducted at 37C°, lower than the T_m, the presence of cholesterol would decrease the liposomal membrane rigidity and therefore decrease the efficiency of endocytosis. This is supported by the observation from the cellular interaction studied by fluorescence microscopy and flow cytometry in **Figure 26b-c**. A reduction in fluorescence signal

from cellular associated liposome was observed when cholesterol content in the liposome lipid bilayer increased from 0% to 30%. However, no further reduction in fluorescence signal was observed when cholesterol increased from 30% to 60%, suggesting the possibility of reaching the lower limit below which further reduction in rigidity would not affect liposome cellular interaction.

Figure 27. Schematic representation of different physical states adopted by a lipid bilayer in aqueous medium. Adapted from Ref^[29]

Figure 28. (a) Nano Differential scanning calorimetry (NanoDSC) analysis of the effect of cholesterol on liposome T_m . (b)-(c) Study of effect of cholesterol on cellular uptake by (b) flow cytometry and (c) fluorescence microscopy

Table 14. Thermodynamic parameters of HSPC 60% cholesterol, HSPC 30% cholesterol, HSPC 10% cholesterol, and HSPC liposomes characterized by nanoDSC

Models			
	Model	Variable	Value
HSPC 60% cholesterol	TwoStateScaled	Aw	0.98186
		Tm (°C)	52.7029
		dH (kJ/mol)	184.6
HSPC 30% cholesterol	TwoStateScaled	Aw	1.0906
		Tm (°C)	51.8991
		dH (kJ/mol)	364.02
HSPC 10% cholesterol	TwoStateScaled	Aw	1.00045
		Tm (°C)	51.9806
		dH (kJ/mol)	1411.62
HSPC	TwoStateScaled	Aw	1.00161
		Tm (°C)	52.7439
		dH (kJ/mol)	1443.3

5.3.2 Insulin characterization

Circular dichroism Conformation (CD) was performed to analyze the folding and conformation of insulin in a buffered solution. A plane of polarized light consisting of 2 rotating circularly polarized component of equal magnitude, one rotating counter-clockwise (L) and one rotating clockwise (R). In CD spectrometry, a plane polarized light is split into its L and R components by passing through a modulator experienced with an alternating electric field, detector measures the difference in absorption by the sample between L and R circularly polarized light and is represented as degree of ellipticity (Θ mdeg).

CD is widely used in the study of structural changes of protein and nucleic acid (helical structure), whose conformation is affected by temperature, concentration, and the chemical environment. The far UV region (240nm and below) measures the absorption due to peptide bond of protein, which reflects its secondary structure such as α -helix, β -sheets, or random coil. The near UV region (250nm and above) measures the adsorption due to the tertiary structure of protein contributed by aromatic amino acids such as Tryptophan (290-305nm), Tyrosine (275-282nm), and Phenylalanine (255-270nm).

The conformational study of human recombinant insulin was studied by CD to find out the effect of different preservatives on the secondary structure of monomeric

insulin in buffered solution. Various preservatives including mercaptoethanol, glycerin, phenol, and M-cresol were each added to sodium phosphate dibasic buffered (Na_2HPO_4) insulin solution, and its CD absorbance was studied in **Figure 29**. The CD spectra of insulin in Na_2HPO_4 reflect the native structure of insulin due to the presence of α -helix peaks at 203 nm and 222 nm. The addition of any preservatives including Mercaptoethanol, glycerin, phenol, and M-cresol resulted in a loss of characteristic α -helix peaks of insulin, suggesting a change in the conformation of the protein. Mercaptoethanol could stabilize insulin in monomer form while preventing the formation of dimer, trimer, or hexamer form. However, it functions as a reducing agent at the same time that may break the disulphide linkage between cysteine residues, causing change in insulin conformation^[30] The addition of phenolic reagents such as phenol or m-cresol, associates itself with insulin via the phenolic binding sites which involves hydrogen bonding between the hydroxyl group of phenol group and the nitrogen and oxygen of the amino acids, promoting the stabilization of insulin in its hexameric form in the presence of Zn^{2+} .^[31] In the absence of Zn^{2+} , these phenolic compounds may induce change in secondary structure by disrupting the stabilizing hydrogen bonding.^[32] To preserve the insulin in its monomeric form and in native conformation, none of the preservatives were added in the preparation of insulin solution for loading of liposomes.

Figure 29. Conformational study of insulin formulated in different buffer compositions by Circular Dichroism. An α -helix have negative peaks at 222 nm and 208 nm and positive peaks at 190 nm, while β -sheets have negative and positive peaks at 218 nm and 198 nm respectively. Random coil have positive and negative peaks at 212 nm and 195 nm respectively.^[33]

5.3.3 Thermal properties of insulin

The thermal stability of insulin was studied by nanoDSC to determine the acceptable temperature range used for rehydration of liposomes based on the measurement of heat capacity change of the protein at constant pressure (ΔC_p). The thermograph in **Figure 30** shows the thermodynamic characterization of the unfolding process of insulin. As the temperature increased from 0°C to 90°C, 504.076 kJ/mol of heat was absorbed by the protein causing it to unfold at temperature range between 60°C to 70°C, as represented by the endothermic peak at about 65°C. This indicates that thermal denaturation of insulin occurs at 65°C, at which the enthalpy increases due to the absorption of energy, causing the disruption of weak hydrogen bonding and the unfolding of the protein's secondary structure. From the nanoDSC data, it suggests that insulin is stable at the rehydration temperature at or below 60°C.

Figure 30. Thermogram of human recombinant insulin studied by nano DSC.

Table 15. Thermodynamic parameters of insulin characterized by nanoDSC

Models		
Model	Variable	Value
TwoStateScaled	Aw	1.00148
	Tm (°C)	64.8275
	dH (kJ/mol)	504.076

5.3.4 Optimization of RP-HPLC method for insulin quantification

High Performance Liquid Chromatography (HPLC) is a widely used method to separate, identify, and quantify analytes in a mixture. Two main types of HPLC are commonly used for separation of analytes namely, normal phase HPLC (NP-HPLC) and reverse phase HPLC (RP-HPLC), based on the type of interactions between analyte and stationary phase. A polar stationary phase is used in NP-HPLC with non-polar mobile phase carrying the analyte with the ability to engage in polar interactions such as hydrogen bonding or dipole-dipole interactions. The analyte gets retained by the stationary phase due to higher affinity and gradually eluted out as the polarity of the solvent increase in a gradient elution method. On the other hand, a non-polar stationary phase is used in RP-HPLC for analyzing analytes that are less polar. The analyte gets elute out when the hydrophobicity of the mobile phase is increased. A C18 column consisting of porous silica beads with silane derivatives was used as a stationary phase for capturing insulin.^[34] During sample injection, insulin carried by mobile phase with high polarity tends to be retained by the stationary hydrophobic

C18 column. During sample elution, as the organic solvent component of the mobile phase increases and polar component of the mobile phase decreases, insulin which initially retained by the C18 column gradually gain affinity to the relative more hydrophobic mobile phase and the protein is eluted out.

The gradient elution method was optimized by varying the experimental runtime and altering the change in solvent composition to reduce the retention time of insulin and improve the resolution of peaks. As the runtime decreased from 30 min to 15 min, the retention time of insulin decreased from 13.5 min to about 9 min as solvent hydrophobicity increased from 10 to 90% (**Figure 31a**). The retention time was further reduced to 6.7 – 7 min by adopting a narrower range of gradient from 20% to 80% over 10 mins (**Figure 31a**). Both reduced runtime and shortened gradient range make the solvent composition to reach the eluting condition faster, thus reducing the retention time of insulin. The calibration chromatograms of insulin are created following a 0.6x serial dilution starting from 0.6mg/ml shown in **Figure 31b**, and the area under the curves was plotted against its corresponding concentration and shown in **Figure 31c**. The detection range established from the standard curve ranged between 0.01mg/ml to 0.6mg/ml.

Figure 31. HPLC method development for human recombinant insulin. (a) spectra peaks for linear gradient from 10-90% 0.1% TFA in ACN over 30 min (grey), 15 min (orange) and linear gradient from 20-80% 0.1% TFA in ACN over 10 min (blue). (b) Standard chromatograms of insulin at different concentrations, (c) Calibration curve for insulin with a linear relation of insulin concentration vs. peak area

5.3.5 Effect of lipid structure on loading and encapsulation efficiency

Loading of hydrophilic drugs is commonly introduced at the vesicle formation step during thin film rehydration. The hydrophilic insulin molecules would ideally be entrapped within the liposome aqueous core as the lipid layers self-assembles into a spherical vesicle. However, the loading of hydrophilic drugs is normally low because of the lower volume of liposome aqueous core compared to the external aqueous environment. Furthermore, the surface-associated drugs tend to make lipid bilayer curve inwards during vesicle formation. As a result, majority of the drugs either

situated on the outer surface due to the larger exterior surface area or remain untrapped in the outer aqueous environment rather than inside the aqueous core.^[35]

To improve drug loading, progressive rehydration was adopted for loading of insulin. Progressive rehydration is a slow rehydration process that introduces hydrophilic drugs step by step at a smaller volume over an extended period. The rationale behind progressive rehydration was to create a higher initial lipid concentration to allow higher chance of interaction with drug and more opportunities for drugs to stay inside the aqueous core. The initial small rehydration volume increased the volume ratio of liposome aqueous core to external aqueous environment as compared to that of direct rehydration.

Loading and encapsulation was studied by HPLC in an indirect method whereby the insulin concentration in the supernatant was quantified and used to calculate the loaded insulin by comparing to the total insulin loaded into the system. The Encapsulation efficiency EE (%) and Loading efficiency LE (%) between lipids of different carbon chain lengths showed no obvious correlation between the lipid structure and entrapment efficiencies as observed in **Figure 32**. This could be due to the hydrophilic nature of insulin, it is less likely to be affected by the thickness in the hydrophobic lipid bilayer.^[36] The structure of lipid acyl chains will affect the loading of hydrophobic drugs because the entrapment of the drug is within the lipid bilayer due to hydrophobic interactions with the lipid acyl chains.^[37] On the contrary, the loading of hydrophilic drugs is more likely to be affected by the charge of the lipid head groups due to the possible interactions between the charged lipid head groups and charged groups of the hydrophilic drug, as proposed by Colletier et al.^[37] Comparing the loading and encapsulation between positively charged HSPC/DOTAP, negatively charged HSPC/DPPG and neutral HSPC liposomes in **Figure 32**, it can be seen that liposomes fabricated with positive charge had the highest LE 0.48% and EE 58%, followed by negatively charged HSPC/DPPG with LE 0.15% and EE 21.8%, and neutral HSPC liposomes with LE 0.14% and EE 13.7%. This could be due to the electrostatic interactions between the charged R groups of the amino acids in insulin and the charged head groups of the lipids. As the insulin is dissolved in SPD buffer with pH adjusted to 7, above the pI of the protein, thus it would be slightly negatively charged and would tend to interact more with the positively charged HSPC/DOTAP liposomes. Comparing the loading and encapsulation between HSPC liposomes

fabricated with increasing cholesterol content, it can be seen that increased cholesterol content correlates to increased loading and encapsulation. This could be because of the buffering effect of the cholesterol in the liposomes. Cholesterol acts as a temperature buffer that increase or decrease the membrane rigidity depends on the environmental temperature. Insulin was loaded into the liposomes during thin film rehydration at a temperature above the T_m of lipids, at which the lipids are more mobile and flexible. Presence of cholesterol would tend to reduce the flexibility of the liposome and make it more rigid. This is because the rigid planar structure hinders the motion and decreases the trans-gauche isomerization of the hydrocarbon chain, thus decreasing the membrane permeability.^[38, 39] Studies have reported that the presence of cholesterol decreased the permeability of Na^+ , K^+ , Cl^- and glucose across liposomal membrane at 36°C regardless of the surface charge and headgroups of lipids.^[40, 41] This could correlate to the increased loading and encapsulation observed when cholesterol is increased from 10% to 60% in HSPC liposomes in **Figure 32**.

Figure 32. Loading and encapsulation of different types of liposomes

5.3.6 Effect of Eudragit S100 on transport of different types of liposomes

Transport studies were done on caco-2 cells grown on inserts in 12-well plates after 21 days for studying the transport of Eudragit S100-coated liposomes. Normalized TEER values increased from 1.5 to 635 ohm.cm^2 during 30 days post seeding, indicating the formation of monolayer and intercellular tight junctions (**Figure 33a**). The transport study was performed on cells with normalized TEER values above 300 ohm.cm^2 . In a parallel study, immunostaining of the monolayer cultured on glass

coverslip with anti-claudin-1 antibodies confirmed the expression of tight junction and the integrity of the monolayer before transport study (**Figure 33a**).

To study the effect of Eudragit S100 on the transport of empty liposomes, lipids of different carbon chain length and degree of saturation were used to fabricate liposomes with different physiochemical properties. The 18C unsaturated lipids EPC and SPC serves as a direct comparison between its saturated form HEPC and HSPC for the effect of rigidity on transport, since liposomes of saturated lipids lack of double bonds in the lipid bilayer is less permeable and more rigid in its structure. The comparison between 18C HSPC, 16C DPPC and 14C DMPC helps to understand the effect of different carbon chain length on the transport of liposomes. DOTAP was incorporated into the liposomes during fabrication to render the surface a positive charge for subsequent surface modifications. Coating of 6 different types of liposomes (EPC/DOTAP, HEPC/DOTAP, SPC/DOTAP, HSPC/DOTAP, DPPC/DOTAP, DMPC/DOTAP) was achieved via electrostatic interaction between negatively charged Eudragit S100 polymer and positively charged DOTAP liposomes in Tween 80 stabilized solution. Eudragit S100 coating of liposomes generally increased the hydrodynamic radius of liposomes by 14-191nm. The variation of coating thickness could be due to the difference in lipid chain structure which in term affects polymer adsorption. All the coated liposomes revealed increased hydrodynamic radius while decreased zeta potential as measured by DLS (**Figure 33b-c**), indicating the successful modification of the liposome surface.

The results of transport study revealed an interesting trend; cells treated with all 6 coated liposomes exhibited significantly higher lipid transport and lower lipid association with cells, when compared to uncoated liposomes (**Figure 33d-e**). TEER values recorded throughout the transport experiment showed a general decreasing trend for the coated liposomes while an increasing trend for the uncoated liposomes except for the drop at 4th hour which could be due to long hours of incubation in medium free environment (**Figure 33f-g**), observed also in HBSS control experiment in **Figure 34g**. This may suggest that the Eudragit S100 polymer, either as liposomal coating or existing as the excess free polymer in solution, played a role in disrupting tight junctions of the Caco-2 cells, which could in turn facilitate the transport of liposomes across the cells. Highest percentage transport and percentage cellular association was observed in rigid HSPC/DOTAP (5x improved cellular association)

and HEPC/DOTAP (12x improved transport) liposomes. Rigid nanoparticles have been reported by various studies regarding their capability to initiate cellular uptake more efficiently than soft ones.^[21-24] In addition to rigidity, particle charge and size also have profound influence in triggering cellular uptake. Cell membrane is slightly negatively charged because of the phospholipid head groups and the presence of glycans on the transmembrane proteins. Cationic particles are therefore preferred over anionic particles for interacting with the cell surface via electrostatic attraction.^[42] Oral bioavailability was only observed when liposome size reduced from 2 μm to 400 nm, further reducing the size of particle from 400 nm to 80 nm resulted in shortening of the duration of hypoglycemic effect from 24 h to 4 h.^[43, 44] Based on the results from transport and cellular association studies, both HSPC/DOTAP and HEPC/DOTAP could be considered for further investigation but HSPC/DOTAP was selected as a model carrier due to its overall performance in terms of particle size, charge, and rigidity. The observations from this transport study suggest that Eudragit S100 could have been involved in enhancing transport of liposomes.

Figure 33. Establishment of Caco-2 monolayer on transwell inserts. DLS characterization of 6 types of liposomes comprising 10 mol% DOTAP, EPC/DOTAP, HEPC/DOTAP, SPC/DOTAP, HSPC/DOTAP, DPPC/DOTAP, and DMPC/DOTAP with and without Eudragit S100 coating. Transport study of the 6 types of empty liposomes with and without Eudragit S100 coating on Caco-2 monolayer. (a) TEER reading of Caco-2 cells cultured on transwell inserts recorded throughout a period of 30 days. Insets show immunostaining of Caco-2 cells cultured on glass coverslip. Green represents Claudin-1, Blue represents DAPI staining. (b) Size of the 6 types of liposomes with and without Eudragit S100 coating. (c) Zeta potential of the 6 types of liposomes with and without Eudragit S100 coating. (d)-(e) Percentage of lipids from the liposomes transported (d) and associated (e) with cells. TEER reading of uncoated (f) and coated (g) liposomes recorded during transport study. The asterisk represents significant difference, determined by *t*-test (* $P < 0.05$) relative to uncoated liposomes.

5.3.7 Investigation of the factor causing transport of liposome

HSPC/DOTAP was chosen as a model carrier for subsequent transport study because of its rigidity, smaller size, and higher zeta potential after surface modification. To study the factor causing the enhanced transport, further transport studies were performed with HSPC/DOTAP liposomes. During the coating of HSPC/DOTAP liposomes with Eudragit S100, Tween 80 was used as a stabilizer to prevent liposome aggregation. The trend observed during transport of different types of liposomes suggested a potential role that Eudragit S100 could have played in promoting the transport of liposomes. To find out which factor in the formulation was responsible for such enhancement in transport, ELISA was used to quantify the amount of insulin transported by Caco-2 cells treated with insulin-loaded liposomes with and without coating or free Eudragit S100 and Tween 80 (EuTw) (**Figure 34a**). Generally, transport was only observed when there is free EuTw present in the suspension of Eudragit S100 coated HSPC/DOTAP liposomes. Absence of either coating or free EuTw resulted in the loss of transport, suggesting the importance of both coating and free EuTw in causing transport. Coating HSPC/DOTAP liposomes with Eudragit in the absence of Tween 80 caused particle aggregation (**Figure 34b-c**); as a result no transport was detected. As the positive charge conferred by DOTAP is necessary for Eudragit S100 assembly on the HSPC liposomes, it is postulated that the same formulation applied to neutral HSPC liposomes resulted only in uncoated bare liposomes with free EuTw. DLS measurement showed that the size increased marginally when either Eudragit S100 or both Eudragit S100 and Tween 80 were present in neutral liposomes, indicating the absence of coating and possible non-specific adsorption of the polymer on the liposomes surface (**Figure 34b-c**). No transport was observed when cells were treated with uncoated neutral HSPC liposomes in the presence of either Eudragit S100 or both Eudragit S100 and Tween 80. An interesting observation from this transport study was that both Eudragit S100 coating and free EuTw have to be present in order to initiate transport of liposomes.

To study the effect of Eudragit S100 concentration in promoting transport of coated liposomes, three concentrations of Eudragit S100 were tested while keeping Tween 80 constant. The three concentrations are 5.5 µg/ml (E10 with EuTw), 8.2 µg/ml (E15 with EuTw) and 16.4 µg/ml (E30 with EuTw). Addition of Tween 80 has no effect on

the size of liposomes but zeta potential decreased significantly as shown in **Figure 34d-e**. This is because the adsorption of non-ionic surfactant Tween 80 on the cationic liposome surface shifted the shear plane to be further away from the particle, decreasing the zeta potential without changing the charge density on the particle surface.^[45-47] Increasing Eudragit S100 resulted in an increase in size and decrease in zeta potential of the coated liposome, indicating the adsorption of the polymer on liposome surface, represented by E10 with EuTw, E15 with EuTw, and E30 with EuTw in **Figure 34d-e**. PDI generally increased as the size increased from 103.6nm to 423.7nm, suggesting that the dispersity of coated particles increased with more Eudragit S100 coating (**Figure 34d**). Eudragit S100 coating appeared as dark spots on the surface of HSPC/DOTAP liposomes (E15) as characterized by Transmission Electron Microscope (**Figure 34h-i**). The size of the coated HSPC/DOTAP liposomes showed a marginal increase in size (~100nm) as characterized by TEM while a hydrodynamic size of 139.8nm as measured by DLS. To allow an optimum uptake, the size of the coated liposomes were kept below 500nm.^[19, 43, 48] An increasing concentration of insulin in the basal compartment was observed with increasing amount of Eudragit S100, as represented by the transport curves of E10 with EuTw, E15 with EuTw, and E30 with EuTw in **Figure 34f**. Highest transport of insulin was observed in E30 with EuTw liposomes with 3.9ng/ml detected in basal compartment 4 hours cumulatively (**Figure 34f**). TEER values decreased drastically from the 2nd hour onwards for E10 with EuTw, E15 with EuTw, and E30 with EuTw. On the contrary, an increasing TEER for free insulin solution and HBSS control was observed for the first 3 hours, followed by a drop at the 4th hour. The reason for the decrease in TEER value could be due to the long hours of incubation in medium free environment. A recovery of TEER was observed in all treated cells including, indicating that the effect of coating formulation on opening of tight junction was temporary (**Figure 34g**). The observations from this transport study confirmed the role of Eudragit S100 in enhancing transport of insulin-loaded liposomes.

Figure 34. Investigation of the co-factors facilitating the transport of liposome. (a) Transport of neutral HSPC liposomes with and without Eudragit S100 or EuTw. Transport of HSPC/DOTAP liposomes with and without Eudragit S100 coating or EuTw. Insulin serves as control. (b)-(c) Particle size and zeta potential of liposomes used for transport study. (d) Particle size comparison of uncoated, Tween 80 stabilized, and HSPC/DOTAP liposomes coated with increasing amount of 0.0125% Eudragit® S100, as represented by E10 with EuTw, E15 with EuTw, and E30 with EuTw. (e) Zeta potential comparison of uncoated, Tween 80 stabilized, and HSPC/DOTAP liposomes coated with increasing amount of 0.0125% Eudragit® S100. (f) Effect of increasing amount of Eudragit S100 on transport of HSPC/DOTAP liposome. (g) TEER values recorded during transport study. (h)-(i) Transmission Electron Microscope image of Eudragit S100 coated HSPC/DOTAP liposomes (E15).

Despite the enhanced liposome transport with the help of Eudragit S100, the amount of insulin reaching the basal compartment could be specifically improved by increasing liposome loading. The most direct way of improving liposome loading was to increase the insulin concentration in the rehydration buffer. However, insulin being a hydrophilic macromolecule with solubility up to 10mg/ml, increasing its concentration in rehydration solution to 10 mg/ml resulted in difficulty during extrusion, presumably caused by undesired complexation between the charged groups of insulin and the oppositely charged lipid head groups. Hence, to improve transport, loading of the liposome was improved with alteration of the traditional rehydration protocol. Schematic drawing of the strategy to improve loading was shown in **Figure 35a**. To allow maximum interaction of drug with the lipid head group, pH of the rehydration buffer was adjusted to slightly alkaline (pH8) to make insulin deprotonate with net negative charge. Insulin concentration was increased from 0.2 mg/ml to 2 mg/ml. This ensured higher loading of insulin in the core of the multi-lamellar vesicles (MLVs) during rehydration. To avoid complexation with oppositely charged HSPC/DOTAP during extrusion, pH of the rehydration buffer was reverted again using HCl to become slightly acidic (pH4) before undergoing extrusion to form unilamellar liposomes. This allows the separation of surface bound insulin and

prevents the formation of large agglomerates which will hinder extrusion and adversely affect the loading. With the altered rehydration method, loading was improved from 0.4% to 3.2% with the compensation of encapsulation efficiency from 55.9% to 17.6% (**Figure 35b-c**). Cumulative transport showed significant improvement (9x) from 3.89 ng/ml to 35.7 ng/ml as measured by ELISA (**Figure 35d**). In physiological condition, the portal vein insulin concentration ranges from 1 ng/ml to 4.6 ng/ml, during fasting and fed state.^[49] With 37.5ng/ml insulin transported across Caco-2 cells, the physiological level of insulin could be reached provided that 1.81mg - 8.36mg of insulin are loaded into 87.5mM - 402.5mM of liposomes in an oral tablet.

Figure 35. Improving the loading of liposomes results in enhanced transport. (a) Schematic illustration of the strategy to improve insulin loading in HSPC/DOTAP liposome. (b)-(c) Encapsulation (b) and Loading (c) of HSPC/DOTAP liposomes rehydrated with 0.2mg/ml, 2mg/ml insulin. The asterisk represents the significant difference, determined by t-test ($* P < 0.05$) relative to HSPC/DOTAP liposomes rehydrated with 0.2mg/ml (d) Transport of Eudragit S100 coated HSPC/DOTAP liposomes (E30 with EuTw) loaded with 0.2mg/ml, 2mg/ml insulin. The asterisk represents significant difference, determined by t-test ($* P < 0.05$) relative to free insulin solution

5.3.8 Investigation of transport pathway

In order to understand the behavior of liposomes in releasing the loaded insulin, release study was performed in PBS pH 7.4 which simulates the neutral pH in the blood. From the release curve, an initial burst release of up to 80% was observed within the first 4 hours of incubation at 37°C, suggesting that majority of the insulin is situated on the outer surface of the liposome. No significant difference between release profiles of coated and uncoated liposomes was observed (**Figure 36a**) suggesting that the Eudragit S100 coating on the liposomes surface could have become partially protonated in PBS pH 7.4 since the polymer have a pK_a value of 6.66 ± 0.05 .^[50]

To investigate the possible transport pathway, transport of free insulin in the presence and absence of free EuTw was compared. The amount of free insulin was kept the same as the amount loaded in E30 liposomes and assessed by HPLC and microBCA prior to testing. Interestingly, the transport of free insulin is only observed when in the presence of free EuTw. The transport of free insulin was accompanied by a TEER reduction suggesting the potential role of free EuTw as permeation enhancers which opens up tight junctions and promoted paracellular diffusion of the free insulin (**Figure 36b**). Next, the role of Tween 80 and Eudragit S100 in assisting the transport of insulin-loaded HSPC/DOTAP liposomes was investigated by comparing the transport between three groups namely insulin loaded HSPC/DOTAP liposomes, insulin loaded HSPC/DOTAP liposome in the presence of Tween 80, and insulin loaded HSPC/DOTAP liposomes in the presence of both Tween 80 and Eudragit S100 (E30 with EuTw liposomes) (**Figure 36b**). Transport of Insulin loaded HSPC/DOTAP liposomes was barely detectable without Tween 80 and Eudragit S100. In the presence of Tween 80, the transport of insulin loaded HSPC/DOTAP liposomes was detected with simultaneous TEER reduction, indicating the potential role of Tween 80 in opening tight junctions. Upon further addition of Eudragit S100 into the Tween 80 stabilized liposomes, the amount of transported insulin detected in the basal compartment further increased by 4x. Notably, the TEER values were reduced to a lesser extent compared to the other two groups (**Figure 36c**). Tween 80 has been found to be effective for promoting transport of nanoparticles across Blood Brain Barrier (BBB); the speculation of Tween 80 for triggering non-specific uptake

and facilitating transcytosis of intact nanoparticles has been reported by various studies.^[51-54] The presence of Tween 80 in the coating formulation as a stabilizer could have contributed to the cellular uptake and transport of coated liposomes. To investigate the role of liposome in transport, transport of free insulin and insulin loaded HSPC/DOTAP liposomes in the presence of both Eudragit S100 and Tween 80 was compared. A 2 fold increase in transported insulin was observed when HSPC/DOTAP is present, indicating the important role of the liposomal carrier in assisting the transport (**Figure 36b**).^[55] Alamar Blue assay was performed immediately after the treatment to investigate cytotoxicity of the formulation at the applied dosage. Results showed that the effect of TEER reduction was only transient as the cell number (% viability) increased post treatment in addition to the 24 hour TEER recovery (**Figure 36d**). This eliminated the possibility of insulin transport due to compromised monolayer and indicated the dosage of formulation as safe for cells *in vitro*. The observations from this transport study support the proposed role of both Eudragit S100 and liposomes in helping the transport of insulin.

Figure 36. Investigation of transport pathway. (a) Release of coated and uncoated HSPC/DOTAP liposomes in PBS 7.4. (b) Transport of insulin loaded HSPC/DOTAP liposomes (Lip) stabilized with Tween 80 (Lip + Tw), surface modified with Eudragit S100 (Lip + Tw + Eu, E30 with EuTw). Transport of free insulin in the absence (Insulin) and presence (Insulin + Tw + Eu) of Tween 80 and Eudragit S100. (c) TEER values recorded during transport study. (d) Alamar blue assay performed immediately after transport experiment for studying cell cytotoxicity. Caco-2 cells treated with HBSS-HEPES transport buffer serves as the control.

To delineate the transport pathway of Eudragit S100 coated HSPC/DOTAP liposomes, cellular uptake was assessed qualitatively by fluorescence microscopy (**Figure 37**) and quantitatively by flow cytometry (**Figure 38a**). In order to obtain information on internalization of the coated liposomes, insulin was tagged with AF546 before thin film rehydration. Caco-2 cells were immunostained with Claudin-1 antibodies for monitoring of tight junction expression. Increasing area of cellular uptake was observed with increasing free Eudragit S100 as represented by the area circled in red. The reduced Claudin-1 expression and presence of AF546-insulin were marked in green and pink respectively as shown in **Figure 37**. This increasing area of insulin uptake correlates well with the increasing insulin transport observed in **Figure 34f**, suggesting that Eudragit S100 played an important role in cellular uptake and transport of Eudragit S100 coated liposomes.

For flow cytometry, Caco-2 cells were cultured for 14 days before treatment. The cells were treated with three different formulations of liposomes namely, AF546-insulin loaded HSPC/DOTAP liposomes, AF546-insulin loaded Eudragit S100 coated HSPC/DOTAP liposomes, and AF546-insulin loaded Eudragit S100 coated HSPC/DOTAP liposomes in the presence of free EuTw. In the absence of free EuTw, limited uptake (1.45 – 1.88 %) was observed for cells treated with either coated or uncoated HSPC/DOTAP compared to HBSS treated negative control. When free EuTw was present in the solution, the population of cells taking up the coated liposome increased almost 10x to a maximum of 6.08 % (**Figure 38a**). The observations from fluorescence microscopy and flow cytometry correlates with the observations from transport study, which support the role of Eudragit S100 in enhancing transcellular transport of liposomes.

To confirm if liposomes were taken up by Caco-2 cells and transported via transcellular pathway, phosphatidylcholine (PC) lipid assay was conducted to measure the amount of transported PC lipids in the basal compartment. From the percentage lipid transport graph in **Figure 38b**, presence of the E30 liposome coating system resulted in 2.4x increase in the amount of PC detected in the basal compartment than uncoated HSPC/DOTAP liposome. The observations from PC assay correlates well with the results obtained from empty liposome transport (3.2x increased transport observed in coated HSPC/DOTAP liposomes) in **Figure 33d-e** and insulin-loaded

coated liposome transport (2x increase in insulin transport) in **Figure 36b**, reaffirming the role of Eudragit S100 in enhancing transcellular transport of liposomes.

Figure 37. Fluorescence images showing the effect of increasing amount of Eudragit S100 on uptake of Eudragit S100 coated HSPC DOTAP liposome. Caco-2 cells cultured on glass coverslip were treated with liposomes loaded with AF546-tagged insulin, immunostained with AF488-tagged anti-Claudin-1 antibodies. Green represents Claudin-1, Blue represents DAPI staining (a)-(c) E10 with EuTw. (d)-(f) E15 with EuTw. (g)-(i) E30 with EuTw. (d-1), (e-1), and (f-1) represent the inset of (d), (e), and (f).

Figure 38. Investigation of liposome transport pathway. (a). Flow cytometry study of Eudragit S100 on uptake of HSPC/DOTAP liposome. Dot plots of caco-2 cells treated with liposomes loaded with AF546-insulin were analyzed in the PE channel. After determination of baseline PE signal using untreated cells (negative control), the gating on AF546-positive cells revealed proportion of cells with insulin uptake for respective liposomes-treated cells. (b) PC assay studying the percentage lipid transport.

5.3.9 Discussion

Oral delivery of insulin has been extensively researched over the years for being the most desirable treatment option for diabetes mellitus. One of the great advantages of orally administered insulin is the ability to directly enter portal circulation and primarily target the liver once having crossed the GI tract barriers. Targeting the hepatocytes directly would facilitate the use of much lower doses of insulin to control the level of glycemia. Studies using intraperitoneal insulin delivery have shown that the physiological route of insulin delivery resulted in a lower fasting plasma insulin levels compared to the conventional subcutaneous insulin injection in type 1 diabetes patient ^[56, 57] and pancreatectomized dogs. ^[58, 59] Reducing the level of peripheral insulin would also result in a much lower risk of hypoglycemic events, a major drawback of current insulin regimens.

Liposome encapsulation provides extended protection of protein and peptide drugs against pH and enzymatic digestion in the GI tract; however, the carrier suffers from low permeability across intestinal epithelium which hinders the effectiveness of its application for oral drug delivery. To overcome the barrier of permeation, a pH sensitive anionic carboxylated polymer Eudragit S100 was used to modify the surface of liposomes.

We have elucidated the role of Eudragit S100 in enhancing the uptake and transport of insulin loaded nanosized liposomes using quantitative ELISA, flow cytometry, and PC assay. The role of Eudragit S100 in enhancing the transport of empty liposomes was first discovered when screening through a panel of 6 different types of liposomes. Increased transport and reduced cellular association of fluorescent lipid was observed when coumarin-6 tagged liposomes were surface modified with Eudragit S100 in the presence of Tween 80 (**Figure 33b-c**). Highest percentage transport and percentage cellular association was observed in rigid HSPC (5x improved cellular association) liposomes and HEPC liposomes (12x improved transport). This is consistent with current literature findings that rigid particles enhance endocytosis more efficiently than softer ones because lower adhesion energy was required to achieve full membrane wrapping around the nanoparticle upon binding onto cell surface, resulting in a higher binding rate and lower dissociation rate compared to the softer nanoparticles.^[22, 23] In addition to rigidity, positively charged particles with smaller

size were found to initiate cellular uptake more efficiently.^[42, 43] HSPC/DOTAP was selected as a model carrier due to its overall performance in terms of particle size, charge, and rigidity.

To evaluate the effectiveness of Eudragit S100 in improving the transport, transport study was performed caco-2 cells treated with insulin loaded liposomes in the presence of increasing Eudragit S100 concentration while keeping Tween 80 fixed. In addition, 3 groups including free insulin, insulin loaded liposomes in the presence of Tween 80, insulin loaded liposomes in the presence of both Tween 80 and Eudragit S100 were designed and tested for transport across Caco-2 cells. In **Figure 34f**, increasing insulin transport was observed with increasing Eudragit S100 concentration while keeping Tween 80 constant, suggesting the role of Eudragit S100 in enhancing transport of Eudragit S100 coated liposomes. Transport of free insulin is only observed with reduction in TEER when both Tween 80 and Eudragit S100 are present, suggesting their role in promoting paracellular transport of insulin (**Figure 36b**). Transport of Insulin loaded HSPC/DOTAP liposomes was observed in the presence of Tween 80, and further enhanced by 4x with the addition of Eudragit S100 (**Figure 36b**), indicating the importance of Eudragit S100 in assisting transport which correlates well with the observations from transport curves in **Figure 34f** and fluorescence images in **Figure 37**. Since Tween 80 has been reported for enhancing cellular uptake and transport of nanoparticles across BBB,^[51-54] it is highly possible that in addition to Eudragit S100, the presence of Tween 80 in the coating formulation as a stabilizer contributed to the enhanced cellular uptake and transport of coated liposomes as observed in **Figure 36b**. In the presence of both Eudragit S100 and Tween 80, a 2 fold increase in transported insulin was observed only when liposome is present, suggesting the importance of carrier in causing the enhanced transport (**Figure 36b**). The detection of PC groups in the basal compartment suggests that liposomes has been taken up and transported via an intracellular pathway across the caco-2 cells. A 2.4x increase in basal compartment PC was observed as compared to uncoated HSPC/DOTAP in **Figure 38b**, correlating well with the 3.2x increase for coated empty liposome transport in **Figure 33d-e** and the 2 fold increase with the help of coated liposome in **Figure 36b**. Moreover, the effect of Eudragit S100 in causing uptake of AF546 insulin loaded Eudragit S100-coated liposomes was further confirmed by the increased area of cellular uptake when increasing Eudragit S100 was

introduced while keeping Tween 80 fixed, as observed by fluorescence images in **Figure 37**. This shows that other than Tween 80, Eudragit S100 played a role in initiating cellular uptake of coated liposomes. Furthermore, the quantitative analysis of cellular uptake by flow cytometry revealed an almost 10x improved uptake of Eudragit S100 coated liposomes as compared to unstained control (**Figure 38a**). The increased PC content in basal compartment observed for Eudragit S100 coated liposomes in **Figure 38b** suggest the transport is highly possible to be transcellular due to the limited tight junctional space. The tight junctional space when fully opened by any enhancers is only 20nm, limiting the paracellular diffusion of any coated liposomes with size 290nm.^[55] Hence, a transcellular pathway is highly plausible for this carrier related enhancement of transport because the tight junctional space is too small for large polymer coated liposomes to diffuse through.^[60] Alamar blue assay showed increased % viability and recovered TEER values 24 hours after treatment suggesting that the applied dosage of formulation was non-toxic and the effect of free EuTw on tight junction is temporary and reversible (**Figure 36c-d**). Taken together, these evidences suggested that Eudragit S100 is effective in assisting transport of insulin-loaded liposomes when present on the surface of liposome with free EuTw. However, this does not exclude the possibility of paracellular transport of insulin with the help of EuTw as evidence from release study suggest the possible release of liposome surface-bound insulin. In summary, the presence of Eudragit S100 seems to promote both paracellular insulin release and transcellular transport of insulin loaded liposomes.

Our overall finding is summarized in **Figure 39** based on all the experimental results was that Eudragit S100 triggers cellular uptake of coated liposomes in the presence of EuTw, and transports insulin loaded liposome via transcellular pathway. Free insulin released from the liposome surface was able to cross the caco-2 cell layer due to the permeation enhancer effect of EuTw via paracellular pathway. The coating could have been condensed on the liposome surface as the polymer transformed from coil to globule, the resulting pH-induced conformational change caused localized instability of endolysosome membrane, resulting in release of liposome in late endosomes. Intracellularly, free Eudragit S100 destabilized lysosomal membrane causing release of endocytosed liposomes which eventually was exocytosed to the basal compartment. The protocol has the potential of reaching physiological insulin levels

provided that 35.7 ng/ml of insulin crossed the caco-2 monolayer during 4 h transport study.

Figure 39. Overall schematic drawing summarizing possible mechanism of intracellular events during transport of liposomes

5.3.10 Conclusion

In an attempt to enhance permeability of insulin loaded liposomes, a simple, fast, and facile method of coating anionic polymer Eudragit S100 onto positively charged nano-sized HSPC/DOTAP liposome was developed for the first time in an aqueous medium based on electrostatic interactions. When Eudragit S100 is present as coating on the liposome surface and free in the solution, it enhances cellular uptake and transport of 6 different types of liposomes tested in caco-2 cell model simulating the

intestinal barrier. The successful *in vitro* studies of uptake and transport showed promising results of Eudragit S100 to serve as potential coating material for other types of carriers for oral delivery of insulin. The liposomal coating process developed is fast and simple, producing nanometer sized coated liposomes with minimal toxicity within minutes. The coated liposomes demonstrated enhanced permeability via transcellular pathway *in vitro*, thus opening new opportunities for oral delivery of macromolecular drugs.

5.4 References

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Chapter 6

Layer-by-layer approach: Surface modification of liposome with multilayers of chitosan/insulin

This chapter describes the experimental results from a multilayered system consisting of alternating layers of chitosan and insulin on the surface of HSPC/DPPG liposome. The multilayered liposome was synthesized through repeated cycles of coating with oppositely charged polymer and drug. Areas including particle fabrication, parameter optimization, loading and release, cellular uptake and transport, and long term stability were investigated and will be discussed in this chapter. The insulin loaded on the surface of liposome via electrostatic interactions demonstrated sustained release up to three months and excellent stability in PBS pH 7.4 over one month of study.

6.1 Introduction

Liposomes are ideal carriers for loading both hydrophobic and hydrophilic drugs in their bilayer and aqueous core. Despite the protection that it offers for orally ingested insulin, the loading and release remains to be improved. Layer-by-layer (LbL) assembly of polyelectrolytes on solid substrate has been explored by several groups over recent years because of the superiority of this system in terms of controlled release and high drug loading for applications in areas such as drug and gene delivery, and tissue engineering. Multilayered films are generated by sequentially depositing oppositely charged polyelectrolytes via electrostatic interaction. A key advantage of this system lies in the ability to control the release of loaded therapeutics using polymer layers. LbL assembly not only improves the loading capacity to hydrophilic peptide drugs such as insulin, but also the stability of liposomes by forming a polyelectrolyte complex (PEC), giving rise to controlled release over extended period of time. Oral formulation with controlled release profile could potential avoid multiple injections required by subcutaneous injection, thus offers better patient compliance.

One of the widely used natural cationic polymers is chitosan, a linear polysaccharide derived from deacetylation of chitin. The backbone of chitosan is rich in primary amino groups, making them highly pH responsive and soluble in acidic solutions. The polycationic polymer has the ability to interact with negatively charged mucus membrane, thus are popular bioadhesive material to increase residence time of nanocarriers. Furthermore, chitosan has been found to function as permeation enhancer which interacts with the tight junction of intestinal epithelial cells result in transient opening of intercellular tight junctions, improving bioavailability of poorly permeable hydrophilic drugs. These superior properties of chitosan make them excellent choice for LbL assembly. It not only serves as layers of adhesives to hold insulin on the surface of liposomes, but also contributes to enhance transport and retention time of the liposomes.

6.2 Objectives

This study focuses on using layer-by-layer technique to improve loading and prolong the release of insulin by coating the drug on the surface of nano-liposomes based on

electrostatic interaction between negatively charged insulin and positively charged chitosan. The objectives of the study are:

1. To optimize average particle sizes, PDI and zeta potentials for LbL coated liposomes
2. To investigate drug loading through release study in PBS
3. To assess the stability of LbL coated liposomes in both neutral (PBS) and acidic (SGF) medium

Figure 40. Schematic diagram illustrating the objective of chapter 6. Coating multilayers of chitosan/insulin onto liposomes for improving the release, loading, and stability.

6.3 Optimization of chitosan and insulin coating layers

To obtain homogeneous multilayer coated liposomes, an optimization step was performed on HSPC/DPPG liposomes. For all the odd layers of chitosan, increasing amount of chitosan solution was added to a series of fixed volume of liposome solution. For all the even layers of insulin, increasing amount of insulin solution was added to a series of fixed volume of chitosan coated liposomes. Size and zeta potential were measured after each addition and plotted against the volume of chitosan/insulin added to the liposome solution shown in **Figure 41b** and **d**. Increasing the amount of chitosan added to HSPC/DPPG liposomes caused the charge

to increase continuously from -40mV, crossing the zero point and eventually stabilized at +40mV. Size increased gradually as the zeta potential increases until reaching a maximum size of 3500 nm at -20mV, a decrease in size was observed when more chitosan was added to the liposome solution **Figure 41c**. Insulin coating was optimized in a similar way on chitosan coated HSPC/DPPG liposomes. Increasing the amount of insulin added to chitosan coated liposomes caused the charge to decrease continuously from +40mV, crossing the zero point and eventually stabilized at -300mV **Figure 41e**. Size increased gradually as the zeta potential decreases until reaching a maximum size of close to 3500 nm at around -20mV, a decrease in size was observed when more insulin was added to the solution. Optimization was continued after charge reversal was observed and stopped when increasing chitosan/insulin does not induce any further change in zeta potential and smallest size was achieved with low PDI value.

The rationale behind this optimization can be explained by classical theory of Derjaguin, Landau, Verwey, and Overbeek (DLVO), which describes the stability of colloid in suspension from the perspective of intermolecular forces.^[1-3] When small amount of polyelectrolyte is added to a suspension of oppositely charged particles, polyelectrolytes quickly adsorb onto the surface of the particle due to electrostatic interaction. This adsorption reduces the surface potential as the surface charge is partially neutralized by the oppositely charged polyelectrolytes. As the amount of polyelectrolyte continues to increase, the surface charge approaches a close to net zero value which renders the colloidal system unstable. At this stage, Van Der Waals forces dominate over repulsion forces between particles, results in rapid aggregation. Further addition of polyelectrolyte into the suspension introduces more charges on the particle surface, leading to a complete charge reversal which provides stabilization of the particle due to the intermolecular repulsion force. In order to have a stable colloidal system, the electrostatic repulsion contributed by particle surface potential must be sufficiently high to overcome the Van Der Waals attraction force. From **Figure 41b** and **d**, the optimal sizes of coated liposomes were in the range of 100-200 nm when surface charges are either below -30 mV or above +30 mV for both chitosan and insulin.

Figure 41. Optimization of LbL coating on HSPC/DPPG liposomes. (a) Schematic drawing of LbL assembly on the surface of HSPC/DPPG liposomes. (b)-(c)) Optimization of chitosan coating on negatively charged HSPC/DPPG liposome. (b) Effect of increasing amount of chitosan on the zeta potential of HSPC/DPPG liposomes. (c) The increasing zeta potential caused by chitosan attachment on the HSPC/DPPG liposome surface corresponded to an increase followed by a decrease in size. (d)-(e) Optimization of insulin coating on positively charged chitosan coated HSPC/DPPG liposome. (d) Effect of increasing amount of insulin on the zeta potential of HSPC/DPPG liposomes. (e) The increasing zeta potential caused by insulin attachment on the chit-HSPC/DPPG liposome surface which corresponded to an increase followed by a decrease in size.

Chitosan is a linear chain of polysaccharide extended when the amino groups are ionized in aqueous solution. Solubilized chitosan exhibits as flexible rod-like macromolecule with size range from 6nm to 22nm.^[4] A schematic representation of the process of chitosan coating onto negatively charged liposomes surface is described in **Figure 42**. Addition of the polymer onto weakly negatively charged liposome surface initially induces bending of the polymer as it accommodates the curvature surface of the lipid membrane. During adsorption, the organization of the polymer first flattens as the long polymer chain completely adsorbs on the surface of the liposomes via electrostatic interaction. As the amount of polymer increase, the

charge on the liposome surface are neutralized and reversed to positively charged, stabilizing the particles based on electrostatic repulsion (**Figure 42B**). In the case of a strongly negatively charged liposome surface, binding strength between chitosan and liposome is lower, resulting in lower chain bending and organization when the polymer adsorbs on the surface of liposome (**Figure 42C**). As a result, additional polymer would not be able to cover the surface completely due to the existing polymer that is already present. As a result, the newly added polymers partially attracted to the remaining available space between the already adsorbed flattened polymers, with its chain extending in the solution, forming bridges between the adjacent liposomes and leading to particle aggregation (**Figure 42D-E**). In this case, the amount of chitosan is not enough to neutralize the highly negatively charged liposome surface for particle stabilization. ^[4]

Figure 42. Schematic drawing of process of chitosan interaction with negatively charged liposomes: (A) At low chitosan concentration, short ranged weak electrostatic interaction between positively charged chitosan and neutral or slightly negatively charged liposome. (B) Strong and tight binding occurred when chitosan concentration increased, resulting in full coverage of outer membrane of a neutral or slightly negative liposome with charge reversal stabilizing the coated liposome. The negative charged membrane was neutralized by the binding of positively charged chitosan. (C) At low chitosan concentration, weak electrostatic interaction between positively charged chitosan and highly negatively charged liposome. (D) Increased chitosan concentration results in more polymers partially adsorbing onto the remaining free spots on the highly negatively charged liposome surface. (E) Aggregation occurred as the partially bound chitosan crosslink the unbound portion of its polymer chain with surrounding liposomes when chitosan concentration further increased. Adapted from Ref^[4]

6.3.1 Understanding the thermodynamics of LbL binding

NanoITC provide essential information about the type of interactions between molecules that drives a reaction. Binding between molecules occur because the affinity between the molecules is greater than that between the solvent. Enthalpic contribution to the binding event is primarily related to increased number of hydrogen

bonds, electrostatic interactions, dipole-dipole interactions, and Van Der Waals forces between the interacting molecules. Entropic contribution is related to primarily conformational changes of macromolecules (such as folding and unfolding of proteins) and release of bound solvent due to interaction between hydrophobic groups. The entropy gained from releasing of ordered water molecules from the surface of the binding entity is the main driving force to the hydrophobic binding between molecules and is sufficient to compensate for the loss of conformational and rotational freedom following binding.

NanoITC was performed to examine the thermodynamics behavior during chitosan coating onto HSPC/DPPG liposomes. Following stepwise injection of chitosan into HSPC/DPPG liposomes, energy change of the system was reflected in the thermogram as a function of mole ratio between titrant (chitosan) and titrate (HSPC/DPPG) concentration. The integrated area of the data points were fitted using independent binding site model and normalized by subtracting heat of dilution. The thermodynamic parameters of the reaction were summarized in **Table 12**. The Gibbs free energy ΔG , entropy ΔS , and enthalpy ΔH are related by the classical thermodynamic equation $\Delta G = \Delta H - T\Delta S$. The binding of chitosan to HSPC/DPPG liposomes appear to be governed by electrostatic interactions, but in fact many other non-covalent interactions are present (i.e., hydrogen bonding, hydrophobic and van der Waals interactions). From the nanoITC results, it is possible to predict whether the reaction is enthalpically driven or entropically driven. Building of new bonds or interactions such as electrostatic interaction or other non-covalent interactions are enthalpically favorable, meaning exothermic process. This is often coupled with displacement of counterions associated on the liposomes surface, which is enthalpically unfavorable. As the counterions or the water molecules gets released from the surface of the liposome, the entropy of the system increase. Chitosan changes conformation when binding to the surface of liposomes, the polymer chain rearrangement brings more freedom and disorder, but when it is bound to the surface, the conformational rigidity decreases the system entropy^[5] The overall balance between enthalpy and entropy of the reaction is reflected by the Gibbs free energy ΔG which was negative, indicating that the process of chitosan binding to liposome is spontaneous.

Binding of chitosan onto HSPC/DPPG liposomes was studied by nanoITC to determine the thermodynamic driving force for the interaction between chitosan monomers and PG lipids **Figure 43a**. Heat of dilution was performed by titrating 0.1% acetic acid into the HSPC/DPPG liposomes and used as a blank (**Figure 43c**). Raw data was fitted using the independent binding site model (**Figure 43e**) to obtain the stoichiometry parameters. The number of binding sites N represents the number of chitosan monomer units bound to each PG lipid at saturation. N value of 0.493 suggests that only about half of the PG binding sites were bound with chitosan at saturation. A strong negative Gibbs free energy ΔG (-26.23 kJ/mol) and enthalpy ΔH (-7.66 kJ/mol) suggests that hydrogen bonding and electrostatic interaction between positively ionized amine groups of chitosan and negatively charged PG groups of liposomes drives an exothermic and energetically favorable reaction, leading to stabilization of the coated liposomes. Contribution of entropy (62.3 J/mol·K) may arise from the release of water molecules from the surface of PG as a result of chitosan binding, changes in water structure, and alteration in ion distribution during the molecular interaction of binding, which leads to higher degree of freedom and increased number of possible energy states.

The thermograms in **Figure 43b** representing the binding of insulin with chitosan-HSPC/DPPG liposomes reflected the thermodynamic driving force for the reaction. Heat of dilution was performed by titrating DI into the Chi-PG liposomes and used as a blank (**Figure 43d**). Raw data was fitted using the independent binding site model (**Figure 43f**) to obtain the stoichiometry parameters. N value of 0.568 suggests that only about half of the binding sites in chitosan were bound with insulin at saturation. A strong negative Gibbs free energy ΔG (-32.38 kJ/mol) and enthalpy ΔH (-15.21 kJ/mol) suggests that hydrogen bonding and electrostatic interaction between positively ionized amine groups of chitosan and negatively charged amino acids of insulin drives an exothermic and energetically favorable reaction, leading to stabilization of the coated liposomes. Contribution of entropy (57.6 J/mol·K) may arise from the release of water molecules from the surface of chitosan as a result of insulin binding, which leads to higher degree of freedom and increased number of possible energy states.

Figure 43. Study of binding of multilayers onto liposome surface by Nano Isothermal Titration Calorimeter (ITC) (a) ITC data for titration by stepwise injections of 0.1% chitosan into HSPC/DPPG liposomes (PG 2 mM) in 0.1% acetic acid at 25 °C. (b) ITC data for titration by stepwise injections of 1mg/ml insulin (0.172mM) into chitosan-HSPC/DPPG liposomes (PG 0.02 mM) in DI water at 25 °C. (c) - (d) Heat flow and respective integrated binding heat corrected for heat of dilution are plotted as a function of mole ratio. (e) - (f) The solid curves were fitted using the independent binding site model.

Table 16. Thermodynamic parameters of chitosan-HSPC/DPPG binding and insulin-chi/PG biniding characterized by nanoITC

Models			
Model	Variable	Chit-PG	Ins-Chit/PG
Blank (constant)	blank (μJ)	0.18	-0.517
Independent	N	0.493	0.568
	ΔH (kJ/mol)	-7.66	-15.21
	K_a ($10^5 M^{-1}$)	0.394	4.707
	$-T\Delta S$ (kJ/mol)	-18.58	-17.18
	ΔG (kJ/mol)	-26.23	-32.38
	ΔS (J/mol·K)	62.3	57.6

6.3.2 Characterization of chitosan/insulin LbL coated liposome

Using the formulation obtained from the optimization experiments, alternate coating of chitosan and insulin layers onto HSPC/DPPG liposome surface was achieved. Successful fabrication of LbL coating on liposomes was indicated by the negative zeta potential values observed in even layers (Layer 0, Layer 2 and Layer 4) and positive zeta potential values observed in odd layers (Layer 1, Layer 3 and Layer 5) as shown in **Figure 44a**. The anionic liposome surface was completely covered with cationic polyelectrolyte chitosan, with charge reversal observed from -41.77 mV to 33.7 mV after first layer of chitosan coating. Subsequent coating with anionic polypeptide insulin completely reversed the charge from 33.7 mV to -36.07 mV. The procedure of coating alternating layers of oppositely charged polymer was repeated until desired number of layers was achieved. 5 Layers of polymer deposition was accompanied by a simultaneous increase in size as measured by DLS from 101.8 nm to 164.26 nm (**Figure 44b**). Polydispersity index (PDI) from DLS measurement indicates that the particles generally get increasingly polydisperse as the number of layers coated increase. The PDI increased from 0.07 before coating, to 0.28 after 5 layers of coating.

In **Table 17**, it was observed that coating of chitosan layers caused a larger increase in size than that of the insulin layers. An increase of ~ 24 nm, ~ 22 nm, and ~ 10 nm was observed when coating chitosan layer 1, 3, and 5, while only ~ 5 nm and ~ 2 nm was observed when coating insulin layer 2 and 4. This could be due to the higher molecular weight and bigger size of chitosan compared to insulin which contributes to a thicker coating layer with polymer chains extending out into the solution. Additionally, zeta potential values give important information about the stability of the colloidal suspensions. Generally, particles are sufficiently charged and considered stable when zeta potential values are above $+30$ mV or below -30 mV. The LbL coated liposomes has a zeta potential range of -40 nm to $+30$ nm with the range becoming smaller after 5 layers of coating. This is in consistent with the increasing PDI values which indicate that the system is getting less stable and aggregation occurred during the process of coating.

Table 17. Particle size and zeta potential measurements of LbL coated liposomes.

Layer	Layer-by-Layer nanoparticles	Mean particle size (nm)	Poly Dispersity Index	Zeta potential (mV)
0	DPPG	101.8 ± 1.76	0.07 ± 0.01	-41.77 ± 2.4
1	DPPG/Chit/	125.87 ± 1.56	0.15 ± 0.01	33.7 ± 0.36
2	DPPG/Chit/Ins/	130.7 ± 0.98	0.21 ± 0.03	-36.07 ± 3.75
3	DPPG/Chit/Ins/Chit/	152.8 ± 3.90	0.19 ± 0.03	25.67 ± 1.12
4	DPPG/Chit/Ins/Chit/Ins/	154.13 ± 5.45	0.28 ± 0.01	-37.53 ± 4.36
5	DPPG/Chit/Ins/Chit/Ins/Chit/	164.26 ± 6.05	0.28 ± 0.02	20.6 ± 0.82

Figure 44 Characterization of size, zeta potential and PDI of LbL by Dynamic light scattering. (a) Charge reversal of LbL upon successful coating of each layer. (b) Size and PDI measured after each layer of coating.

6.3.3 Loading and release of chitosan/insulin LbL coated liposomes

Cumulative drug release from both 3 and 5 layers coated liposomes were studied over a period of 85 days. The release profile of both 3 and 5 layers coated liposomes showed a pulsatile release pattern. A rapid release was observed for both liposomes during the initial 13 days, the 3 layers coated liposomes released 129ng of insulin while about 90ng was released for 5 layers coated liposomes. Following which, the release for 3 and 5 layers coated liposomes was slowed down for about 2 months and 1 month, respectively. In the next phase, both liposomes started to release substantially again with 133 ng released during a period of 20 days for 3 layers coated liposomes, while 331 ng released during a period of 43 days for 5 layers coated liposomes.

The loading of insulin on the surface of liposomes achieved by the LbL approach ensures the protein to be tightly entrapped within the chitosan layers. Diffusion of the protein was slowed down due to the size of the protein and electrostatic attraction by the chitosan. The glycosidic bonds that connects chitosan monomer gradually undergoes hydrolysis results in the slow degradation of the polymer in aqueous environment. Insulin slowly diffuses out from the layers as chitosan degrades. The pulsatile release system characterized by repeated phases of rapid and sustained release could be explained by the differences in bonding strength between insulin and chitosan. As the outermost layer of chitosan slows down the diffusion of the insulin which was trapped within the multilayers, a slow and sustained release was observed. **Figure 45a** appears to be a surface eroding system whereby the coating erodes after a lag time when the system comes into contact with an aqueous medium. As chitosan backbone undergoes hydrolysis in aqueous environment over time, the bonding gradually weakens until the chitosan layer dissolves exposing the underneath insulin layer, resulting in rapid release following the sustained release period. Such a release system prolongs the stability of liposome in simulated physiological conditions, allowing insulin to be released for an extended period of time. Such a formulation could potentially avoid multiple injections and protect insulin for a longer time in the body.

Loading is a crucial factor for drug carrier because it has a direct relationship between the bioavailability of the drug. For orally administered drugs, more drugs being loaded inside the carrier means more drugs will be able to reach the portal vein with the same number of particles transported across the intestinal epithelial cells. Secondly, it determines the efficiency and cost effectiveness of its commercial value. Coating insulin on the surface of liposomes improved the loading significantly. Liposome loading increased from 0.15% to 0.4% after the first insulin layer and further increased to 1.25% after the second insulin layer (**Figure 45b**). An enhancement factor of more than 8x was observed after just 2 layers of insulin coating (**Figure 45c**).

Figure 45. Release and loading of LbL coated liposomes (a) release of LbL-liposomes after 3 and 5 layer of coating with oppositely charged chitosan and insulin in PBS 7.4 at 37°C. (b) Loading of 3 and 5 layers coated LbL-liposomes assessed by the ratio between cumulatively released weight of insulin and lyophilized weight of insulin loaded particle. (c) Fold of increased loading calculated based on the ratio between the loading of LbL-liposomes and loading of uncoated HSPC DPPG liposome.

6.3.4 Cellular uptake and transport of chitosan/insulin LbL coated liposomes

Transport of LbL-liposomes was performed on Caco-2 cells cultured on permeable transwells after 21 days. The 3-layers coated HSPC/DPPG liposomes (Lip-chitosan/insulin/chitosan) has only one more layer of insulin compared with 1-layer HSPC/DPPG coated liposomes (Lip-chitosan). The 1- and 3- layers coated liposomes (L1 and L3) were further modified with Eudragit S100 coating (L1 E30 with EuTw and L3 with EuTw) and the amount of insulin transported was measured by ELISA.

From **Figure 46a**, it can be seen that with only 1 layer of insulin loaded on the surface of liposomes, the transport of insulin across Caco-2 cells was enhanced by 2.6 fold from 5.7ng/ml to 15ng/ml. The increased transport of L3 compared to L5 is because more insulin is loaded in the layers on the surface of liposome. Therefore, with the same number of carriers transported, more insulin would be able to be transported due to the increased loading. A comparison between the transport of L1 E30 with EuTw

and L1 shows that the transport was similar for the first 3 hours but L1 exhibited a higher transport after the third hour. However, a comparison between the transport of L3 E30 with EuTw and L3 shows that LbL-liposomes have higher transport without Eudragit S100. From the transport graph, it can be concluded that the effect of Eudragit S100 coating and free EuTw in enhancing transport of particles is comparable to that of the previously known permeation enhancer chitosan.

The TEER reading for cells treated with LbL-liposomes (L1 and L3) decreased whereas formulations involving Eudragit S100 caused TEER reading to increase then decrease during the 4 hours of transport in **Figure 46b**. Immunostaining of insulin with AF488 tagged anti-insulin antibodies was represented by green channel, while immunostaining of tight junctional protein claudin-1 with AF568 tagged anti-claudin-1 antibodies was represented by orange channel in the fluorescence images in **Figure 46c**. Claudin-1 expression seem to be more prominent in Caco-2 cells treated with HBSS, whereas the green fluorescence from insulin was only observed in Caco-2 cells treated with L3, indicating the possibility of insulin release from endocytosed L3, which could have been captured by anti-insulin antibodies during immunostaining (**Figure 46c**).

Figure 46. Transport and cellular uptake of LbL coated liposomes. (a) Transport of insulin loaded LbL-liposomes across caco-2 cells with Eudragit S100 coated at the outermost layer. (b) TEER readings measured during transport study (c) cellular uptake of LbL-liposomes imaged by fluorescence microscopy. Green channel represent AF488 anti-insulin antibody staining of insulin. Red channel represent AF568 anti-claudin-1 antibody staining of claudin-1 of the tight junction.

6.3.5 Stability of chitosan/insulin LbL coated liposomes

Stability of liposomes coated with 0, 1, 3 and 5 Layers of chitosan/insulin was studied over a period of 30 days in both neutral (PBS) and acidic (SGF) medium and results

were shown in **Figure 47**. Size, PDI, and zeta potential were recorded weekly by DLS to study the effect of the environmental pH on LbL-liposomes. Only odd layers coated liposomes were subjected to the test to avoid direct exposure of insulin to the release medium.

The stability of LbL-liposomes in PBS solution reflected a relative stability over a period of 4 weeks. Size was generally increasing in all the layers. The sizes are within 250nm by the end of 4 weeks with PDI ranged from 0.2-0.3. Interestingly, the zeta potential measured for L1, L3, and L5 is different from uncoated HSPC/DPPG liposomes. The charge started to reverse from positive to negative in all the odd layers coated liposomes after 1 week in release medium while remains negative for uncoated layer 0 liposomes throughout the 4 weeks of study. This could be due to the dissociation of chitosan and the release of insulin as the particles get released over time. The charges for all the layers of coated liposomes in Week 0 generally show a decrease in zeta potential.

On the other hand, LbL-liposomes were relatively unstability in SGF as indicated by the large size and PDI values. The size of all the layers of coated liposomes increased drastically to micron sized particles after immersed in SGF for a week, and keep increasing as the time progress. PDI increased to be close to 1, indicating non homogeneous distribution of particles. Zeta potential reversed from negative to positive for uncoated HSPC/DPPG liposomes while remains positive for all the other layers of coated liposome. This could be due to the association of H^+ ions on the surface of the aggregated particles. Low pH results in a high concentrations of H^+ ions which could potentially facilitate the fragmentation of chitosan, destabilizing the LbL-liposome.^[6] Furthermore, the degradation of chitosan is accelerated in acidic environment due to acid hydrolysis of its glycosidic bonds. The degradation of chitosan reduces the surface charge of the LbL-liposomes and renders the particles vulnerable and prone to aggregation.

Figure 47. Stability of LbL coated HSPC/DPPG liposomes in SGF and PBS. The size, zeta potential, and PDI changed following 4 weeks of incubation at 37°C in SGF and PBS were represented by (a), (c), (e) and (b), (d), (f), respectively.

6.4 Discussion

Chitosan has been widely used in the field of oral drug delivery due to its excellent biocompatibility, mucoadhesiveness, and ability to function as permeation enhancer by interacting with intestinal epithelial cellular tight junctions.^[7] Numerous studies have development nanocarriers for oral delivery of insulin using chitosan or its derivatives.^[8, 9] Chitosan-insulin nanoparticles have been synthesized based on polyelectrolyte complexation between chitosan and insulin with the help of ionotropic gelation with sodium tripolyphosphate (TPP) anions, the synthesized particles has shown to be enhancing transcellular uptake and transport by Caco-2 cells.^[10] Chitosan have been used as coatings on the surface of liposomes to improve their mucoadhesiveness.^[11] Furthermore, chitosan nanoparticles encapsulating insulin was

demonstrated to cause concentration-dependent internalization and simultaneous reduction in TEER values in Caco-2 monolayer.^[12]

In the present study, insulin has been loaded onto the surface of negatively charged HSPC/DPPG liposomes via a LbL approach. The pH was adjusted to be above the pI of the protein to render the protein a net negative charge. In between the insulin and liposome, an oppositely charged poly-cationic chitosan was used to conveniently hold the insulin layer in place via strong electrostatic attraction. The LbL coated HSPC/DPPG liposomes exhibited extended release of up to 3 months and significant improvement in loading as compared to liposomes without any modification. The loading was significantly improved by more than 8 times with only 2 layers of insulin coating. Release was significantly prolonged and sustained over a period of 89 days compared to insulin loaded in liposomes without LbL assembly, which showed burst release of up to 80% within the first few hours in the same release environment in Chapter 5 **Figure 36a**. Fluorescence imaging and ELISA studies showed that the LbL-liposomes has been taken up by the Caco-2 cells and transported across the cell monolayer. The size of the LbL-liposomes generally increased but was below 250nm over a period of 30 days in PBS pH 7.4, indicating superior stability of the particle in aqueous medium.

6.5 Conclusion

In this study, we have successfully loaded multilayers of insulin on the surface of HSPC/DPPG liposomes for the first time. The LbL-liposomes has demonstrated improved transport and uptake by Caco-2 cells *in vitro*. With only 1 layer of insulin loaded on the surface of liposomes, the transport of insulin across Caco-2 cells was enhanced by 2.6 fold from 5.7ng/ml to 15ng/ml. The chitosan layers used in between insulin layers could serve as a reservoir of permeation enhancers for the slow dissolving insulin to pass through intestinal epithelium via paracellular transport. In addition, the nanometer sized LbL-liposomes have demonstrated cellular uptake and transport by Caco-2 cells *in vitro*. This multilayered liposome-polymer nanoparticle has been developed with controlled size distribution and superior improvement on the stability, loading, release, uptake, and transport, which are important characteristics for a good candidate for oral insulin delivery.

6.6 References

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Chapter 7

Conclusions and Future works

This chapter describes the summary of research findings and proposes with studies to further investigate the bioactivity of transported insulin and to improve the efficiency and effectiveness of the LbL system. A glucose uptake study using 3T3 cells cultured at the basal compartment of the transport set-up is proposed to study the bioactivity of transported insulin. It is hypothesized that the transported insulin retains its functionality and is able to bind to the insulin receptor on the 3T3 cell surface to trigger glucose uptake. In addition, a fluidic flow system consisting of a series of purifying columns that are capable of separating the free coating material from the coated nanoparticle is proposed to improve the efficiency and effectiveness of fabricating LbL nanoparticles. Such a device is hypothesized to minimize the loss of nanoparticles during the purification step of each coating layer and up scale the synthesis of LbL nanoparticles.

7.1 Overall conclusion

An oral route of insulin delivery has been on the top list of alternative treatments for diabetic patients owing to its non-invasiveness and liver targeting effects. The need for better protection of insulin in the GI tract and improved transport across intestinal epithelial cells drives the motivation of this project. This thesis focuses on the studies of insulin transport across intestinal epithelial cells with the help of surface modified liposomal carriers. The first objective was set to optimize the coating parameters for a gelatin capsule coated with pH-sensitive polymers for intestinal targeted release. The second objective was set to investigate the physiochemical properties of liposomes in affecting the loading and transport of insulin across intestinal epithelial cells and enhance the transport via surface modification of liposomes using Eudragit S100 and Tween 80. The third objective was set to improve the loading and release of insulin by coating insulin on the surface of liposomes via a Layer-by-Layer approach.

The first hypothesis that liposomes can assist the transport of insulin across Caco-2 cells with the help of appropriate surface modification using Tween 80 and Eudragit S100 was proposed and investigated based on the literature finding which suggests the potential role of Tween 80 as enhancer for nanoparticle transport across the blood brain barrier (BBB) and Eudragit S100 in promoting membrane destabilization and endosomal escape. In an attempt to test the hypothesis, a novel coating of Eudragit S100 onto liposomes with the help of Tween 80 in aqueous solution was discovered, which ensured a final size within the nanometer sized range and has eliminated the use of organic solvent which was previously used to coat Eudragit S100 onto MLV. A novel insight into the pathway of insulin transport with the help of Tween 80 stabilized Eudragit S100-coated liposomes was studied; the coated liposomes demonstrated improved the cumulative transport of insulin from 0.139ng/ml to 3.9ng/ml via both paracellular and transcellular pathway compared to unmodified insulin loaded liposomes. A further increase of insulin transport from 3.89ng/ml to 35.7ng/ml was achieved by increasing liposome loading via a modified rehydration protocol.

The second hypothesis that insulin can be coated onto the surface of negatively charged HSPC/DPPG liposomes with the help of positively charged chitosan was proposed and investigated based on the literature finding that the charge of insulin can

be adjusted to become either positive (pH below pI) or negative (pH above pI). Chitosan has been reported as a permeation enhancer by many groups for its ability to transiently open up the tight junction between adjacent intestinal epithelial cells. However, no studies have been found loading of insulin on the surface of liposomes based on electrostatic interaction between chitosan polymers. In this study, insulin was successfully loaded onto the surface of HSPC/DPPG liposomes with the help of positively charged chitosan layers.

The third hypothesis that loading and sustained release of insulin can be improved by coating insulin on the surface of liposome via a Layer-by-Layer approach was proposed and tested based on the literature finding of the current status of LbL coated nanoparticles which has been investigated by Paula et al. using LbL system to carry siRNA for anticancer treatment. The LbL coated particle has been reported for its prolonged sustained release, improved loading, and tumor cell uptake *in vivo* and *in vitro*. In an attempt to test the hypothesis, a Layer-by-Layer approach to coat insulin on the surface of HSPC/DPPG liposomes was developed which significantly prolonged the release of insulin for up to 3 months. The Layer-by-Layer coated liposomes demonstrated stability for up to 1 month in PBS. With only 1 layer of insulin loaded on the surface of liposomes, the transport of insulin across Caco-2 cells was enhanced by 2.6 fold from 5.7ng/ml to 15ng/ml.

The current research findings provide invaluable insights into the potential application of liposomes based surface modifications for oral delivery of protein drugs. The amount of insulin transported across Caco-2 monolayer with surface modified liposome *in vitro* is 35.7ng/ml, which is higher than the concentration of insulin required during fasting (1 ng/ml) or fed state (4.6 ng/ml) in physiological conditions.^[1] Given that the insulin that crossed the intestinal epithelial cells entering portal circulation is able to sensitize hepatic glucose uptake at much lower insulin dose as compared to conventional subcutaneous insulin injection^[2-4], our findings provide a more efficient treatment option for diabetics. The research direction coincides with one of Diasome Pharmaceuticals's technologies which focuses on developing hepatic-directed vesicles-insulin (HDV-I) targeting delivery of insulin loaded nanoliposomes to the liver. This technology has shown notable progress and has been approved by FDA for clinical trial Phase III.^[5]

Last but not least, LbL coating of insulin on the surface of insulin-loaded liposome has achieved significant improvement of loading and release compared to the insulin-loaded liposome alone. This technology could be potentially incorporated into a mucoadhesive intestinal patch that adheres to the luminal wall of small intestine for localized sustained release of insulin loaded LbL-liposomes.^[6]

7.2 Future research directions

In this section, the future research directions are planned and proposed to expand the knowledge and understanding of current liposome based oral delivery system. A closed loop fluidic system is proposed for upscale and improve the efficiency of synthesizing Layer-by-Layer liposome (LbL-liposomes). Bioactivity of transported insulin can be further investigated by using 3T3 adipocyte cell model to study glucose uptake. The multi-layers on the LbL surface could be optimized for co-delivery of GLP-1 and insulin for enhanced glucose lowering effect. The outermost layer of the LbL-liposomes could be replaced by a PEG capping layer to improve the blood circulation time of liposomes.

7.2.1 Improving the efficiency of LbL synthesis with closed loop fluidic system

Efficiency of the LbL-liposome fabrication process is limited due to the centrifuge-based purification method. Loss of liposomes is expected during repeated cycles of coating, centrifugation, transfer, and resuspension. This can be improved by switching the sample purification system from ultracentrifugation to a continuous fluidic system that includes dynamic control with purifying columns to dialyze, purify, and concentrate the coated liposomes (**Figure 46**). By using a closed system with a series of dialysis columns, the loss of liposomes could be minimized and the efficiency of the coating process is expected to be improved. Additionally, a recycled solvent reservoir system reduces the demand and wastage of insulin, making the process more sustainable, affordable and less time consuming. A schematic drawing of how such purification system could be designed is shown **Figure 46**. A Pellicon® Ultrafiltration system from Millipore could be considered with some modifications to suit the need of the closed system design. Such a set up could potentially allow scaling up of LbL-liposomes production.

Figure 48. Schematic drawing a continuous fluidic system for improving the efficiency of LbL synthesis.

7.2.2 Bioactivity study by using 3T3 cell line

The next evaluation of transported insulin in the basal compartment would be the bioactivity assessment, which measures the functionality or ability of the transported insulin to bind to the insulin receptor and trigger the desired glucose lowering effect. A differentiated adipocyte cell line 3T3-L1 serves as an excellent experimental model for testing the effect of drugs on cellular uptake of glucose, because the fully differentiated 3T3-L1 is particularly responsive to insulin and glucose uptake can be highly activated in the presence of insulin.^[7] Bioactivity of the transported insulin could be investigated by adopting a layer of 3T3 adipocyte cell line at the basal compartment of the transwell, allowing direct measurement of glucose uptake in the basal compartment (**Figure 47**).

Figure 49. Design of the bioactivity study of transported insulin using 3T3 cell models

7.2.3 Modifying the layers to enhance loading and transport

The chitosan layer used in this model serves as a platform for loading other positively charged polymers with potential permeation enhancing properties for oral delivery of protein drugs. The release could be modulated by adopting polymers with different strength of interaction with the protein drug. For example, the release of insulin could be further extended by using a polymer that has a stronger binding strength to insulin, or shortened by using a polymer that has a weaker affinity to insulin. The binding strength of the polymer to insulin could be studied by nanoITC. Glucagon-like peptide-1 (GLP-1) is a 30 amino acid peptide hormone secreted by the intestinal enteroendocrine L-cells into the portal circulation to lower the blood glucose level in a glucose dependent manner.^[8] By using the LbL approach, co-delivery of insulin and GLP-I could be achieved by incorporating the peptide in the multilayers so that the glucose lower effect could be enhanced. Outermost layer of the LbL could be designed with a layer of PEG to provide the liposomes with stealth properties, allowing for extended blood circulation time and reduced protein corona formation.^[9]

7.2.4 Modifying the liposome surface with Fc ligand to enhance transport

In addition, Fc receptor (FcR) is expressed on the neonatal intestinal cells for receptor mediated uptake and transcytosis of IgG antibody from mother to offspring during breast milk feeding.^[10] As a future research direction, the modification of liposome surface with Fc is proposed to improve the uptake and transport of insulin loaded liposomes. Preliminary studies of Fc modified liposome showed successful conjugation of Fc onto HSPC/DOTAP liposomes surface via the chemistry between DSPE PEG Maleimide lipids, as represented by an increase in both size and zeta potential in **Figure 48**. Liposome modified with Fc showed a 2x increase in cellular uptake compared to negative control (HBSS) (**Figure 49**). When Eudragit S100 coating was applied onto the Fc modified liposome with the help of Tween 80, up to 13.1% increase in cellular uptake was observed by flow cytometry (**Figure 49**). Removing the free EuTw caused the cellular uptake to decrease to 3.7%, which is still more than 7x higher than negative control (HBSS) and almost 4x higher than Fc modified liposome (**Figure 49**). These results supported the effectiveness of Fc in enhancing the cellular uptake of liposomes and thus could be further investigated with transport studies.

Figure 50. DLS measurement of liposome size before and after Fc ligand modification

Figure S1. Flow cytometry study of uptake of liposomes surface modified with Fc and Eudragit S100 coating

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