

**ENCAPSULATION OF BIOACTIVE SALMON PROTEIN HYDROLYSATES
WITH CHITOSAN-COATED LIPOSOMES**

by

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For my parents, without whom none of this would have been possible.

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ABSTRACT

Bioactive low molecular weight protein hydrolysates need to be protected, transported to the targeted absorption site, and released in a controlled manner to optimize their effectiveness during oral administration. The focus of this research was to develop a chitosan-coated liposomal oral delivery system with milk fat globule membrane (MFGM) phospholipids for antidiabetic Atlantic salmon protein hydrolysates (SPH). The size, zeta potential, entrapment efficiency, stability during freeze-drying and freeze thawing, and long-term storage abilities were investigated as a function of phospholipid concentration and chitosan coating concentration. Chitosan coating greatly improved the stability of MFGM liposomes. The maximum encapsulation efficiency (71.3%) and physical stability were achieved with 10% MFGM and 0.4% chitosan. Chitosan coating significantly prolongs the release of SPH in simulated biological fluids. In conclusion, liposomes with “optimal” chitosan-coating-concentration show great promise as a potential new delivery system for protein hydrolysates. However, the bioactivity of encapsulated SPH need to be tested.

LIST OF ABBREVIATIONS USED

CH	Chitosan
EE	Encapsulation efficiency
FD-RH	Freeze-drying and rehydration
FT	Freeze thawing
MFGM	Milk fat globule membrane
SEM	Scanning electron microscopy
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SPH	Salmon protein hydrolysates
TEM	Transmission electron microscopy
T_c	Phase transition temperature
w/v	weight per volume
w/w	weight per weight

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CHAPTER 1 INTRODUCTION

Commercial interest in functional foods and nutraceuticals has been growing quickly in the global market. Low molecular weight (LMW) protein hydrolysates have been found to provide exceptional health benefits and have been developed into nutraceuticals (Mine *et al.*, 2010). By virtue of their small size, LMW hydrolysates are highly digestible and as such, are less likely to elicit an immune response, unlike the proteins from which they have been derived. Many hydrolysates have been found to provide specific bioactivities, such as antioxidative, antihypertensive, antidiabetic, etc., depending on the protein source (Yang *et al.*, 2012). However, the likelihood of reduced hydrolysate potency as a result of over-digestion, poor absorption and the potential production of unpleasant bitter flavors may restrict the application of protein hydrolysates in food systems (Chen *et al.*, 2006; Rocha *et al.*, 2009).

Bioactive peptide delivery systems are as important as the peptides themselves. A good carrier system should provide controlled release and enhancement of bioavailability while minimizing side effects and toxicity (Rekha & Sharma, 2010). Several approaches have been applied to improve the intestinal absorption of peptides, such as the use of enzyme inhibitors, permeation enhancers and encapsulation in nano- and submicron-sized vesicles (Rekha & Sharma, 2010). The use of enzyme inhibitors and absorption enhancers will resist degradation by enzymes present in the stomach and intestine and/or will increase peptide membrane permeability (Aungst, 2000; Bernkop-Schnürch, 1998). However, long-term use of these additives has been found to permit the absorption and accumulation of unwanted peptides and a general disturbance of digestion (Shaji & Patole, 2008). On the other hand, nano- and submicron-particles, by virtue of their small size and high surface area, are believed to enhance the bioavailability of these proteinaceous drugs (Solaro, Chiellini, & Battisti, 2010).

Liposomes and polyplexes are the most studied self-assembly carriers for peptide delivery. Both liposomal and polymeric carriers can be biodegradable and non-toxic if

proper formulation and preparation methods are chosen (Thompson *et al.*, 2009; Vauthier & Labarre, 2008). In addition, liposomes can carry both hydrophilic and hydrophobic components in one single vesicle, and they can be easily produced from food grade materials (Mufamadi *et al.*, 2011). This enables liposomes to encapsulate both polar and non-polar amino acids from low molecular weight protein hydrolysates. However, liposomes are often degraded by the active pepsin in the acidic environment in the stomach as well as the enzymes in the small intestine, and they may be oxidized during processing and storage (Soltero, 2005). These problems can be resolved by adding an extra coating of chitosan, a biocompatible, biodegradable and mucoadhesive polysaccharide (Sihorkar & Vyas, 2001). The 2-component (polysaccharide and lipid) coating is intended to:

- 1) mask any unpalatable flavors of bitter hydrophobic peptides;
- 2) act as a barrier to help prevent acidic and enzymatic degradation and;
- 3) enhance the intestinal absorption of bioactive peptides (Mozafari, Khosravi-Darani *et al.*, 2008; Singh *et al.*, 2010).

The focus of this research was to develop a polymer-coated liposomal delivery system for oral administration. Atlantic salmon protein hydrolysates (SPH) known to contain antidiabetic peptides (Pilon *et al.*, 2011) were encapsulated in chitosan-coated liposomes prepared from milk fat globule membrane-derived phospholipids. A better understanding of this encapsulation technique will contribute to the development of bioactive peptide carrier systems and imparting functional properties associated with the lipid membrane and polysaccharide that ease incorporation into a variety of food products.

CHAPTER 2 LITERATURE REVIEW

2.1 Bioactive Fish Peptides as Functional Food Ingredients

2.1.1 Introduction to Bioactive Peptides

Bioactive peptides are defined as “food derived components (genuine or generated) that, in addition to their functional value, exert a physiological effect in the body” (Vermeirssen, Camp, & Verstraete, 2007). These small peptides usually have only 2-20 amino acid residues and molecular masses less than 6000 Da (Sun, He, & Xie, 2004). Many protein sources such as those derived from milk (Florisa *et al.*, 2003), eggs (Yoshikawa *et al.*, 2000), fish (Harnedy & FitzGerald, 2012), cereal grains (Matsui, Li, & Osajima, 1999) and soybeans (Chen *et al.*, 2002), have been shown to contain bioactive peptides which are inactive when intact in the parent proteins. However, when they are released by enzymatic hydrolysis during either gastric digestion or simulated gastric digestion in a processing facility, these peptides may produce a measurable health benefit, particularly during fermentation in which microorganisms and enzymes are involved (Korhonen & Pihlanto, 2006).

In recent years, more research aimed at the liberation of bioactive peptides from food protein sources has been carried out in order to find potential functional foods or nutraceutical candidates (Ryan *et al.*, 2011). During the manufacturing process of many foods, these physiologically active components are sometimes added to enrich and modify the products. Table 2-1 lists some commercially available products containing fish peptides or hydrolysates that have been approved as functional ingredients in Japan. Among these, Valturon® has been shown to reduce blood pressure and has been incorporated into 33 different products including beverages, jelly, powdered soup and dietary supplements (Harnedy & FitzGerald, 2012). This sardine peptide product has been shown to be a safe food ingredient at a level of 0.6 g/serving by the European Food Safety Authority (EFSA) on Dietetic Products, Nutrition and Allergies (EFSA, 2010). Fish bioactive peptides/hydrolysates are absorbed through the intestine into the blood,

exerting multiple effects, which may include immunomodulatory, antimicrobial, antioxidant, antithrombotic, hypocholesterolemic and antihypertensive actions (Erdmann, Cheung, & Schröder, 2008; Hartmann & Meisel, 2007; Je *et al.*, 2007).

Table 2-1. Some commercially available marine protein hydrolysate and peptide products (Harnedy & FitzGerald, 2012).

Product	Activity	Source	Manufacturer
PeptACE™	Antihypertensive	Bonito peptides	Natural Factors Nutritional Products Ltd., Canada
Vasotensin®	Antihypertensive	Bonito peptides	Metagenics, US
Levenorm®	Antihypertensive	Bonito peptides	Ocean Nutrition Canada Ltd.
Peptide ACE 3000	Antihypertensive	Bonito peptides	Nippon Supplement Inc., Japan
Lapis Support	Antihypertensive	Sardine peptides	Tokiwa Yakuhin Co. Ltd., Japan
Valtyron®	Antihypertensive	Sardine peptides	Senmi Ekisu Co. Ltd.
Stabilium® 200	Relaxing	Fish autolysate	Yalacta, France
Protizen®	Relaxing	Fish hydrolysate	Copalis Sea Solutions, France
AntiStress 24	Relaxing	Fish hydrolysate	Forté Pharma Laboratories, France
Nutripeptin™	Lowers glycemic index	Cod hydrolysates	Nutrimarine Life Science AS, Norway
Seacure®	Improves gastrointestinal health	Pacific Whiting hydrolysate	Proper Nutrition, US
Fortidium Liquamen®	Antioxidant, lowers glycemic index, anti-stress	Fish autolysate	Biothalassol, France

2.1.2 Functions of bioactive peptides from marine fish waste

Although the demand for fish is increasing, the fish stocks worldwide are static (Gildberg, 2004). It is estimated that among the 140 million tonnes of fish and shellfish produced each year, only 50 to 70% of the tissues have the potential for human consumption. The waste, including carcasses, frames, heads, intestinal organs and trimmings, is often dumped or used as animal feed and fertilizer (Guérard & Decourcelle, 2010). Recent studies have shown that a number of bioactive peptides have been isolated from various fish body parts including muscle from discarded fish frames and cut offs, collagen and gelatin from fish skin waste, bones, as well as from gills and innards (Senevirathne & Kim, 2012). Therefore, it is sensible to promote better utilization of fish waste and these by-products for foods and nutraceuticals (Gildberg, 2004). Table 2-2 is a summary of bioactive protein hydrolysates and peptides derived from fish.

Table 2-2. Bioactive peptides and protein hydrolysates derived from fish waste (Harnedy & FitzGerald, 2012).

Common name	Origin	Biological activity	Peptide(s) sequence
Cod	Frame	Antioxidant ACE inhibitory	-
Herring	Body, Head, Gonads	Antioxidant	-
Yellowtail	Bone, Scale	Antioxidant ACE inhibitory	- -
Tuna	Frame	Antioxidant Antihypertensive	VKAGFAWTANQQLS GDLGKTTTVSNWSPPKYKDTP
Sole	Frame	Anticoagulant Antioxidant	- N-terminal RPDFDLEPPY
Pollack	Skin Frame	ACE inhibitory ACE inhibitory Antioxidant Ca-binding	GPL, GPM FGASTRGA LPHSGY VLSGGTTMAMYTLV

Antioxidative peptides have been recovered from various fish sources and can reduce peroxidation of lipids, scavenge free radicals, and chelate transition metal ions (Rajapakse, Mendis, Byun, & Kim, 2005). Hydrolysates released from mackerel with Protease N were found to inhibit the autoxidation of linoleic acid and reduced Fe³⁺ to Fe²⁺ *in vitro* according to the ferric thiocyanate method (Wu, Chen, & Shiau, 2003). In another study, seven antioxidant peptides were isolated from the processing waste from

sardinelle (Bougatef *et al.*, 2010). All of the seven peptide fractions (Table 2-3) obtained were less than 600 Da, and they displayed different free radical α,α -diphenyl- β -picrylhydrazyl (DPPH) scavenging abilities, with the highest being 63% at a peptide concentration of 150 $\mu\text{g}/\text{mL}$ (Bougatef *et al.*, 2010). More recently, peptides derived from Atlantic salmon frame hydrolyzates have been shown to possess anti-oxidative properties (Girgih *et al.*, 2013).

Table 2-3. Peptides identified by MS/MS in fractions P4-1, P4-2, P4-3, P4-4 and P4-5 separated by RP-HPLC (Bougatef *et al.*, 2010).

Fractions	MW (Da)	Sequence	DPPH scavenging activity (%)
P ₄₋₁	471.3	Leu-Ala-Arg-Leu	51 \pm 1.31
P ₄₋₂	263.08	Gly-Gly-Glu	38 \pm 1.27
P ₄₋₃	431.2	Leu-His-Tyr	63 \pm 1.5
P ₄₋₄	283.1	Gly-Ala-His	52 \pm 1.44
	403.1	Gly-Ala-Trp-Ala	
	528.2	Pro-His-Tyr-Leu	
P ₄₋₅	538.2	Gly-Ala-Leu-Ala-Ala-His	54 \pm 1.38

Antimicrobial activity was also discovered in bioactive peptides derived from fish. Iijima *et al.* (2003) isolated three isoforms of a novel C-terminally amidated peptide from the gills of red sea bream, *Chrysophrys (Pagrus) major*. Due to the amphiphilic and highly cationic properties of the peptides, they exerted a broad spectrum bactericidal effect against Gram-negative and Gram-positive bacteria, including *Escherichia coli*, and *Bacillus subtilis*. Meanwhile, a cysteine rich antimicrobial peptide was identified in oyster muscle (Liu *et al.*, 2008). Pleurocidin, a 25-residue linear peptide isolated from the skin mucous secretions of the winter flounder, was found to possess antimicrobial properties (Cole, Weis, & Diamond, 1997). Furthermore, protamines, arginine-rich small

linear antimicrobial peptides commonly isolated from the nuclei of fish spermatozoa, were reported to possess broad-spectrum antimicrobial properties, and Gram-negative bacteria are shown to be the most susceptible (Hansen & Gill, 2000). McClean (1931) first discovered the antimicrobial activity of protamine, however it wasn't until the 1980's that protamine was identified as a natural source of antimicrobials (Gill *et al.*, 2006). It is believed that the cationic protamine first binds with the anionic cell envelope, and then exerts its antimicrobial effect through membrane disruption and leakage of potassium ions, adenosine tri-phosphate (ATP) and intracellular enzymes (Potter, Truelstrup Hansen, & Gill, 2005).

Antihypertensive peptides (ACE inhibitory peptides) have been isolated from Alaska pollock frame protein which is usually treated as a waste by-product during fish processing (Je *et al.*, 2004). Another ACE inhibitory peptide was isolated from waste yellowfin sole frame protein by Jung *et al.* (2006). More ACE inhibitory peptides were found in chum salmon muscle (Ono *et al.*, 2003), dark tuna muscle (Qian, Je, & Kim, 2007) and shark meat (Wu *et al.*, 2008).

Antitumor and antiproliferative fish peptides have been discovered from fish proteins and have been shown to induce cancer cell death, indicating a high potential for therapeutic applications (De Vries & Beart, 1995). Recently, antiproliferative activity was found in hydrolysates from tuna dark muscle. The bioactivity was demonstrated in tissue culture experiments using a human breast cancer cell line, MCF-7 (Hsu, Li-Chan, & Jao, 2011). Several fractions of a peptide mixture from the tuna dark muscle, with a molecular weight range of 400 to 1400 Da, were identified as having the ability to reduce tumor growth. Picot *et al.* (2006) also found antiproliferative peptides active against the breast cancer cell line MCF-7. These peptides were isolated from blue whiting, cod, plaice and salmon species, with various sizes up to 7 kDa.

In addition, marine bioactive peptides have been shown to have antidiabetic activity. These peptides are found in fish and fish by-products such as cod (*Gadus morhua*) (Chiasson *et al.*, 2003; Ouellet *et al.*, 2007; Zhu *et al.*, 2010), shark liver (Huang & Wu,

2010), and salmon (Pilon *et al.*, 2011). Lavigne *et al.* (2000, 2001) found that cod proteins have the potential to improve glucose tolerance and insulin sensitivity in high fat fed rats, and this might be attributed to certain amino acids. Zhu *et al.* (2010) found that marine collagen peptides (MCPs) dramatically reduced the level of hs-CRP, a free fatty acid that is related to diabetes. Therefore, MCPs could be a potential protective source for diabetic patients. Ouellet *et al.* (2007) reported that dietary cod protein significantly improved insulin sensitivity in insulin-resistant men and women compared to a similar diet containing lean beef, pork, veal, eggs, and milk products. In Huang & Wu's (2010) study, a new peptide S-8300 was purified from shark livers and deemed to have antidiabetic functions. S-8300 contained 17 amino acids and the N-terminus had the sequence of NH₂-Met-Leu-Val-Gly-Pro-Ile-Gly-Ala-Ala-Lys-Val-Val-Tyr-Glu-Gln. S-8300 was found to have an adverse effect on streptozotocin (STZ), a naturally occurring chemical that damages insulin-producing cells, which decreases the secretion of insulin by preventing apoptosis of pancreas cells (Chen, Yu, & Shen, 2004). Pilon *et al.* (2010) demonstrated the effect of salmon protein hydrolysates (SPH) on insulin sensitivity in high fat fed rats. Pilon *et al.* (2010) supplemented the diets of high fat- and high sucrose-induced diabetic rats with various fish protein diets containing casein, bonito, herring and mackerel. After 28 days, they found that the salmon-protein fed group showed the most significant decrease in weight, improved whole-body insulin sensitivity and insulin and C-peptide secretion. They reported that the bioactive peptides prepared in the Jin (2012) study were responsible for the anti-diabetic effects and were derived from Atlantic salmon muscle digested with pepsin, trypsin and chymotrypsin and had molecular weights of under 1 kDa. This low molecular weight salmon protein hydrolysate (SPH) improved the glucose uptake in mouse myocytes, decreased the glucose production of rat hepatocytes and attenuated the macrophage inflammatory profile (Pilon *et al.*, 2010).

These findings emphasize the potential value of fish by-products as potential sources of bioactive peptides for both functional food and nutraceutical industries.

2.1.3 Bitterness of protein hydrolysates

Bitterness has been related to the enzymatic production of protein hydrolysates from gelatin and casein as early as 1952 (Murray & Baker, 1952). The bitterness is caused by the adsorption of hydrophobic peptides onto the human hydrophobic bitter taste receptors of the tongue (Ishibashi *et al.*, 1988). Both children and animals have been observed to reject casein hydrolysates from fermented milk products because of bitterness (Figueroa *et al.*, 2008; Kimball *et al.*, 2008; Kimball *et al.*, 2005; Mennella *et al.*, 2004). Recently, it was shown that the human bitter taste receptors T2Rs are activated by synthetic bitter dipeptides Gly-Phe and Gly-Leu (Maehashi *et al.*, 2008). Similarly, Upadhyaya *et al.* (2010) found *in vitro* that di- and tripeptides derived from food proteins can activate the human bitter receptor T2R1. Some of the peptides with ACE-inhibitory activity were also found to be able to activate the T2R1 receptor.

New methods need to be developed to improve the palatability of bitter protein hydrolysates. Simply adding sugar and salt to minimize the bitter sensation does not work for products that have high peptide content or peptides that have high ratio of bitter components (Mozafari *et al.*, 2008). Many techniques have been applied to reduce the bitterness, such as treatment with activated carbon, hydrophobic interaction chromatography and hydrolysis with exopeptidases and the plastein reaction, a reversal of enzymatic protein hydrolysis (Lin *et al.*, 1997; Pedersen, 1994; Stevenson *et al.*, 1998). Nonetheless, these methods have several drawbacks. They mainly focused on the cleavage of peptide bonds and removal of end-chain hydrophobic amino acids such as phenylalanine and tryptophan. These methods will also increase the free amino acid content, leading to a high osmolality and low yield (Pedersen, 1994; Stevenson *et al.*, 1998). Encapsulation is a promising method to eliminate bitterness of protein hydrolysates/peptides because it coats the bioactive hydrolysates/peptides and prevents the adsorption of bitter peptide to taste receptors without altering peptide structure. However, the release mechanisms of various encapsulated peptides has not been studied in detail, and the effect of encapsulation on bioavailability needs to be further investigated (Li Chan & Cheung, 2010; Mine *et al.*, 2010).

2.1.4 Fate of protein and peptides in human GI tract

Pepsin digestion begins in the stomach at a pH range of 1.5-3. Pepsin is activated in an acidic pH environment, and mainly catalyzes the hydrolysis of Phe, Tyr and Leu, producing long polypeptides, oligopeptides and some free amino acids (Erickson & Kim, 1990). Then, the digested products enter the small intestine and are further digested by enzymes released by the pancreas, such as trypsin, chymotrypsin, elastase and carboxypeptidase A and B. The pancreatic enzymes are activated in neutral or slightly alkaline environments in the duodenum, wherein gastric pepsins are inactivated (Erickson & Kim, 1990). Trypsin cleaves basic amino acids Arg and Lys on the carboxyl terminal of the peptide chain. Chymotrypsin hydrolyzes amino acids with aromatic carbonyl groups, such as Tyr, Phe and Trp. Elastase cleaves interior peptide bonds (endopeptidase) between amino acids containing aliphatic carbonyl groups, such as Ala, Leu, Gly, Val and Ile. Carboxypeptidases A and B digest single amino acids from the carboxyl terminals of peptide chains (exopeptidases). These pancreatic enzymes hydrolyze peptide chains into short oligopeptides (2-6 amino acids) and single amino acids (Erickson & Kim, 1990).

The small intestine is the main absorption site for protein digestion products. Small peptides, such as di- and tripeptides and free amino acids can be absorbed directly into the intestinal epithelium (Roberts *et al.*, 1999). However, only some fragments can enter the blood stream intact in physiologically active amounts without being hydrolyzed by cytoplasmic lysosomal peptidases, depending on their terminal amino acid composition (Lee, 2002; Robert & Zaloga, 1994). For example, some dipeptides and short oligopeptides from casein hydrolysates that contain proline and hydroxyproline in the C-terminal are resistant to lysosomal peptidase digestion (FitzGerald & Meisel, 2000). On the other hand, peptides of more than three amino acids are further digested extracellularly into peptides containing 2 or 3 amino acids by the peptidases embedded in the microvilli of the intestinal epithelium, also known as brush border enzymes, before absorption (Segura-Campos *et al.*, 2011).

Generally speaking, the absorption of molecules can be divided into two different pathways, transcellular and paracellular (Blanchette, Kavimandan, & Peppas, 2004). Dipeptides and tripeptides can be absorbed actively through the epithelia membrane with the help of a proton gradient (Yang, Dantzig, & Pidgeon, 1999), whereas oligopeptides are transferred through other routes such as pinocytosis or paracellular channels, depending on their size and hydrophobicity (Robert *et al.*, 1999). The uptake of peptides through trans-cellular pathways decreases with increasing peptide size and decreasing hydrophobicity (Lee, 2002). Tight epithelial junction proteins control the paracellular pathway by modulating the tight junctions between cells. This pathway is most favourable for the uptake of small hydrophilic peptides (Robert *et al.*, 1999).

2.2 Encapsulation of Peptides by Nano-carriers

2.2.1 Challenges in oral delivery of bioactive peptides

Bioactive peptides must remain active during digestion and absorption before entering the blood stream at significant levels in order to exert potential physiological effects, even though there are various barriers once they enter digestive systems (Segura-Campos *et al.*, 2011). The main objective of oral delivery is to protect bioactive peptides against the gastrointestinal environment and enhance absorption. Bioactive peptides may be denatured and lose their bioactivities in the acidic environment of the stomach; proteolytic enzymes in the stomach and intestine will also degrade small peptides; mucin acts as a barrier for intestinal adsorption; and as discussed above, unpalatable bitterness of hydrophobic peptides also hinders the application of protein hydrolysates into functional food products (Rekha & Sharma, 2012).

2.2.2 Enhanced bioavailability of functional peptides: Oral approaches

In order to protect bioactive peptides against acid and enzymatic degradation in the GI tract and provide high transfer efficiency across the epithelium mucosa, various approaches have been studied, such as adding permeation enhancers or protease inhibitors, and chemical modification. Recently, micro-, submicro- and nano-particles

have been designed to overcome the intestinal barriers and improve bioavailability of orally administered bioactive peptides (Rekha & Sharma, 2010).

Enzyme inhibitors are sometimes co-administrated with peptides to prevent proteolytic degradation, thereby increasing the bioavailability (Aungst, 2000). However, since the inhibitors are co-administered with peptides, they are not restricted to the absorption site, thus enzyme inhibitors will cause deleterious side effects in long-term application. Once the intestinal enzyme activity is inhibited and digestion is retarded, it will lead to poor digestion of food proteins. As a result of the feedback mechanism, the pancreas will overproduce enzymes, leading to pancreatic hypertrophy and hyperplasia (Rekha & Sharma, 2010).

Permeation enhancers alter paracellular and transcellular pathways to enhance peptide absorption, by opening the cell-cell tight junctions, changing mucus layer viscosity, and modifying cell membrane structure to facilitate receptor mediated endocytosis (Salama, Eddington, & Fasano, 2006). The most commonly used permeation enhancers are bile acids, fatty acids and dicarboxylic acids (Rekha & Sharma, 2010). However, the activity mechanisms of permeation enhancers are highly correlated with induced toxicity and serious side effects, especially for patients with chronic diseases, for example diabetes, as they need a daily intake of permeation enhancers (Sweson & Curatolo, 1992). In addition, permeation enhancers not only improve the absorption of bioactive peptides through the tight junctions, they also open the up-take route for pathogenic viruses, toxic peptides etc., that may be naturally present in the GI tract (Goldberg & Gomez-Orellana, 2003).

Chemical modification is the conjugation between peptides with other polymeric moieties, such as polyethylene glycol (PEG) or ligands that are related to receptor-mediated endocytosis (Hinds & Kim, 2002; Shah & Shen, 1996). The bioactive peptides which have been PEGylated have longer systemic circulation, elicit less immune response, and are less likely to be digested by enzymes (Hinds & Kim, 2002). Conjugation of ligands such as transferrin, a natural protein that facilitates iron transport,

has been found to increase the uptake of insulin and prevent proteolysis (Kavimandan *et al.*, 2006; Shah & Shen, 1996). However, the exact mechanism is not fully understood.

The most practical approach to protect the bioactive peptides from digestion is by applying an enteric coating and forming a capsule (Martinho, Damg e, & Reis, 2011). The inactivation, degradation and metabolism phenomena of peptides and proteins are minimized in transit. Once the capsule reaches its desired absorption site (wall of the small intestine), it will be triggered to release its bioactive payload (Esser-Kahn *et al.*, 2011).

2.2.3 *Current approaches to encapsulate peptides in nanoparticles*

Nanoencapsulation is a technique whereby solids, liquids or gases are enclosed within a thin film of wall material to generate nanoscopic particles (Esser-Kahn *et al.*, 2011). The particles are usually free flowing powders, made up of a liquid or solid functional core and a polymeric continuous outer coating and have a particle size less than 1 μm . Depending on the morphology and internal structure of the final product, they are named “nanoparticle”, “nanocapsule”, or “nanosphere” (Saze, Hernandez, & Peniche, 2007). Nanosized carriers improve transit across biological barriers that would otherwise be difficult for peptides to accede to the site of interest. Moreover, they have a high surface area to volume ratio, providing improved solubility (Solaro *et al.*, 2010). The following aspects are the basic prerequisites for designing appropriate carriers for peptide delivery (De Jong & Borm, 2008):

- Peptide incorporation and release
- Biocompatibility
- Formulation stability and shelf life
- Bio-distribution and targeting
- Functionality
- Residual material

This class of carriers includes complexes based on protein or colloidosomal aggregates of latex particles, polyplexes, liposomes, colloidal gold, silica, and superparamagnetic particles (Solaro *et al.*, 2010). Based on the above criteria, biodegradable nanoparticles would be optimal. Liposomes and polyplexes are the most studied self-assembly

nanoparticles for peptide delivery, which are formed through intermolecular forces (Solaro *et al.*, 2010).

2.2.4 Polymeric nanoparticles

Polymeric nanoparticles contribute to a versatile delivery system, as most of the polymers can be easily modified. This allows the particles to cross biological barriers and deliver the peptide core into intracellular compartments (Solaro *et al.*, 2010). However, only a limited number of polymers can be used for nanocarriers to deliver peptides (Table 2-4) (Qiu & Bae, 2006). A proper coating polymer must be eliminated from the body to avoid accumulation, especially for repeated administration. In addition, the polymer and its degradation products must be non-toxic and non-immunogenic. Finally, the polymeric particles must be able to encapsulate the selected peptide and endure the GI environment (Vauthier & Labarre, 2008). One problem generally associated with nanoparticles is their low absorption efficiencies, as the proportion of intact particles is usually found to be below 5% (Shaji & Patole, 2008).

Mucoadhesive polymeric nanoparticles are one of the most promising approaches among all the polymeric nanocarriers (Shaji & Patole, 2008). The mucoadhesive property enables the carriers to come in close contact with the mucosa at the absorption site, preventing the elimination of peptides on the way to the absorption membrane in the gastrointestinal tract. Moreover, it increases the residence time of peptides at the uptake site, resulting in increased bioavailability of peptide core material (Mahato *et al.*, 2003). Only a limited number of polymers can be used for the formulation of mucoadhesive particles. Examples of polyacrylic acid-based polymers are polyacrylic acid, poly(isohexacyanoacrylate) and poly(isobutylcyanoacrylate). Two semi-natural mucoadhesive polymers are chitosan and poly(vinyl alcohol) (Shaji & Patole, 2008; Solaro *et al.*, 2010).

Table 2-4. Most widely used polymers as nano-sized drug carriers (Vauthier & Bouchemal, 2009).

Material	Full name	Abbreviation
Synthetic homopolymers	Poly(lactide)	PLA
	Poly(lactide-co-glycolide)	PLGA
	Poly(ϵ -caprolactone)	PCL
	Poly(isobutylcyanoacrylate)	PICBA
	Poly(n-butylcyanoacrylate)	PBCA
	Polyacrylates and Polymethacrylates	Eudragit (commercial name)
Natural polymers	Chitosan	
	Alginate	
	Gelatin	
	Albumin	
Copolymers	Polyactide-poly(ethylene glycol)	PLA-PEG
	Poly(lactide-co-glycolide)-poly(ethylene glycol)	PLGA-PEG
	Poly(ϵ -caprolactone)-poly(ethylene glycol)	PCL-PEG
	Poly(hexadecylcyanoacrylate-co-poly(ethyleneglycol)cyanoacrylate)	Poly(HDCA-PEGCA)

A variety of methods have been developed for polymer carrier preparation. Generally, these methods include two main steps: emulsified system preparation and particle carrier formation (Vauthier & Bouchemal, 2009). The first step is to prepare an emulsified system. The emulsified system requires two immiscible phases and a surfactant to form a

dispersion of one phase in the other. Most of the emulsification methods require high-energy mechanical processes, such as the use of a colloidal mill and extrusion processing. These mechanical processes deliver uniform-sized droplets and can be easily scaled up to meet industrial needs (Vauthier & Bouchemal, 2009). The colloidal mill uses shear stress formed by a rotor and stator to induce the breaking of a pre-emulsion that contains larger parent droplets into uniformed small daughter droplets (Figure 2-1) (Stork *et al.*, 2003). In an extruder, the coarse dispersion is passed through a microfiltration device in a continuous manner (Charcosset, El-Harati, & Fessi, 2005). Meanwhile, novel types of emulsification methods have been introduced: microemulsification and miniemulsification. In both processes, they are composed of two immiscible organic phases (Bouchemal *et al.*, 2004). The formation of nanoparticles is usually based on the gelation of polymers or a monomer. Some nanoparticles are formed at the same time as emulsification, while a few others do not require the prior preparation of an emulsion to obtain nanoparticles. The latter methods are based on the spontaneous dispersion formation or on the self-assembly of macropolymers (Vauthier & Bouchemal, 2009). Table 2-5 summarizes the most common methods to prepare nanoparticles.

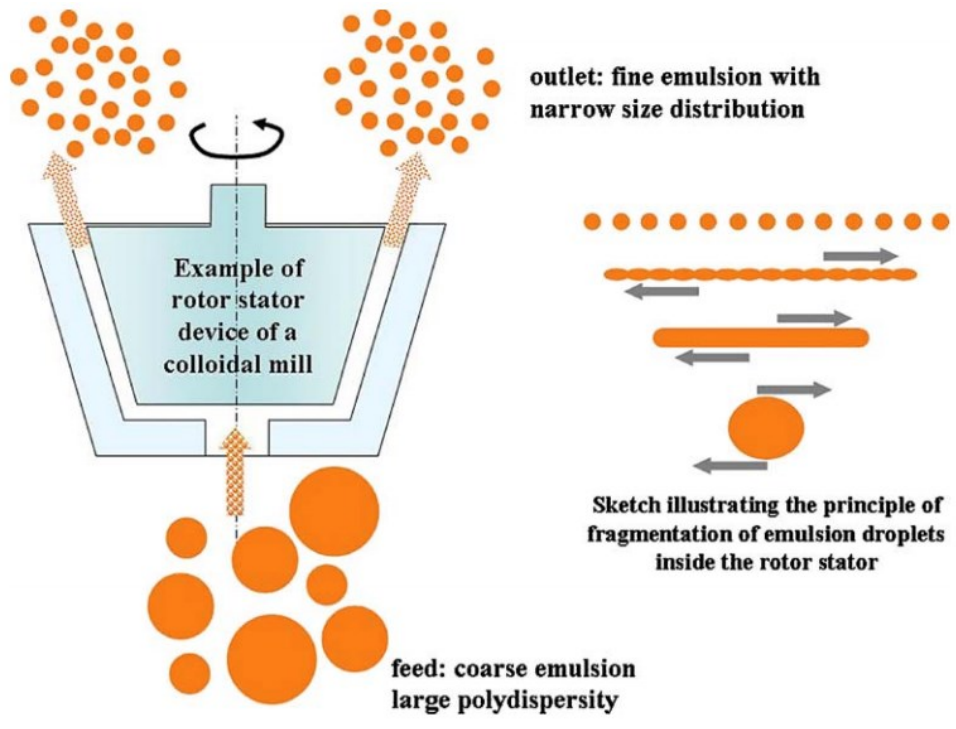


Figure 2-1. Schematic showing the principle of fine emulsification using a colloidal mill (Vauthier & Bouchemal, 2009).

Table 2-5. Summary of nanoparticle preparation methods (Vauthier & Bouchemal, 2009).

Method	Advantages	Disadvantages
Emulsification-solvent evaporation	Possibility to encapsulate both hydrophilic and hydrophobic drugs	Possible coalescence of the nanodroplets during evaporation
Emulsification-solvent diffusion	Control of nanoparticle size. Easy to scale-up	High volume of water to be eliminated Leakage of water soluble drug into the saturated-aqueous external phase
Emulsification-reverse salting out	Minimal stress to fragile drugs. High loading efficiency. Easy to scale-up	Possible incompatibility between the salts and drugs. Purification needed to remove electrolytes
6 Gelation of emulsion droplets	Possibility to use natural macromolecules, hydrophilic and biocompatible	Limited to hydrophilic drugs
Polymerization of alkyl cyanoacrylates	Easy control of particle size by surfactant	Purification needed
Interfacial polycondensation	Low concentration of surfactants. Modulation of the nanocapsule thickness by varying the monomer concentration	Limited to the encapsulation of lipophilic drugs
Nanoprecipitation of a polymer	Simple, fast and reproducible. Low concentrations of surfactants. Easy to scale up	Low polymer concentration in the organic phase
Formation of polyelectrolyte complexes	Easy to achieve.	Necessity to optimize the ratio between negatively and positively charged molecules

Table 2-5. Summary of nanoparticle preparation methods, continued (Vauthier & Bouchemal, 2009).

Method	Advantages	Disadvantages
Formation of nanoparticles from neutral nanogels	Organic solvent free. Controlled core material release	Not yet applicable to hydrophilic drugs
Methods based on ionic gelation	Organic solvent free. Possibility to control core material release upon variation of pH or ion concentration	Possible ion disintegration due to the weakness of the ionic interactions

2.2.5 Liposomes

Liposomes have great cell membrane biocompatibility, low toxicity and are able to incorporate both hydrophilic and hydrophobic components simultaneously (Thompson *et al.*, 2009). Hydrophilic components are captured within an aqueous core and hydrophobic components are entrapped using a phospholipid bilayer(s) that surround the aqueous core (Thompson *et al.*, 2009). However, liposomes might be degraded rapidly in the body, thus limiting the release of the bioactive payload over a prolonged period. Meanwhile, there might be loss of bioactivity during the formation of liposomes due to exposure to heat and organic solvents. Liposomes are also prone to aggregate with each other to form large vesicles, which may lead to leakage of core material during reformation of vesicles (Taylor *et al.*, 2005). Therefore, the stability of liposomes has always been a major problem with this delivery system.

2.2.5.1 Structure and size

Liposomes are spherical capsules consisting of one or more phospholipid bilayers enclosing an aqueous core (Figure 2-2). The main chemical ingredients of liposomes are lipid and phospholipid molecules. Lipids are subjected to conversion by gastrointestinal lipases to their constituent fatty acids and head groups. Triacylglycerols are lipids made from three fatty acids and a glycerol molecule. Mono- and diacylglycerols are glycerol mono- and di-esters of fatty acids. Phospholipids are similar to triacylglycerols except that the first hydroxyl of the glycerol molecule has a polar phosphate-containing group in place of the fatty acid. Phospholipids are amphiphilic, possessing both hydrophilic and hydrophobic groups. The head group of a phospholipid is hydrophilic, and its fatty acid tail (acyl chain) is hydrophobic (Mozafari, 2010). In order to reach a thermodynamic equilibrium, phospholipids self-aggregate into a sphere, with polar head groups oriented toward the aqueous phase and non-polar tails away from the water region (Solaro *et al.*, 2010).

Liposomes usually range in size from 20 nm to 5000 nm, consisting of one or more lipid bilayers. They can be divided into three classes based on their size and number of bilayers (Mozafari *et al.*, 2008):

- Large multilamellar vesicles (MLV). These liposomes contain several small vesicles within one large vesicle.
- Large unilamellar vesicles (LUV). These usually range from 100-500 nm with one lipid bilayer.
- Small unilamellar vesicles (SUV). These liposomes are the smallest in size (20-100 nm) surrounded by a single phospholipid bilayer.

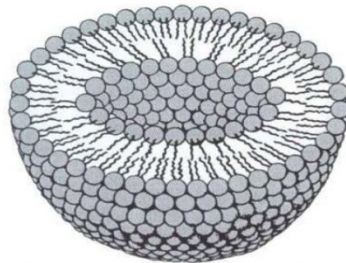


Figure 2-2. Liposome structure formed by phospholipids (Mozafari *et al.*, 2008)

The size and lamellarity of liposomes depends on the amount of energy input during the dispersion process (Mozafari *et al.*, 2008). González-Rodríguez *et al.* (2007) added a buffer to evaporated samples after preparing liposomes by the thin film method. Then the samples were left in a sonicator or treated with other mechanical treatments such as agitation, homogenization and extrusion. The results showed that the size and number of lipid layers decrease with the increasing of mechanical stress.

Nanoliposomes are nanometric versions of liposomes and have the same chemical, structural and thermodynamic properties as liposomes (Mozafari *et al.*, 2008). However, compared to liposomes, nanoliposomes offer numerous advantages over microliposomes or any larger sized liposomes such as providing more surface area, increasing solubility, enhancing particle uptake efficiency, and improving controlled release and precision targeting (Mozafari, 2010).

2.2.5.2 Phase transition temperature (T_c)

Lipid based delivery systems generally exhibit low encapsulation efficiency due to the low permeability of core material (Mozafari, 2010). Nonetheless, when the temperature is raised beyond the phase transition temperature (T_c) of the lipids, the permeability alters. Phase transition temperature also known as gel to liquid transition temperature, is the temperature at which the liposomes are less rigid and their fluidity increases. In general, T_c is increased by increased chain length, the saturation of acyl chains and the decrease in the number of branched chains (Mozafari, 2010).

Knowledge of T_c is essential for liposome preparation, as it controls the permeability and stability of liposomes. If the temperature is below the T_c , liposomes made of pure phospholipids will not form (Mozafari, 2010). For instance, the suggested liposome preparation temperature for dipalmitoyl phosphatidylcholine ($T_c = 41^\circ\text{C}$) is 51°C , 10°C higher than its T_c . This temperature ensures all lipids are dissolved in the suspension and have sufficient suppleness to form spherical vehicles. However, liposome formation temperature can be lowered by the addition of cholesterol (Leserman, Machy, & Zelphati, 1994).

2.2.5.3 Preparation methods

In laboratories, liposomes are generally made by the *thin film method*. Phospholipids and other hydrophobic compounds are dissolved into an organic solvent, such as methanol or chloroform, followed by evaporation and the production of a thin film. The dried bilayer sheet is then treated with mechanical agitation or heat to incorporate the hydrophilic compounds. However, due to the introduction of organic solvent, liposomes produced this way cannot be used in food systems. This is a gentle preparation procedure for peptides and proteins with relatively low encapsulation efficiency (Mozafari *et al.*, 2008).

The *reverse phase evaporation* enables the entrapment of a large percentage of aqueous material (Akbarzadeh *et al.*, 2013). This method is based on the formation of inverted micelles, which are formed upon sonication of a buffer with a water-soluble core material

and an organic phase with phospholipid coating material. The organic material is removed under reduced pressure, forming a gel. The liposomes are formed by the removal of residual solvent using rotary evaporation under reduced pressure (Akbarzadeh *et al.*, 2013). This method provides an encapsulation efficiency of up to 65% and can be used to encapsulate small, large and macromolecules. The disadvantages of this method are the exposure to organic solvent and the formation of a heterogeneous size dispersion (Akbarzadeh *et al.*, 2013).

Sonication of phospholipid dispersions is another way to manufacture liposomes (Mozafari, 2010). It is perhaps the most extensively used method to prepare SUVs (Akbarzadeh *et al.*, 2013). There are two techniques: one is to immerse the tip of a sonicator into a MLV dispersion, and the other is to put the dispersion into a beaker and then place it into a bath sonicator. Tip sonication is the most widely used method for the preparation of liposomes on a small scale. The main disadvantages of this method are low encapsulation efficiency, possible degradation of phospholipids and core material due to hot sonicator tips (probe sonicator), metal pollution from the probe tip and co-production of MLVs and SUVs (Akbarzadeh *et al.*, 2013).

Liposomes can also be prepared by *high-pressure homogenization*. The two types of homogenizers used are the microfluidization (Microfluidics, Inc.) and piston-gap homogenizers (e.g., APV Gaulin, Avestin, etc.) (Keck & Müller, 2006). The high-pressure homogenization method is suitable for food industries as it does not require organic solvents or detergents, and can be easily carried onto commercial scale (Farhang, Kakuda, & Corredig, 2012; Thompson & Singh, 2006). Microfluidizers separate the coarse liposomal suspension into two streams which then collide frontally, leading to particle collision and shear forces (Figure 2-3) (McClements, 2005). In recent research, small monodispersed liposomes with diameters of 100-130 nm were obtained with soybean phospholipids (Alexander *et al.*, 2012). Meanwhile, liposomes produced using microfluidization showed high encapsulation efficiencies for commercial enzymes (Nongonierma *et al.*, 2009). A schematic diagram for piston-gap homogenizers is shown in Figure 2-4. The homogenization process involves forcing the suspension through a

small orifice. The suspension is stored in a cylinder before entering the thin gap. When the liquid enters the homogenization gap, according to Bernoulli's law, the reduction in the diameter decreases the static pressure below the vapor pressure of water at room temperature. Water starts boiling and generates gas bubbles, which explode when the suspension leaves the gap (cavitation) (Patravale, Date, & Kulkarni, 2004). In addition, the high energy dissipating in the suspension during homogenization leads to collision of the liposome particles under intense turbulence. The implosion forces during cavitation and collision are sufficiently high to reduce the sizes of liposomes (Patravale *et al.*, 2004).

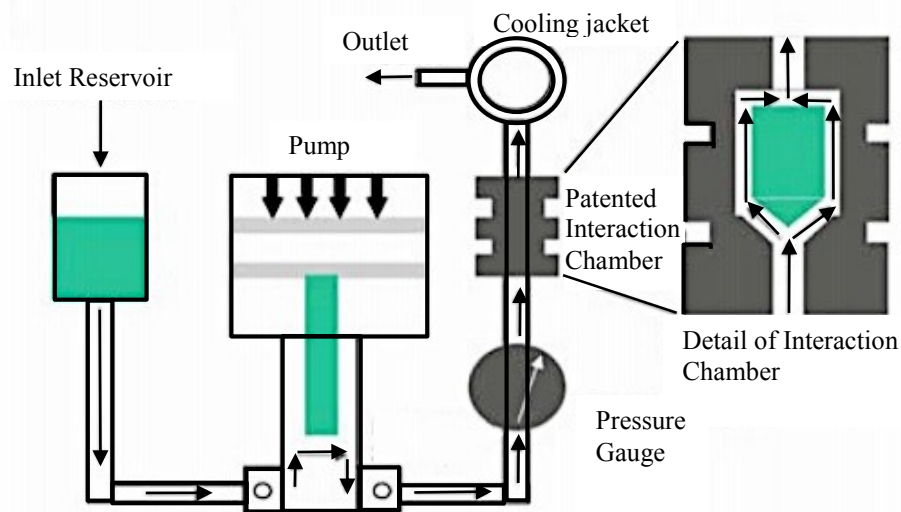


Figure 2-3. Microfluidizer mechanism diagram (Spence, Venditti, & Rojas, 2010).

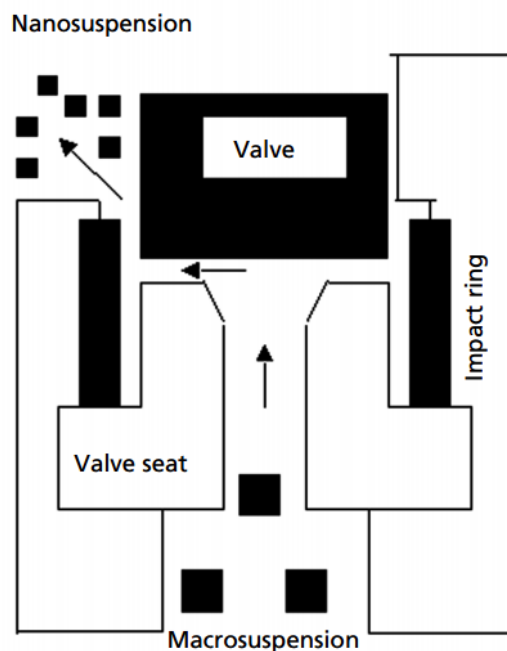


Figure 2-4. High-pressure homogenizer mechanism diagram (Patravale *et al.*, 2004).

The recently developed *heating method* is a rapid process for liposome production that does not use any organic solvent (Mozafari, 2010). Liposome components are wetted and heated with the presence of 3% (v/v) of glycerol in a temperature range of 40-120°C. Glycerol is soluble in water, and it stabilizes the liposome structure and does not need to be removed. No degradation of lipids was observed during heating (Mozafari, Reed, Rostron, Kocum, & Piskin, 2002). The heating method has three advantages: 1) no need to sterilize the final product if high temperature is used; 2) non-toxic, biodegradable glycerol is used during preparation – it prevents sedimentation and coagulation; 3) glycerol also improves the stability during freezing and thawing. Therefore, it is ideal for the formation of dry powder by freeze-drying (Mozafari, 2005). Heat sensitive drugs can be incorporated into liposomes after the liposomes are formed by incubation at room temperature (Mozafari *et al.*, 2004). Mozafari and his team showed that nano-liposomes prepared by the heating method are completely non-toxic towards cultured cells while nano-liposomes prepared by a conventional method using volatile solvents showed significant levels of cytotoxicity (Mozafari, Reed, & Rostron, 2007).

The *Mozafari method* (Mozafari, 2010) is an improved version of the heating method. It is believed to be one of the easiest methods for preparing liposomes. It allows the formation of liposomes in one step, without using any toxic organic solvents. It can be used for laboratory and industry scale production. This method has been successfully applied for encapsulation of nisin, a peptide-based antibacterial, with up to 54% encapsulation efficiency and good storage life (14 months at 4°C) (Colas *et al.*, 2007).

2.3 Encapsulation of Bioactive Peptides with Chitosan Coated Milk Lipid-Derived Liposomes

Polymer-coated liposomes integrate the advantages of both liposomes and polyplex techniques while eliminating the shortcomings. Polymer-based systems provide excellent stability and controlled release of core materials (Solaro *et al.*, 2010). On the other hand, liposome-based systems are able to encapsulate both hydrophilic and lipophilic components. Based upon composition, liposomes are stabilized by the polymer and gain the ability to gradually release the bioactives. Furthermore, this composite system may offer increased efficacy when compared with pure liposome or polymer-based systems (Mufamadi *et al.*, 2011).

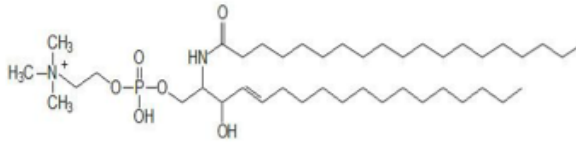
2.3.1 Liposomes prepared from milk fat globule membrane phospholipids

Milk fat globule membrane (MFGM) is the membrane surrounding the milk fat globules produced when the lipid droplets are secreted from epithelial cells of the mammary gland (Thompson *et al.*, 2009). It is not a simple layer of surface-active material. Instead, MFGMs mainly contain phospholipids (73%), including 25.2% of sphingomyelin (a type of glycolipid containing amino alcohol), cholesterol (0.032%), enzymes and proteins (6.6%), moisture (5.6%) and ash (14.8%) (Liu *et al.*, 2012; Thompson, 2005). Its unique structure prevents coalescence and flocculation of fat droplets in milk and acts as a barrier against enzyme degradation. Both MFGM proteins and lipids have been shown to have health promoting properties (Singh, 2006).

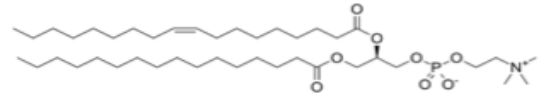
MFGFs can be obtained on laboratory and industrial scales of production (Keenan & Mather, 2002). Large quantities of MFGMs can be manufactured from buttermilk by microfiltration-based techniques (Gallier *et al.*, 2010). Table 2-6 shows the typical composition of phospholipids from soy, egg and MFGM. Figure 2-5 shows the structure of the major phospholipids of MFGMs.

Table 2-6. Phospholipid composition (% w/w) from different food sources (Burling & Graverholt, 2008).

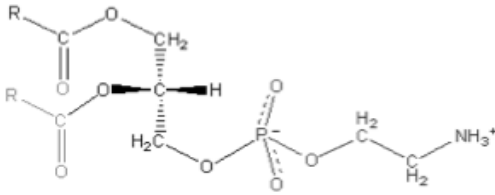
	Soy (%)	Egg (%)	Milk (%)
Phosphatidylcholine	34	75	27
Phosphatidylethanolamine	21	15	25
Phosphatidylinositol	18	0.4	8
Phosphatidylserine	0.5	0	12
Sphingomyelin	0	1.5	24
Phosphatidic acid	9	0	0
Others	17.5	8.1	4



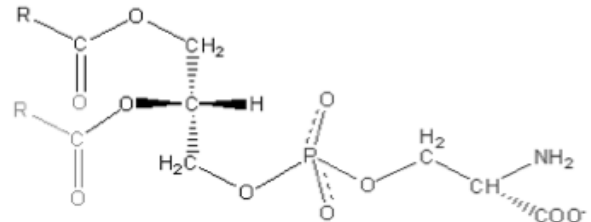
Sphingomyelin



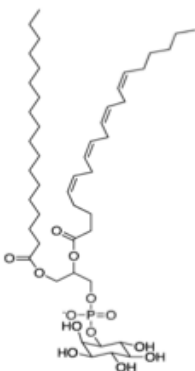
Phosphatidylcholine



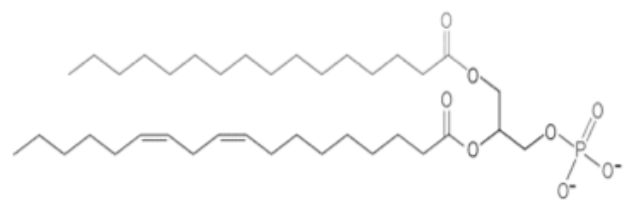
Phosphatidylethanolamine



Phosphatidylserine



Phosphatidylinositol



Phosphatidic acid

Figure 2-5. Structure of major phospholipids in MFGM (Farhang, 2013).

The health improving function of MFGM components distinguish them from the conventional liposome materials from egg or soy. MFGM-derived lipids contain sphingomyelin, which has been shown to be anti-carcinogenic (Singh, 2006), inhibit intestinal cholesterol absorption (Noh & Koo, 2004) and reduce the incidence of colon tumors (Hellhammer, Waladkhani, Hero, & Buss, 2010).

Thompson *et al.* (2006) illustrated that liposomes prepared from MFGM phospholipids were significantly different from the ones made from soy phospholipids. MFGM liposomes have a higher phase transition temperature, thicker membrane and lower

permeability when compared with the ones made from soy. MFGM liposomes exhibit higher stability during heating at all pH levels (Thompson *et al.*, 2006). The stability of liposome membranes is mainly due to the distinct composition between MFGM and soy phospholipids (Thompson *et al.*, 2006). For example, sphingomyelin in MFGMs contribute to a more structured gel phase, which stabilizes the membrane. Moreover, MFGMs have better storage ability between 4 and 35°C and are less likely to aggregate during thermal processing (55-141°C), which is an advantage for liposomes to be applied to food systems (Thompson *et al.*, 2006). In addition, MFGM-derived phospholipids were demonstrated to have higher encapsulation efficiency for both hydrophilic (potassium chromate) and hydrophobic (β -carotene) molecules than soy liposomes (Thompson *et al.*, 2009). The β -carotene entrapment plateau values are ~ 6 μg per gram of MFGM-derived phospholipid and ~ 3.5 μg per gram of soya-derived phospholipid. The maximum ratio of entrapment efficiencies is 0.79 ± 0.12 soya liposome/MFGM liposome. Thompson *et al.* (2009) concluded that the differences in entrapment between the two liposomal dispersions were most likely due to differences in the composition of the phospholipid fractions.

2.3.2 Chitosan coated liposome particles

Lipid-based delivery systems are not suitable for oral delivery of peptides because of their instability in an acidic gastric environment of bile salts and lipase (Page & Cudmore, 2001). Many attempts have been made to overcome the stability problem of liposomes. Mucoadhesive polymer systems, like chitosan, are the most promising approach for enhancing liposomal delivery of bioactive peptides orally (Shaji & Patole, 2008). The stability of liposomal carriers in the GI tract can be greatly enhanced by a chitosan coating layer. Absorption efficiency can be improved by prolonging the retention time at the site of absorption (Channarong *et al.*, 2011; Mady *et al.*, 2009).

Chitosan is a positively charged biodegradable hydrophilic polymer derived from deacetylated chitin (Malaekheh-Nikouei *et al.*, 2008). If the degree of deacetylation is greater than 60%, the material can be called chitosan rather than chitin (Figure 2-6).

Chitosan can bind to liposomes via electrostatic interactions as chitosan is cationic and phospholipid head groups are anionic (Figure 2-7). Liposomes can also be modified to be anionic using an inducer such as diacetyl phosphate and sodium deoxycholate (Channarong *et al.*, 2011).

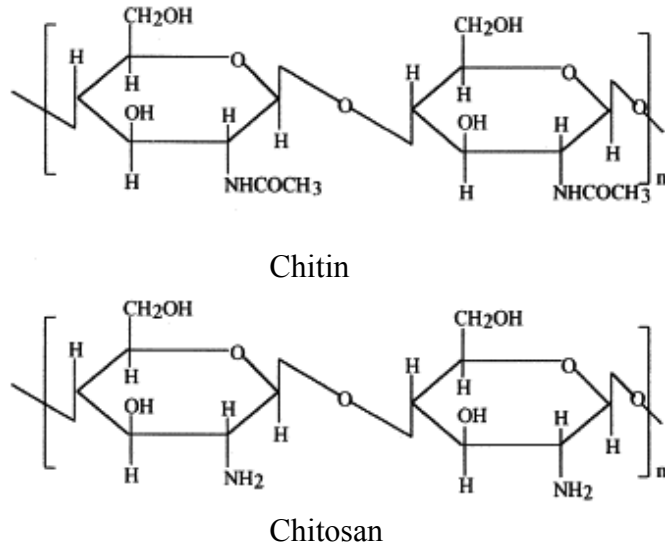


Figure 2-6. Structure of chitin and chitosan (Kuma, 2000).

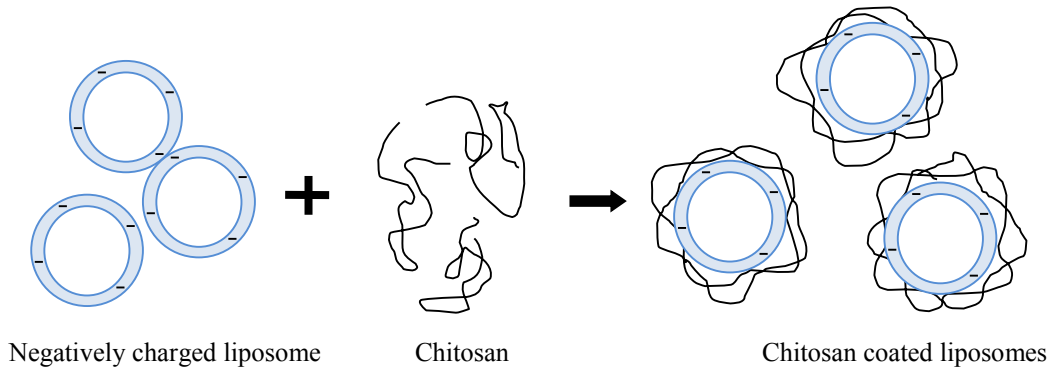


Figure 2-7. Stabilizing liposomes via surface coating with chitosan (Channarong *et al.*, 2010).

Chitosan has also been found to possess some health related properties. For example, chitosan has been made into tablets and used as a dietary supplement to reduce body fat

and control cholesterol absorption (Shahidi & Abuzaytoun, 2005). Chitosan is likely to entrap fat droplets in the stomach, forming micelles, thereby preventing the interaction between bile salts and fatty acids. Therefore, fats are excreted without digestion and absorption (Agullo *et al.*, 2003). In animal trials, chitosan has been shown to possess hypocholesterolemic activities (Hirano *et al.*, 1990; Ylitalo *et al.*, 2002). Ylitalo *et al.* (2002) described the mechanism of chitosan lowering serum cholesterol levels. The amino group of chitosan is positively charged, which enables chitosan to bind with the negatively charged X-COO⁻ group of dietary fats and lipids. This binding inhibits the adsorption of lipids; thus, the cholesterol content of liver cells is reduced leading to the improvement of the ratio between low-density cholesterol and high-density cholesterol.

Chitosan coated particles have been shown to absorb through paracellular transport mechanisms, based on the finding that chitosan interacts with the F-actin in the tight junctions of intestinal epithelium (Bakhru & Furtado, 2013). Mooren *et al.* (1998) suggested that chitosan microspheres pass through the epithelium mainly through paracellular transport, using the junction protein complex as a docking site, while the transcellular pathway only plays a minor role. This unique transport mechanism of chitosan facilitates the preservation of bioactivities of functional peptides, preventing the di- and tripeptides from being broken down by intracellular lysosomal peptidases in intestinal epithelial cell lines (CIBA Foundation Symposium, 1972).

2.3.3 *Liposome encapsulation of food ingredients*

The unique properties of liposomes have been used in numerous scientific and therapeutic applications as well as in food products. Functional food manufacturers have been utilizing liposomes to incorporate health promoting bioactive ingredients into food ingredients and nutraceuticals (Mozafari *et al.*, 2008). Encapsulation brings numerous advantages for food industries by masking any adverse taste and odor, protecting against potential payload degradation in the GI tract by acid and enzymes and transforming liquid ingredients into solid particles (Mozafari *et al.*, 2008). Meanwhile, due to the liposomal capsules' small size, there is an increase in surface area, which thereby increases the solubility and bioavailability.

Liposome delivery has been applied to encapsulate enzymes and enhance cheese ripening during curd formation (Kirby, Brooker, & Law, 2007; Wilkinson & Kilcawley, 2005). The advantages of lipid capsules are that the capsular material can be extracted naturally from ingredients that come from cheese, and it is possible to scale up to meet industrial scale liposome production (Mozafari *et al.*, 2008).

There have been several reports on encapsulation of antioxidants with nanoliposomes (Hood *et al.*, 2011; Mozafari *et al.*, 2006). Vitamin E (α -tocopherol) is a natural lipid-soluble antioxidant. α -tocopherol has low solubility with water, thus making it difficult for it to be incorporated into food systems. Instead, upon using nanoliposomes, α -tocopherol can be effectively added into functional foods as a natural antioxidant, thereby providing consumers with potential health effects, as well as retarding the oxidation of other nutrients such as omega-3 unsaturated fatty acids (Mozafari *et al.*, 2006).

CHAPTER 3 MATERIALS AND METHODS

Liposomes were prepared by the heating method as described by Thompson *et al.*, 2007, followed by high pressure homogenization. These two methods have previously been used to prepare food-grade lipid capsules and are suitable for applications on an industrial scale. MFGM and SPH were first hydrated in distilled water and heated to 60-80°C in the presence of glycerol. The crude liposomes were homogenized to reduce their size to submicron level. It was hypothesized that varying the stoichiometric ratio of salmon protein hydrolysates (SPH) to phospholipids and chitosan could result in different encapsulation efficiencies. Concentrations of milk fat globule membrane (MFGM) phospholipids (Phospholac 700, Fonterra Cooperative Group Ltd., New Zealand) (Table 3-1) and chitosan (CH) (75-85% deacetylated; low molecular weight, 50 – 190 kDa, Sigma-Aldrich (St. Louis, MO, USA)) were varied in the preparation of liposomes in order to optimize encapsulation efficiency. All other chemicals and materials were obtained from Sigma-Aldrich (St. Louis, MO, USA). The SPHs were prepared according to Jin (2012). Atlantic salmon protein were dissolved in 1 M NaOH and then digested with pepsin, trypsin, and chymotrypsin. Peptides with molecular weight less than 1 kDa were collected by ultrafiltration and freeze-dried. The lyophilized samples were desalted at Laval University, Quebec City, Canada. The amino acid content of SPH was determined by analytical HPLC and is shown in Table 3-2 (Girgih *et al.*, 2013).

Table 3-1. Analysis of milk fat globule membrane phospholipids (Phospholac 700) as supplied by Fonterra Co-operative Ltd., (New Zealand).

Parameter	Units of Measure	Test Method	Mean Result
Total lipids	%	Gravimetric	83.6
Phosphatidyl serine	%	HPLC 201nm	4.24
Phosphatidyl choline	%	HPLC 201nm	32.4
Phosphatidyl ethanolamine	%	HPLC 201nm	8.91
Sphingomyelin	%	HPLC 201nm	16
Moisture	% m/m	Gravimetric	1.79
pH	pH units	5% TS 20°C	5.0
Lactose monohydrate	% m/m	Pheno Sulphuric	6.3
Protein			(Not given)

Table 3-2. Amino acid composition of salmon peptide fraction dissolved in 1 M NaOH and digested with pepsin, trypsin, and chymotrypsin (g/100 grams amino acids). BCAA – Branched chain amino acids; EAA – Essential amino acids (Girgih *et al.* 2013).

Amino Acid	Salmon Peptide Fraction	Casein Hydrolysate
Alanine	6.54	2.84
Arginine	6.69	3.84
Aspartic acid	9.80	7.59
Glutamic acid	13.79	21.68
Glycine	4.80	2.00
Histidine	3.20	2.36
Isoleucine	4.89	5.42
Leucine	8.74	9.27
Methionine	3.43	2.64
Lysine	9.99	8.44
Phenylalanine	5.10	4.32
Proline	3.24	9.43
Serine	4.02	5.38
Threonine	4.92	3.94
Tyrosine	4.99	3.59
Valine	5.84	7.27
BCAA	19.47	21.96
EAA	41.01	39.34

3.1 Preparation of Chitosan-Coated Salmon Protein Hydrolysate Liposomes

Crude liposomes were prepared by the heating method of Thompson *et al.* (2007). MFGM and SPH were hydrated in 2 mL distilled water containing SPH at a concentration of 10 mg/mL (1%). However, it is recommended by Agrawal *et al.* (2013) that the optimized peptide (insulin) loading was 10% for their liposomal formulation with 5:5 phosphatidylcholine: cholesterol mole ratio. The low SPH concentration was chosen in this study due to limited amount of desalted SPH obtained. The concentration of MFGM used is shown in Table 3-3. Hydration was performed for 1 h at room temperature under a nitrogen atmosphere. Then, the mixture volume was made up to 10 mL with 3% (v/v) glycerol in phosphate-buffered saline. The pH of the mixture was adjusted to 7.30 ± 0.10 with 2M NaOH, and heated to 60°C with continuous shaking for 60 min under a nitrogen atmosphere. After cooling to room temperature, the crude liposomes were homogenized using an Emulsi-Flex-C3 high pressure homogenizer (Avestin, Ottawa, Canada) at a pressure of ~17,000 psi (~117.2 MPa) for 5 cycles (Thompson *et al.*, 2009), to create a liposomal suspension.

Chitosan-coated (CH-coated) liposomes were prepared by mixing the liposome suspensions with an equal volume of chitosan solution. The CH solution was prepared by dissolving CH in acetic acid at 1% (v/v), with continuous stirring overnight at room temperature and filtering using a Whatman 0.45 µm syringe filter (GE Life Sciences, Uppsala, SWE) and adding drop-wise into an equal volume of liposomal suspension. The mixture was stirred with a stirring bar at room temperature (200 rpm) for 1 h and incubated at 4°C overnight (Karn *et al.*, 2011). Various formulations were tested by varying the amount of CH at a defined SPH and phospholipid concentration and by varying the amount of phospholipid at a fixed SPH and CH concentrations (Table 3-3).

Table 3-3. Summary of liposomal ingredients and chitosan coating concentrations used.

MFGM phospholipid % (w/v)	SPH mg/mL	Glycerol % (v/v)	Chitosan coating % (w/v)
3	10	3	0, 0.025, 0.05,
5	10	3	0.075, 0.1, 0.2,
10	10	3	0.3, 0.4, 0.5, 0.6

3.2 Characterization of Chitosan Coated Liposomes

3.2.1 Scanning Electron Microscopy (SEM)

A Hitachi Model S-4700 SEM in the Dalhousie University Department of Process Engineering and Applied Science was used to perform these experiments. Particle shape and surface morphology were characterized using SEM following the methods described by Yokota *et al.* (2012). Samples were fixed onto slides with 2% osmium tetroxide and were critical point dried with ethanol using an automated critical point dryer (Leica, Model EM CPD300, Germany). The fixed and dehydrated samples were then coated with gold in a low vacuum coater (Leica, Model EM ACE200, Germany) at a deposition rate of $0.51 \text{ \AA}\cdot\text{s}^{-1}$ for 180 s, using 3-5 mA of current at a pressure of 0.2 Pa. SEM images of liposome samples were recorded at an accelerating voltage of 15 kV.

3.2.2 Transmission Electron Microscopy (TEM)

Morphological characteristics were also characterized using transmission electron microscopy following the methods described by Mady *et al.* (2009) with modification. Single drops of each diluted coated and uncoated liposomal dispersions were applied onto formvar-coated grids (400 mesh, Electron Microscopy Sciences, USA). Extra liquid was drawn off using filter paper by delicately touching the edges of the grid. A drop of 2% uranyl acetate was added as negative staining agent and allowed to react for two min. The TEM (Philips, Model Tecnai-12, Netherlands) in the Dalhousie University Department of Biology was used to examine the samples.

3.2.3 *Dynamic Light Scattering*

Measurements of the mean particle diameter and polydispersity index (PDI) of both coated and uncoated liposomes were determined by using dynamic light scattering (DLS) on a Zetasizer Nano Model ZS (Malvern Instruments, Derbyshire, UK) in the Dalhousie University Department of Civil Engineering. The particle sizes for 1 mL samples were measured at 25°C with an angle of 90° after 120 s of autocorrelation.

3.2.4 *Zeta Potential Measurements*

Zeta potential is a physical property of particles in suspension that can be used to optimize suspension stability. It is related to the surface electrical charge of the particles. In general, higher zeta potential indicates greater repulsion between particles, thereby increasing the stability of the colloidal system. In other words, if all liposomes have high positive or negative zeta potential, they will repel each other instead of forming aggregates (Mady *et al.*, 2009). It was anticipated that the liposomes would exhibit a negative zeta potential due to the head groups on the phospholipid membrane, whereas the CH-coated liposomes would display positive zeta potential because of the cationic CH coating layer.

Zeta potential was measured on the same instrument used for particle size analysis using the Zetasizer Nano Model ZS (Malvern Instruments, Derbyshire, UK). Samples were loaded into the universal dip cells, and measurements were made at 20°C in triplicate. The zeta potential is calculated from the electrophoretic mobility of individual particles, measured using laser doppler velocimetry (Malvern Instruments Ltd., 2004).

3.2.5 *Encapsulation Efficiency*

The encapsulation efficiency was defined as the ratio of encapsulated SPH to free SPH \times 100 as determined by using the Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific, Waltham, MA, USA) to monitor the amount of free SPH peptide material remaining as free and unencapsulated by the chitosan. The encapsulated (bound) SPH was determined by difference as total SPH – free SPH. This method utilized the

reduction reaction of Cu^{+2} to Cu^{+1} by protein in alkaline environment. BCA provides highly sensitive colorimetric detection of Cu^{+1} (green to purple) by chelation of two molecules of BCS with one cuprous ion (Walker, 2002). This water-soluble complex exhibits strong absorbance at 562 nm that is linearly related to protein concentration over a range of 20-2000 $\mu\text{g}/\text{mL}$ (Walker, 2002). The number of peptide bonds and the presence of cysteine, cystine, tryptophan, and tyrosine are responsible for the color formation (Wiechelman *et al.*, 1988).

In this assay, 1 mL of freshly prepared CH-coated liposome samples were centrifuged at $8000 \times g$ for 10 min and the supernatant collected (Channarong *et al.*, 2011). In the case of uncoated liposomes, samples were ultracentrifuged (Hitachi, CP100WX, Japan) at $400,000 \times g$ for 1 h at 4°C (Liu *et al.*, 2013), and the supernatant collected. A 0.5 mL volume of supernatant was filtered using a 3 kDa ultrafiltration centrifugal filter (EMD Millipore, Temecula, CA) separate any un-encapsulated SPH and remove free chitosan. Then 0.1 mL of filtrate was mixed, 20:1, with the prepared BCA reagent. Samples were left at room temperature for 2 h before measuring the absorbance at 562 nm using a UV-visible spectrophotometer (Agilent Technology, Model 8453). Liposomes prepared in the absence of SPH were centrifuged, filtered, and used as blanks for the spectrophotometer. Peptide concentrations were calibrated using a standard curve prepared from the dried SPH. Encapsulation efficiency was determined as the ratio of the unbound SPH to the total SPH. SPH that wasn't removed by centrifugation and washing was assumed to be 100% encapsulated into the liposomes.

3.3 *In Vitro* SPH Release Studies

In order to assess the stability and protective effects imparted by the CH-coating layer, coated and uncoated liposome formulations were incubated in simulated-gastric-fluid (SGF) and simulated-intestinal-fluids (SIF) (Jain *et al.*, 2012 and Agrawal *et al.*, 2014). SGF was prepared by dissolving 100 mg of pepsin in 5 mL of water containing 0.35 mL concentrated HCl followed by 100 mg of NaCl and adjusting to a final volume of 50 mL with distilled H_2O . Finally, the pH was adjusted to 1.2 using concentrated HCl. SIF was

prepared by dissolving 340 mg of monobasic potassium phosphate in 10 mL of water followed by the addition of 3.85 mL of 0.2 M NaOH and 500 mg of pancreatin. The final volume was made up to 50 mL, and the pH was adjusted to 6.8 using NaOH. A volume of 20 μ L of coated and uncoated liposome formulations was diluted to 1 mL in microcentrifuge tubes with both simulated fluids and incubated at 37°C with continuous shaking at 800 rpm in a Thermomixer 5436 (Eppendorf, Hamburg, Germany). Incubation times of up to 2 h for SIF, and up to 4 h for SGF were used. At each interval, the SPH content was estimated following the description in Section 3.2.5, following the BCA Protein Assay.

3.4 Physical Stability Tests

3.4.1 Freeze-thaw

Volumes of 0.5 mL of each freshly made liposomal suspension (coated and uncoated) were rapidly frozen at -30°C for four weeks in the absence of additional cryoprotectant. Before use, the samples were allowed to thaw at room temperature for 4 h with repeated vortex mixing. The particle size and encapsulation efficiencies of the dispersed liposomes were determined as in Section 3.2.5.

3.4.2 Freeze Dry-Rehydration

Different formulations of CH-coated liposomes suspensions were subjected to freeze-drying and rehydration (FD-RH) with distilled H₂O to determine their stability. One mL of each formulation was cooled from 25 to -40°C and then maintained at -40°C for 8 h; primary drying was performed at -40°C for 48 h; the samples were heated from -40 to 25°C and dried at 25°C for 10 h. The chamber pressure was maintained at 20 Pa during the drying process. The freeze-dried samples were stored at 4°C for 4 weeks. Then, the freeze-dried cakes were hydrated with 1 mL of distilled water and vortex-mixed until the cake was optically clear. The particle size and SPH trapping efficiency of the dispersed liposomes were obtained as mentioned above.

3.4.3 Long Term Storage

The size and the SPH trapping efficiency of different formulations were monitored at 20°C and 4°C over a period of 4 weeks. At predetermined time intervals, samples were extracted and the particle size and SPH trapping efficiency of the dispersed liposomes were obtained.

3.5 Statistical Analysis

All experiments were carried out in triplicate. Results were expressed as means \pm standard deviation. Analysis of variance (ANOVA) and Tukey's multiple comparison tests were performed using the GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered to be significant at $p < 0.05$ with Tukey test throughout this study.

CHAPTER 4 RESULTS

4.1 The Formation of Liposomal Carriers

MFGM and SPH were dissolved after 10-15 min of heating, depending on the MFGM phospholipid concentration. After homogenization, the apparent viscosity of the suspension was dramatically lowered and the appearance changed from opalescent creamy yellow to clear yellow. The addition of chitosan was visually confirmed, as the appearance of the solution changed from clear yellow to opaque ivory white. However, at low concentrations of chitosan ($< 0.2\%$ (w/v)), the coated liposomes formed very large aggregates, which were followed by a phase separation with a complete clear serum layer with milky white precipitate.

4.2 Characterization of Chitosan-Coated and Uncoated Liposomes

4.2.1 Morphology

The visualization of coated and uncoated liposomes containing SPH was performed by TEM (Figure 4-1) and SEM (Figure 4-2). The size of the coated liposomes was larger than that of uncoated liposomes, which indicated the successful attachment of CH onto the liposome surface. Both types of liposomes possessed a spherical morphology. The existence of a CH coating layer surrounding the liposome surface was well visualized (Figure 4-1B). Furthermore, no significant surficial morphological differences between liposomes with and without CH coating layer were observed. This might be because of the strong ionic bonds formed between the cationic CH layer and the negatively charged liposomal surface, leading to a smooth appearance of CH-coated liposomes, making it difficult to observe the presence of polymer on the liposomal surfaces (Henriksen *et al.*, 1994).

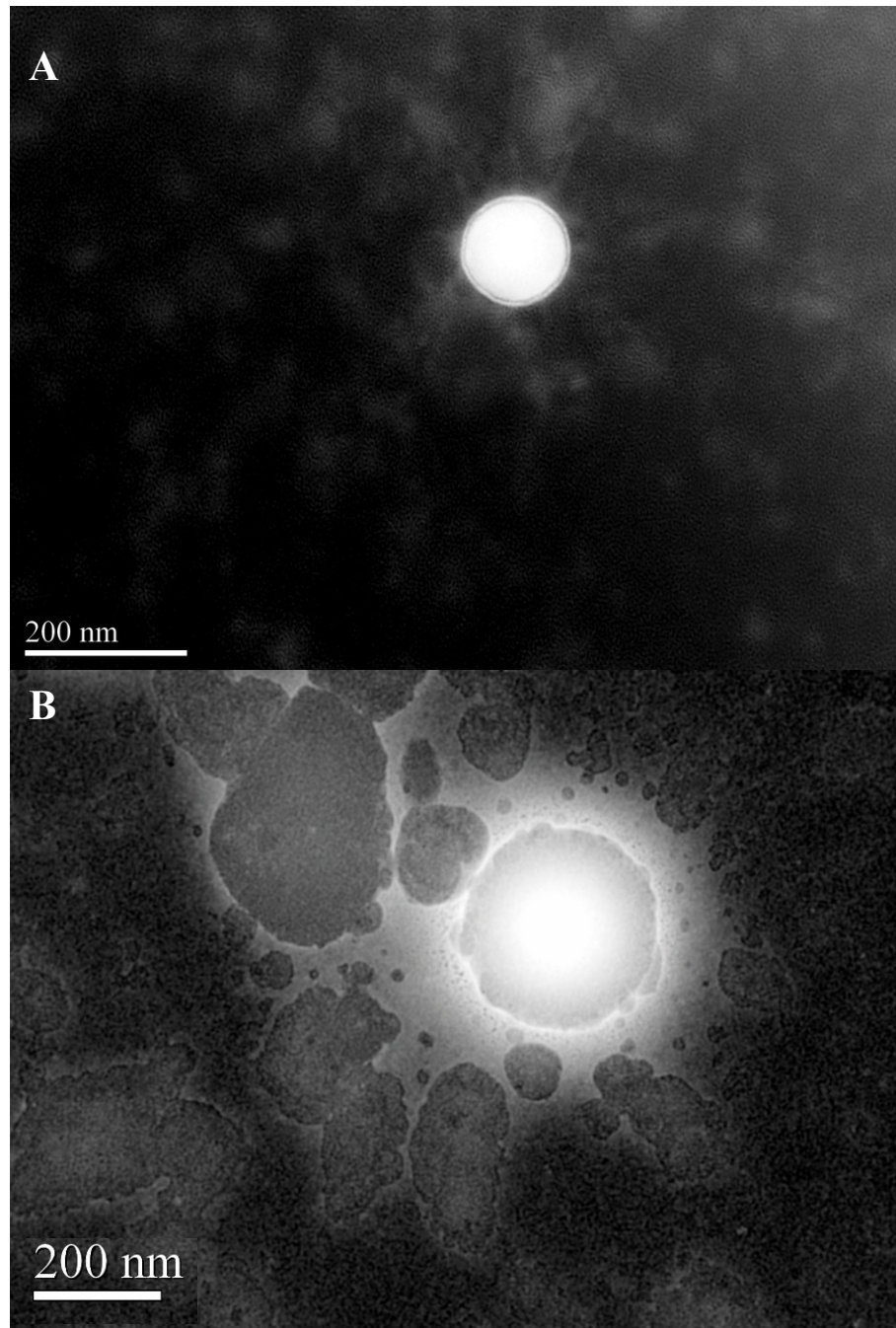


Figure 4-1. Negatively stained TEM images of chitosan uncoated (A) and coated (B) MFGM phospholipid liposomes. “A” is an image of an uncoated liposome with 1% (w/v) SPH; “B” is an image of a chitosan-coated liposome with 1% (w/v) SPH.

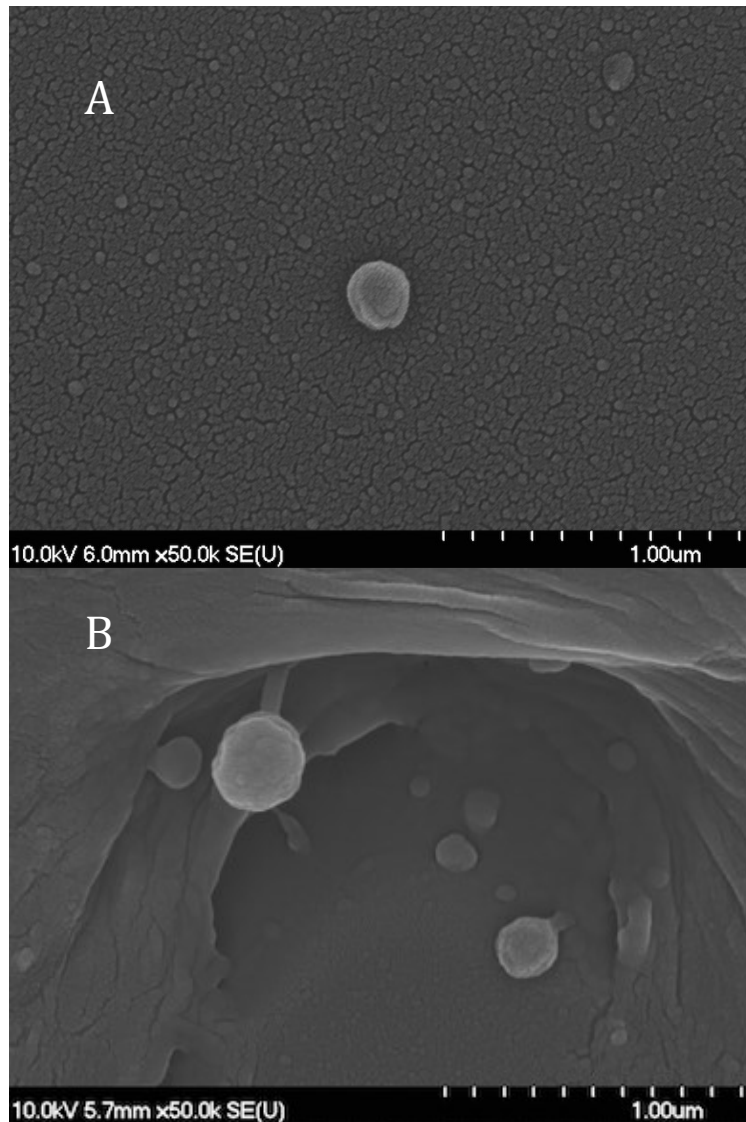


Figure 4-2. SEM images of chitosan uncoated and coated MFGM phospholipid liposomes. (A) represents an uncoated MFGM liposome with 1% (w/v) SPH; (B) represents chitosan-coated MFGM liposome with 1% (w/v) SPH.

4.2.2 Particle Size Determination Using Dynamic Light Scattering (DLS)

DLS was employed to measure the hydrodynamic diameter and size distribution of the liposome. The influence of MFGM percentage and CH concentration on the size and PDI of liposomes are summarized in Figure 4-3 and Table 4-2. The sizes of uncoated liposomes with 1% (w/v) SPH were 85.1 ± 2.3 nm, 101 ± 3.2 nm and 105 ± 8.4 nm for

the 3%, 5% and 10% (w/v) MFGM, respectively. The size of coated-liposomes was highly dependent on the amount of chitosan added (Figure 4-3). At lower chitosan concentrations, the particle diameter ranged from 100 nm to above 4000 nm regardless of the MFGM concentration, indicating extensive aggregation. At 5% MFGM and a CH concentration of 0.05% (w/v), the particle diameter increased to 5700 nm. These aggregates eventually precipitated, showing clear phase separation between the particles and the media. However, by increasing the chitosan concentration, the particle diameter decreased and reached a relatively small size. For example, with an increase in CH concentration from 0.025% to 0.2% (w/v), the 3% (w/v) MFGM liposome diameter dropped from 5234 ± 1101 nm to 272 ± 25.4 nm. Furthermore, the liposome size remained stable until the CH concentration reached 0.6% (w/v). No significant size change was observed ($p > 0.05$). This dramatic size reduction was also observed in 5% and 10% (w/v) MFGM liposomes. For 5% (w/v) liposomes, their size remained stable between 0.2% to 0.6% (w/v) CH ($p > 0.864$), while for 10% (w/v) liposomes, there was no significant size change after the CH concentration was above 0.4% ($p > 0.05$).

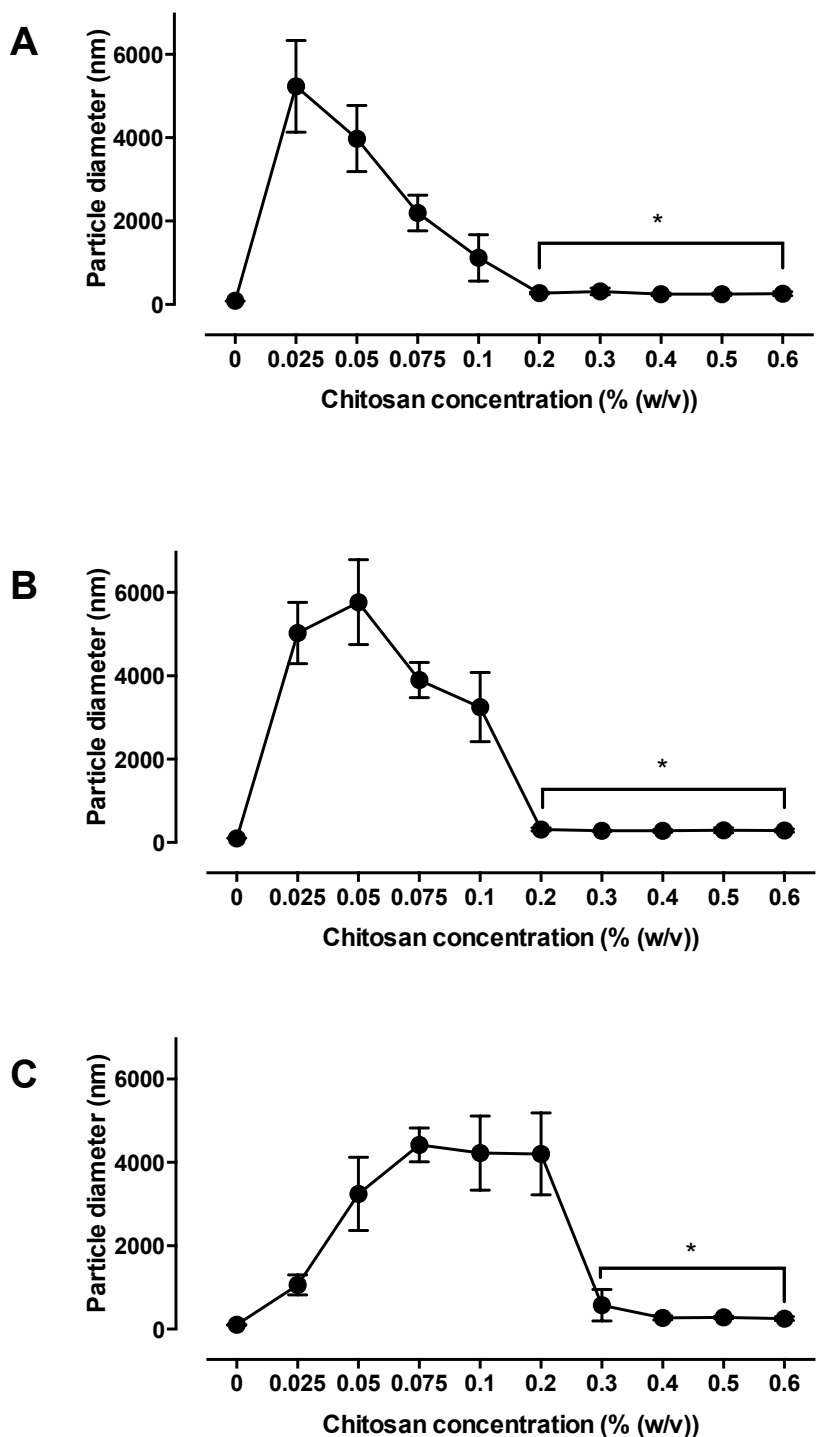


Figure 4-3. The influence of chitosan concentration on the average particle diameter of (A) 3% (w/v), (B) 5% (w/v) and (C) 10% (w/v) MFGM phospholipid liposomes. All formulations were loaded with 10 mg/mL SPH. * indicates the change in particle size was not significant ($p > 0.05$). Values are presented as mean \pm SD ($n = 3$).

The polydispersity index (PDI) is a measure of the size distribution of a sample, ranging from 0-1. A high PDI value indicates a broad size distribution, and may indicate that the sample contains large particles and aggregates (Romero-Pérez *et al.*, 2010). As shown in Table 4-1, the PDI of the three uncoated MFGM phospholipid liposome formulations were all below 0.190, indicating the liposomes were distributed and even in size. This is consistent with the liposome size measurements (Figure 4-3). As for the chitosan-coated liposomes, the PDI values were significantly higher ($p > 0.05$) than those of the uncoated liposomes. Such large PDI values indicate a broad size distribution, and were likely due to the aggregation of liposomes.

Table 4-1. Effect of chitosan coating layer concentration on MFGM phospholipid liposome polydispersity index (PDI) (n = 3).

Chitosan concentration (% (w/v))	3% MF liposome PDI	5% MF liposome PDI	10% MF liposome PDI
0	0.184 ± 0.001	0.118 ± 0.014	0.175 ± 0.020
0.025	0.301 ± 0.034	0.393 ± 0.232	0.925 ± 0.130
0.05	0.768 ± 0.202	0.187 ± 0.186	0.205 ± 0.093
0.075	0.709 ± 0.252	0.118 ± 0.084	0.438 ± 0.055
0.1	0.456 ± 0.271	0.280 ± 0.086	0.286 ± 0.049
0.2	0.519 ± 0.069	0.573 ± 0.043	0.357 ± 0.044
0.3	0.630 ± 0.070	0.565 ± 0.071	0.795 ± 0.054
0.4	0.553 ± 0.006	0.573 ± 0.010	0.654 ± 0.077
0.5	0.702 ± 0.085	0.654 ± 0.048	0.581 ± 0.036
0.6	0.549 ± 0.008	0.559 ± 0.034	0.571 ± 0.028

4.2.3 Zeta Potential

The zeta potential for CH-coated and uncoated MFGM liposome dispersions at various chitosan concentrations is shown in Figure 4-4. The control MFGM liposomes and the uncoated liposomes had zeta potentials of -58.2 ± 1.6 mV and -55 ± 2.4 mV, respectively. This is in agreement with the observations of others (Liu *et al.*, 2012; Thompson & Singh, 2006). With the addition of the CH coating layer, the zeta potential of the washed and re-suspended CH-liposome suspensions became less negative. It increased rapidly to above +50 mV regardless of the initial phospholipid content. This change in zeta potential was perhaps caused by the ionic attraction between positively charged chitosan

amino groups and the negatively charged liposome surface, indicating the successful coating of CH onto MFGM liposome surface. However, the amount of CH required for charge reversal increased in proportion to MFGM phospholipid content. For example, to increase charge from -30 mV to +30 mV, 3% (w/v) MFGM phospholipid liposomes required an increase of chitosan concentration from 0.01 to 0.05% (w/v), whereas 10% (w/v) phospholipid required an increase of chitosan concentration from 0.02 to 0.12% (w/v) to cause a similar change in zeta potential.

As observed in Figure 4-4, the rise of zeta potential of all liposome formulations dramatically slowed down at higher CH concentrations. For both 3 and 5% (w/v) liposomes, there was no significant increase in zeta potential beyond 0.2% CH ($p > 0.05$) (Figure 4-4A, B), whereas for 10% liposomes, the zeta potential plateaued beyond 0.4% CH (Figure 4-4C). These points are assumed to be where the anionic liposomal surface is saturated with the cationic polymer coating (Takeuchi *et al.*, 2005; Guo *et al.*, 2003). The CH concentrations necessary for CH to cover the entire surface of the liposomes were referred to as “optimal” CH levels (0.2% CH for 3% and 5% phospholipid liposomal suspensions; 0.4% CH for 10% phospholipid liposomal suspension). These “optimal” CH concentrations were used in further studies.

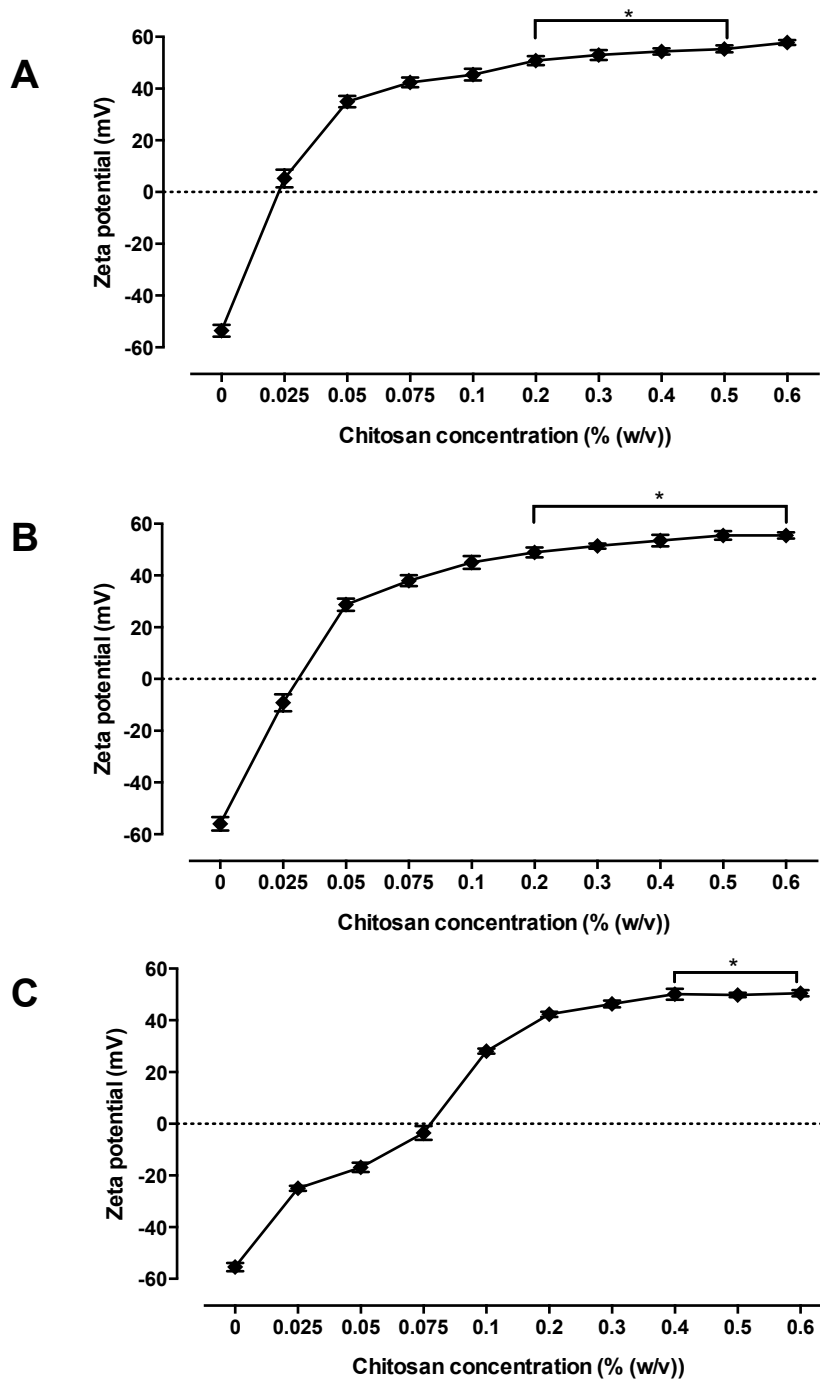


Figure 4-4. The influence of chitosan concentration on the average zeta potential of (A) 3% (w/v) MFGM phospholipid liposomes, (B) 5% (w/v) MFGM phospholipid liposomes, and (C) 10% (w/v) MFGM phospholipid liposomes. All formulations were loaded with 10 mg/mL SPH. Particles were suspended in distilled water, pH ~ 7.1. The square bracket and the * indicate the change in particle zeta potential was not significantly different among adjacent readings. Values are presented as mean \pm SD (n = 3).

4.2.4 Encapsulation Efficiency

The encapsulation efficiency (EE) of SPH in the uncoated 3%, 5% and 10% (w/v) MFGM liposomes was be $43.0 \pm 5.0\%$, $40.2 \pm 5.4\%$ and $50.6 \pm 5.9\%$, respectively. As seen in Figure 4-5, the addition of a low concentration of CH (0.025-0.75% (w/v)) reduced the EE for all three formulations. In contrast, the EE increased with higher CH concentrations. For example, for 3% (w/v) MFGM liposomes, EE increased from $20.5 \pm 5.6\%$ to $48.6 \pm 3.7\%$ by increasing the CH concentration from 0.075 to 0.2% (w/v).

The maximum EE was achieved when the CH concentration was at the “optimal” concentration for each formulation (Section 4.2.3). For 10% (w/v) MFGM phospholipid liposomes, the maximum EE was obtained using a 0.4% CH level. For 3% phospholipid liposomes, the maximum EE was reached with 0.2% CH. However, the 5% MFGM liposomes displayed highest EE at 0.3% CH and not significantly different ($p > 0.05$) from the “optimal” concentration observed for the 0.2% CH-coated liposomes. Meanwhile, there was no significant change in EE when excess CH was added for all three formulations ($p > 0.05$).

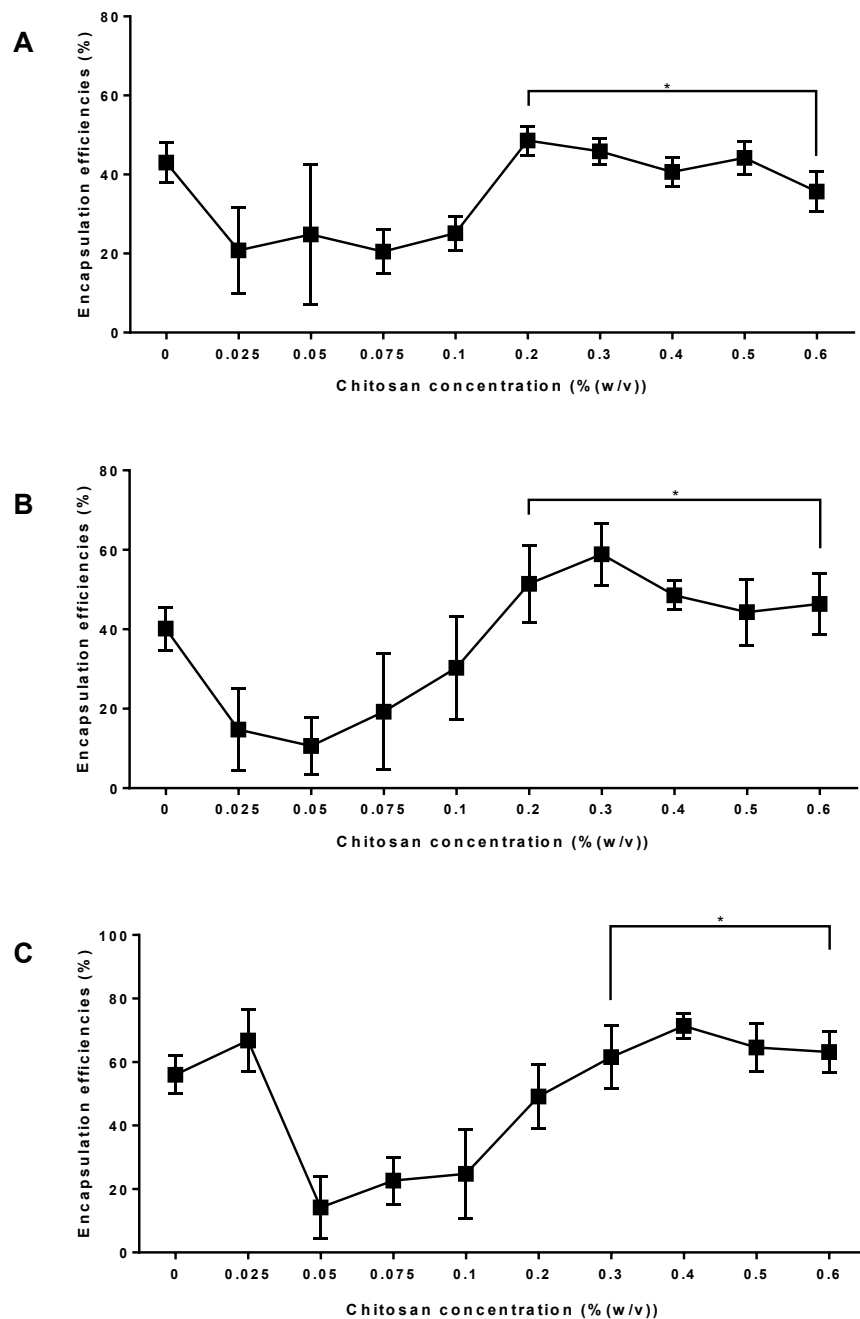


Figure 4-5. The influence of chitosan concentration on the encapsulation efficiency of (A) 3% (w/v) MFGM phospholipid liposomes, (B) 5% (w/v) MFGM phospholipid liposomes, and (C) 10% (w/v) MFGM phospholipid liposomes. The square bracket and the * indicate the difference of encapsulation efficiencies was not significant among adjacent bracketed readings. Readings at lower CH levels were not included during ANOVA and Tukey’s tests. Data are represented as the mean \pm SD (n = 3).

Among all three formulations, 10% MFGM phospholipid liposomes displayed the highest EE. Therefore, 10% MFGM phospholipid liposomes with 0% CH (liposomes without CH coating), 0.4% CH (liposomes with “optimal” coating concentration), and 0.6% CH (liposomes with excess CH coating) were chosen to test the effect of CH coating layer on *in vitro* release profile and physical stability in the following experiments.

4.3 *In Vitro* Release Studies

The goal of these experiments was to determine the release profile in a simulated gastrointestinal environment for encapsulated SPH liposomes. Ten % (w/v) MFGM phospholipid liposomes coated with either 0.4% or 0.6% (w/v) chitosan were tested. The gastric emptying time for a standard meal is ~112 min (Cann *et al.*, 1983), while the mean transition time in the small intestine is about 2 – 4 h (Davis *et al.*, 1986). Therefore, the release tests were designed for 2 h time intervals in simulated gastric fluid (SGF) and 4 h in simulated intestinal fluid (SIF).

The *in vitro* release profiles obtained with different SPH-loaded formulations are shown in Figure 4-6. Figure 4-6A shows the effect of CH coating levels on SPH release rate in SGF at different time intervals. All three formulations displayed a similar release profile. At each time interval, the SPH release rates were significantly reduced by the addition of the CH-coating layer ($p < 0.0001$) (Figure 4-6). To be more specific, the cumulative percentage of SPH release within 2 h approached 48.9% of that of uncoated liposomes, whereas the cumulative release was about 13.2 and 21.3% of liposomes with 0.4 and 0.6% CH coatings, respectively. Moreover, the 2 h cumulative SPH release in 0.4% CH-coated liposomes was significantly lower than that of 0.6% CH liposomes ($p < 0.05$).

Compared to the release profile in SGF, the release rates in SIF were much higher (Figure 4-6B). MFGM liposomes, either coated or uncoated, in SIF were not as stable as in SGF, as approximately 80% of encapsulated SPH was released after 2 h of incubation, increasing to 92.5% after 4 h in SIF. In contrast, the released amount of SPH from 0.4% and 0.6% CH-coated liposomes was significantly lower than that for uncoated liposomes

($p < 0.0002$), about 47.9 and 52.1% within 4 h, respectively. The CH-coated liposomes did not release a significant different amount of SPH after 4 h ($p > 0.05$).

Overall, the chitosan coating was found to prolong the release of SPH from 10% MFGM phospholipid liposomes. The CH-coating layer provided a greater protective effect in SGF than in SIF.

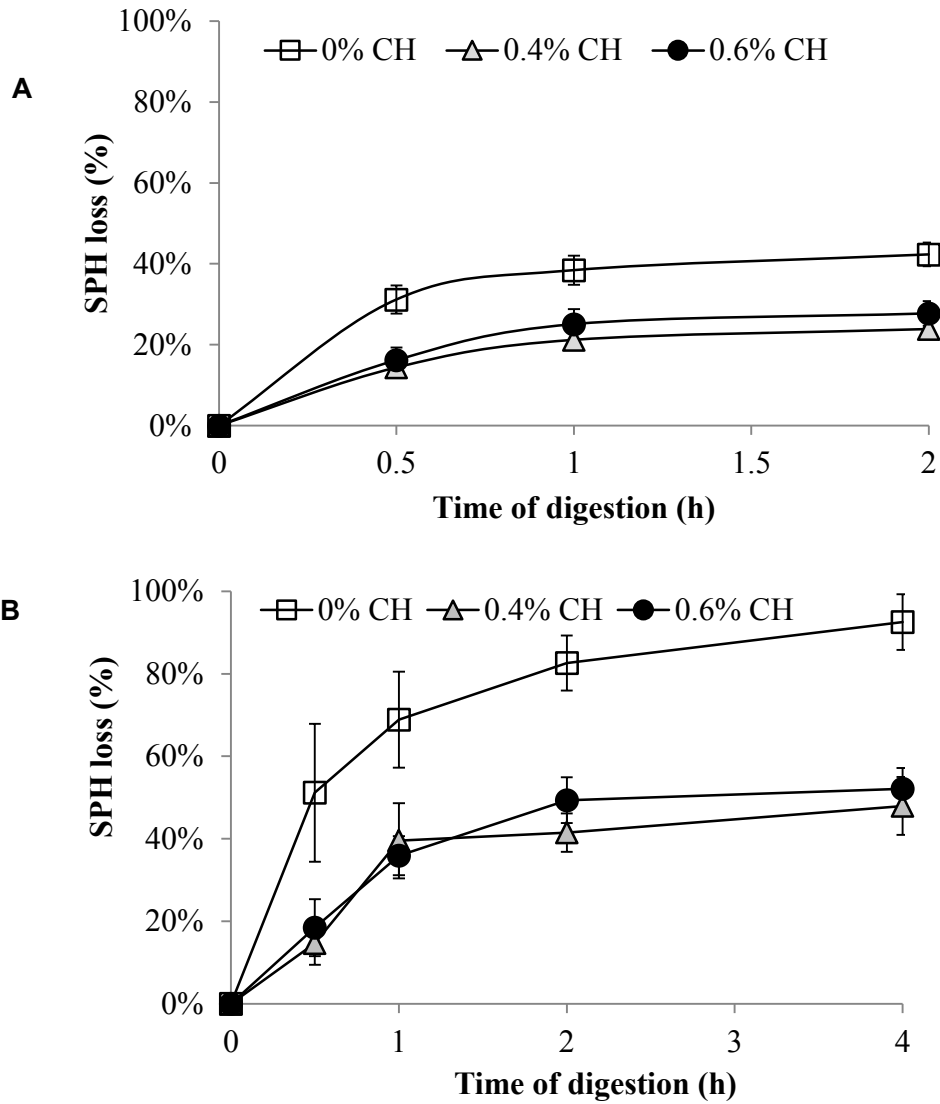


Figure 4-6. Release profile of 10% (w/v) MFGM phospholipid liposomes with 0, 0.4 and 0.6% (w/v) chitosan coatings in (A) simulated gastric fluid (SGF, pH 1.2), and (B) simulated intestinal fluid (SIF, pH 6.8), 37°C. Values are presented as mean \pm SD (n=3).

4.4 Physical Stability Tests

The physical stability of the CH coated and uncoated 10% MFGM liposomes was first evaluated by measuring the change in particle size and encapsulation efficiencies after freezing and thawing (FT) and freeze drying-rehydration (FD-RH). Then the long-term storage ability was tested under two different storage conditions, 4°C and 25°C, for 4 weeks.

4.4.1 Freezing and Thawing (FT)

Liposomes frozen without CH-coating showed the smallest increase in their mean diameter (Figure 4-7A). However, a significant loss of encapsulated SPH (over 80%) was observed ($p < 0.0001$) (Figure 4-7B), which was typically manifested by particle aggregation. Aggregation was confirmed by the increase of PDI values from about 0.175 to above 0.8, and also indicated a decrease in homogeneity.

In order to test the impact of CH coating on the stability during FT, two different CH-coating concentrations were tested. Although the release of encapsulated SPH was similar for both CH-coated liposomes (24% and 24.1% release for liposomes coated with 0.4 and 0.6% (w/v) chitosan, respectively), liposomes with higher CH content experienced larger liposomal diameter changes due to aggregation of small CH-coated liposomes ($p < 0.001$).

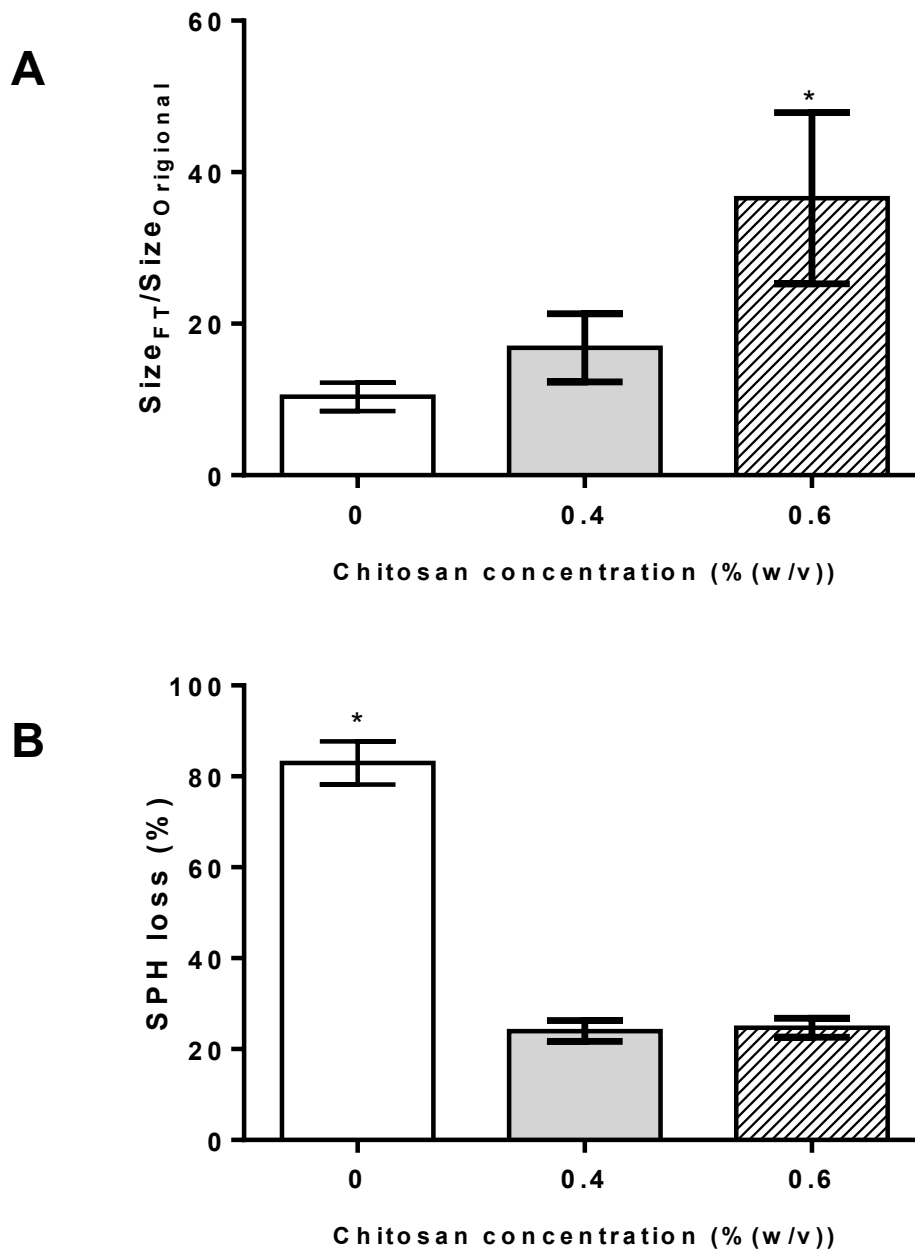


Figure 4-7. (A) Relative particle size and (B) percent loss of encapsulated SPH after freezing and thawing for chitosan coated and uncoated 10% (w/v) MFGM phospholipid liposomes. Relative particle size was determined as the ratio of freeze-thawed particle size to original size. * indicates significantly different means ($p < 0.05$). Data are expressed as the mean \pm SD ($n \geq 3$).

4.4.2 Freeze Drying and Rehydration (FD-RH)

Uncoated and CH-coated 10% (w/v) MFGM liposomes loaded with 1% (w/w) SPH were freeze-dried to test their physical stability and the protective effect of the CH-coating layer. After lyophilization, the uncoated liposomes could not form a dried cake; instead, the sample appeared gluey and difficult to re-suspend properly. This was not surprising as glycerol, added as a dispersant and cryoprotectant, is a liquid at room temperature. Nevertheless, the two chitosan-coated liposomes formed white intact cakes that were the same volume and shape as the original frozen samples.

Figure 4-8A illustrates the ratios of mean liposome diameter for CH-coated and uncoated liposomes after FD-RH, relative to their original values. Uncoated liposomes had significantly higher particle diameter ratios compared to chitosan-coated liposomes ($p < 0.0001$). This indicated a great extent of aggregation of uncoated liposomes and the protective effect of CH-coating layer during FD-RH for MFGM liposomes. About 56%, 13% and 15% of encapsulated SPH was lost for uncoated, coated (0.4% chitosan) and coated (0.6% chitosan), respectively (Figure 4-8B). Overall, chitosan coated liposomes exhibited less aggregation than uncoated liposomes.

Unlike the situation during freeze thawing, CH at a concentration of 0.4% did not show a better stability over chitosan at 0.6%. As the concentration of CH increased from 0.4 to 0.6% (w/v), the two size ratios were not significantly different from one another ($p > 0.05$). Hence, increasing CH content did not compromise the protective effect against the physical stress of FD-RH.

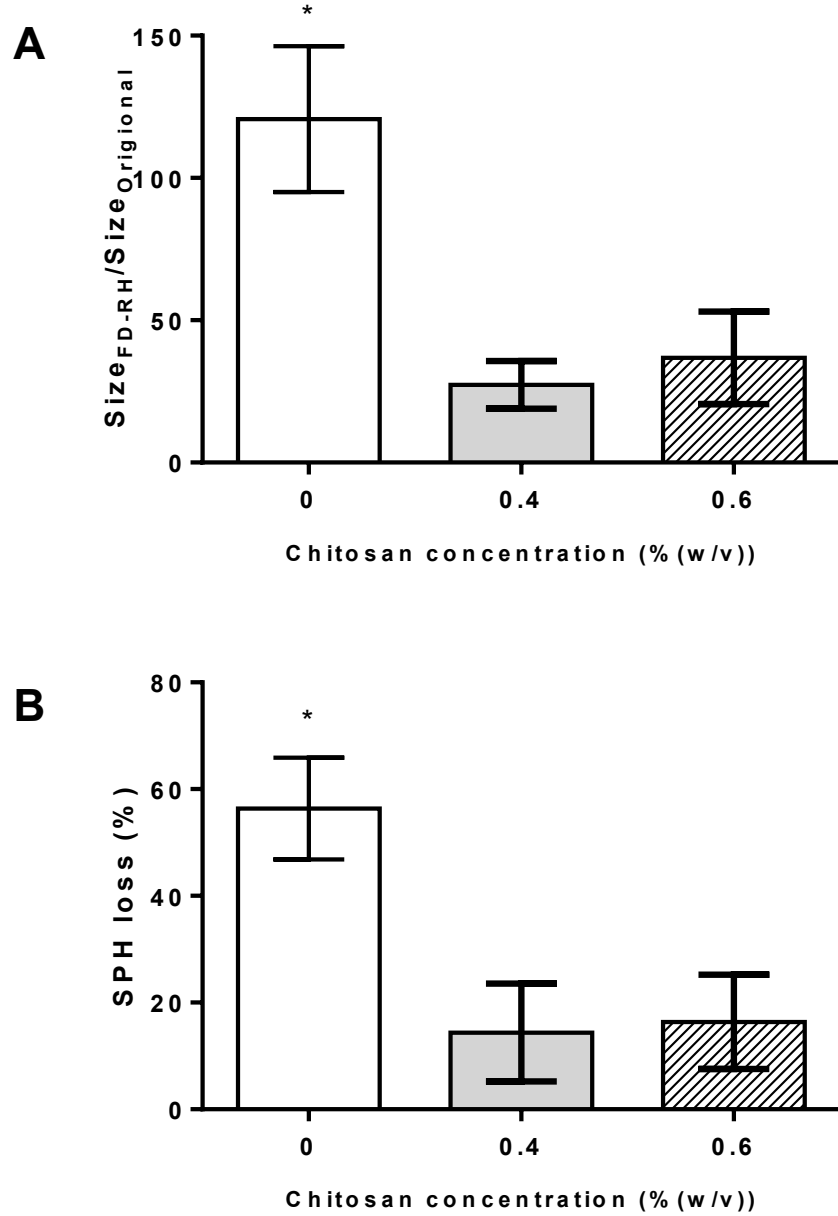


Figure 4-8. (A) Relative particle size and (B) percent loss of encapsulated SPH after freeze-drying and rehydration for chitosan coated and uncoated 10% (w/v) MFGM phospholipid liposomes. Relative particle size is the ratio of freeze-thawed particle size to original size. * indicates significantly different means ($p < 0.05$). Data are depicted as the mean \pm SD ($n \geq 3$).

4.4.3 Long Term Storage

In order to compare the long-term storage stability of CH-coated and uncoated MFGM liposomes, formulations were stored at 4°C and 20°C for 4 weeks, and the particle size and SPH loss were measured.

During 4°C storage (Figure 4-9), uncoated liposomes showed no significant size change after 4 weeks of storage ($p > 0.05$). The average SPH loss after four weeks of storage was $87.31 \pm 7.31\%$. This substantial loss was likely due to diffusion of SPH through the phospholipid bilayer.

As for chitosan-coated liposomes, the liposome sizes of both formulations were not significantly different from the fresh samples ($p > 0.05$). However, in Table 4-2, after 4 weeks of storage, the 0.4% CH liposomes showed a higher homogeneity in size distribution (PDI = 0.587) compared to 0.6% CH liposomes (PDI = 0.633). Meanwhile, the average SPH loss for 0.4% CH liposomes was lower than that for 0.6% CH liposomes, $11.47 \pm 0.89\%$, and $27.89 \pm 7.58\%$, respectively.

It appeared that the addition of chitosan stabilized the liposome suspension for 4 weeks of storage at 4°C, but the homogeneity and the ability to retain SPH depended on the chitosan coating concentration. Hence, the addition of chitosan added protection, but excess chitosan appeared to compromise the stability of the coated liposome suspension. These results are in agreement with the results described in Section 4.4.1.

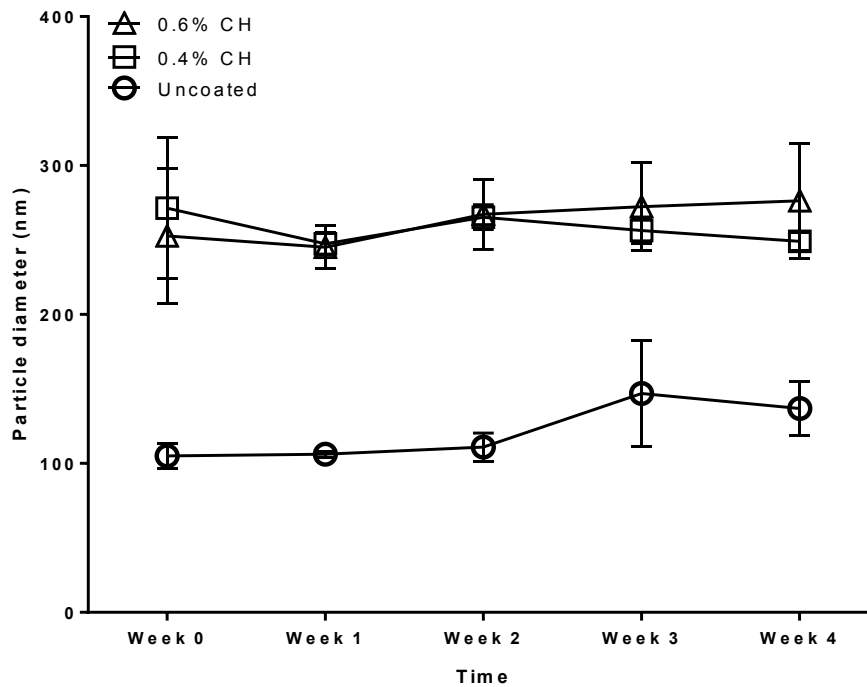


Figure 4-9. Sizes of uncoated MFGM liposomes and 0.4 and 0.6 % (w/v) chitosan-coated MFGM liposomes stored at 4°C. Data are depicted as the mean \pm SD ($n \geq 3$).

The results of long-term storage at 20°C are shown in Figure 4-10. In the case of CH-coated liposomes, neither sample showed significant size change during the first 2 weeks of storage ($p > 0.05$). The particle sizes of 0.4 and 0.6% CH liposomes increased to above 1300 and 6500 nm, respectively, after 4 weeks of storage at 20°C. In addition, the PDI values of both formulations dropped to below 0.5 after 4 weeks (Table 4-2). This increase in homogeneity might be caused by the aggregation of smaller particles, forming more uniform larger aggregates. This could also explain the extensive increase in particle sizes. As for the retention of SPH content, 0.4% CH-coated liposomes released $16.74 \pm 1.54\%$ after 4 weeks at 20°C, whereas 0.6% CH-coated liposome lost $62.05 \pm 3.06\%$ of encapsulated SPH.

On the other hand, uncoated liposomes started to show significant alteration in size only after 4 weeks of storage at 20°C ($p < 0.05$). The particle diameter doubled from $105.0 \pm$

8.4 nm to 226.3 ± 42.9 nm. The liposome diameters were less homogeneous after only 1 week of storage (PDI = 0.353). This change in PDI value was most likely caused by liposome aggregation, thereby inducing the leakage of more than 90% of encapsulated SPH after 4 weeks of storage.

In view of these results, CH coating reduced the SPH loss during long-term storage at both 4 and 20°C. However, excess CH appears to cause aggregation and SPH loss. As for long term storage, 4°C would be a better temperature for both CH-coated and uncoated MFGM liposomes.

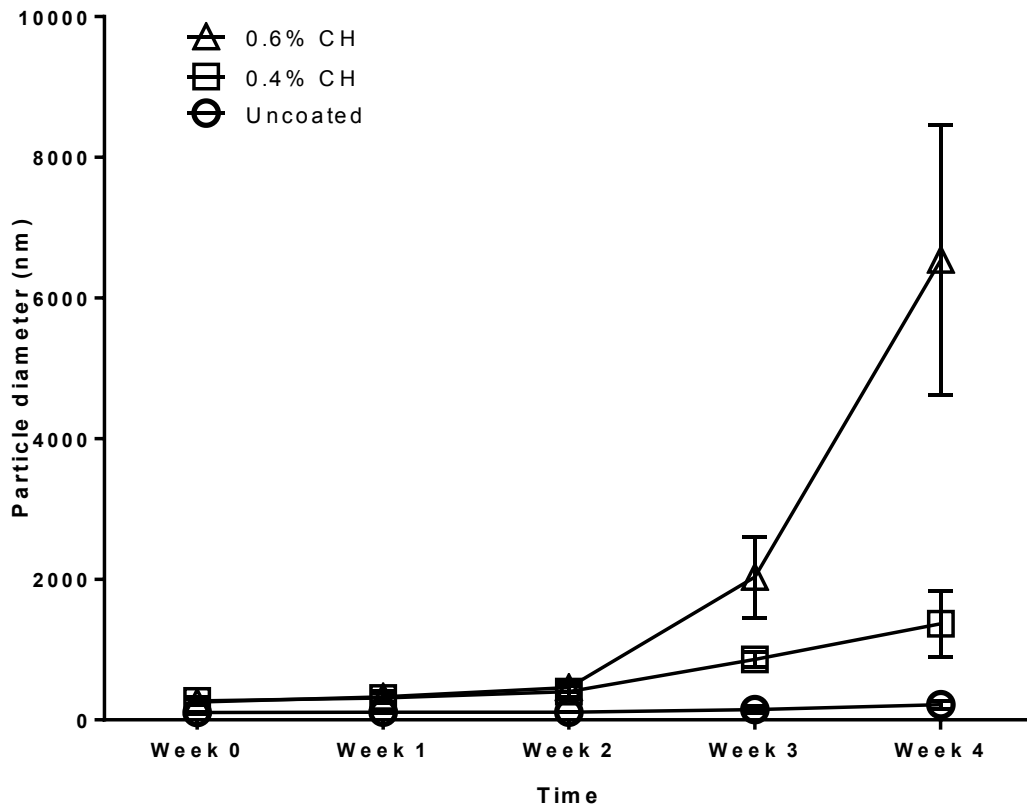


Figure 4-10. Comparison of the sizes of uncoated MFGM liposomes and 0.4 and 0.6 % (w/v) chitosan-coated MFGM liposomes stored at 20°C. Data are depicted as the mean \pm SD ($n \geq 3$).

Table 4-2. Effect of chitosan concentration, storage temperature and storage time on MFGM phospholipid liposome polydispersity index (PDI) (n ≥ 3).

Chitosan coating concentration (w/v %)	Storage time (week)	Storage temperature	
		4°C	20°C
0	0	0.175 ± 0.020	
	1	0.357 ± 0.023	0.353 ± 0.008
	2	0.412 ± 0.099	0.354 ± 0.012
	3	0.429 ± 0.065	0.412 ± 0.086
	4	0.472 ± 0.047	0.525 ± 0.134
0.4	0	0.654 ± 0.077	
	1	0.604 ± 0.013	0.590 ± 0.028
	2	0.555 ± 0.048	0.592 ± 0.038
	3	0.581 ± 0.022	0.601 ± 0.065
	4	0.588 ± 0.059	0.478 ± 0.175
0.6	0	0.571 ± 0.028	
	1	0.552 ± 0.024	0.574 ± 0.042
	2	0.704 ± 0.207	0.575 ± 0.039
	3	0.637 ± 0.156	0.274 ± 0.164
	4	0.633 ± 0.145	0.322 ± 0.075

CHAPTER 5 DISCUSSION

5.1 Effects of MFGM Concentration on Size of Uncoated MFGM Liposomes

The SPH-containing MFGM liposomes were prepared by high-pressure homogenization, using a piston-gap type homogenizer. The mechanism by which piston-gap homogenizers reduce liposome size is by passing the liposomal dispersion through a thin gap at high velocity (Keck & Müller, 2006). The observed homogenized liposome size recorded in this study is supported by other reports in the literature. Sun *et al.* (2008) used a high-pressure homogenizer to produce liposomes with a diameter of 175 nm at 900 bar (13 kpsi) and 101 nm at 1500 bar (22 kpsi) after 5 cycles. Peacock *et al.* (2003) observed a liposome size between 50 to 75 nm after one pass through an Emulsiflex B3 homogenizer (the pressure was unrecorded). Using a Microfluidizer continuous high-pressure homogenizer, Thompson and Singh (2006), produced MFGM liposomes with an average hydrodynamic diameter between 100 and 150 nm after 5 passes at 1100 bar (16 kpsi).

Phospholipid concentration had a significant impact on the size of liposomes. After 5 cycles of homogenization, liposomes with 3% MFGM phospholipid were much smaller than those with 5% or 10% (w/v) MFGM liposomes. This suggested that liposomes with higher phospholipid concentration had greater resistance to the high-pressure shear forces experienced in the homogenization process. Bachmann *et al.* (1993) used a Mini-Lab homogenizer to produce uniform liposomes, and found that repeated circulation led to a reduction in size and increase in homogeneity. The size reduction however, was less effective at high phospholipid concentrations (up to 100 mg/mL). Thompson *et al.* (2006) observed a similar effect when preparing liposomes with a Microfluidizer. They reported that a 10% liposome dispersion showed a smaller size reduction after passing through the Microfluidizer (5 times at 117 MPa) than the 1% and 5% phospholipid dispersions. They concluded that a 10% phospholipid dispersion is resistant to shear forces and turbulence produced by the homogenization process because of its high viscosity. More concentrated

phospholipid dispersions show more resistance to deformation and the breakup of liposomes (Thompson & Singh, 2006).

5.2 Effects of CH-Coating Concentration on Characteristics of Coated MFGM Liposomes

The interactions between MFGM liposomes and CH were investigated by measuring the zeta potential. Zeta potential is related to the stability of suspended particles in a dispersion by characterizing the electrostatic repulsion between them. Generally, if the particles have a smaller (-30 mV to 30 mV) zeta potential, the electrostatic repulsion between particles will be too small to prevent aggregation or flocculation (Malvern Instruments Ltd., 2004). As seen in Section 4.2.3, CH concentration is the main factor affecting the zeta potential. The ability of a charged polyelectrolyte to adsorb to the surfaces of oppositely charged liposomes and cause zeta potential reversal has been well-investigated (Gradauer *et al.*, 2013; Guzey & McClements, 2006; Henriksen *et al.*, 1994; Jain *et al.*, 2012; Kong & Muthukumar, 1998; Meyer, 1998). Stable CH-coated liposomes were formed only when the CH concentration was close to the “optimal” concentration, achieved when the zeta potential reached a relatively constant value (Figure 4-4). The large aggregates formed at suboptimal CH concentrations were mainly caused by charge neutralization and bridging flocculation (Mun, Decker, & McClements, 2005). As liposome surfaces are not saturated with CH, the liposome surface charge consists of both partially negative and positive charges. Consequently, droplets will collide with each other due to charge neutralization (Mun *et al.*, 2005). Bridging flocculation (Figure 5-1) is caused by the extended CH segments of one liposome surface interacting with the vacant surface on another liposome, forming particle-polymer-particle bridges (Pinotti, Bevilacqua, & Zaritzky, 1997). Mun *et al.* (2005) and Laye *et al.* (2008) both reported that stable CH-coated liposome suspensions could only be formed in the presence of sufficient CH. When CH is available to fully coat the liposome surfaces, the coating rate will be faster than the formation of occluding polymer bridges. As a result, the stability of

the liposomes was improved by the CH-coating by maintaining electrostatic and steric repulsion between the coated liposomes (Zhuang *et al.*, 2010).

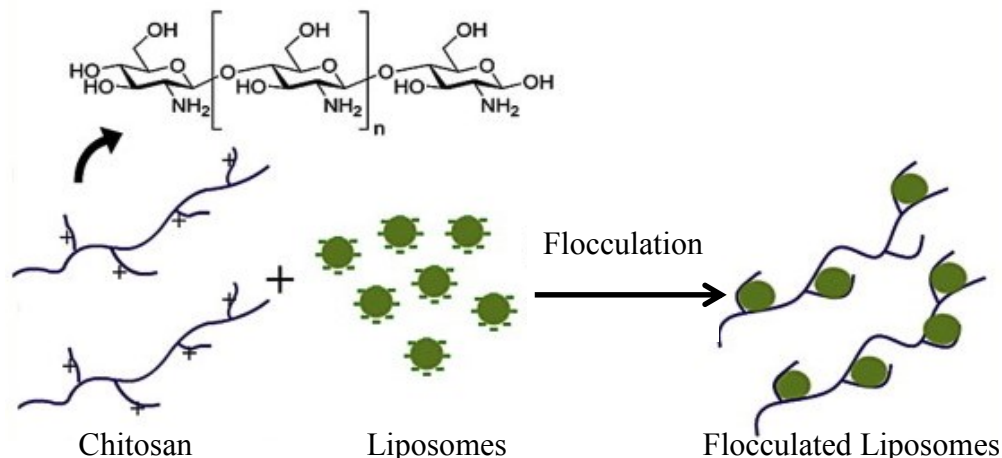


Figure 5-1. Mechanisms responsible for the chitosan flocculation process (Fast, Kokabian, & Gude, 2014).

Bang *et al.*, (2011) observed a liposome size reduction when the CH concentration exceeded the saturation point. This shrink force is produced by the ionic interaction between the CH-coating and the loaded-liposomes. As CH concentration increases, the shrink force also grows, which leads to further size reduction (Bang *et al.*, 2011). In contrast, no obvious size reduction was observed for any of the three formulations.

5.3 Effect of MFGM on Liposome Encapsulation Efficiency

As expected, the EE for uncoated liposomes increased with increasing MFGM concentration (Figure 4-5), with the 10% MFGM liposome formulation as the most efficient. This is not surprising given the fact that hydrophilic entrapment is proportional to the phospholipid concentration and the total internal volume of liposomes (Weiner, 1997). As described in Section 5.1, the average size of an uncoated 10% liposome (105.0 ± 8.4 nm) was also larger than that produced with 3% (85.15 ± 2.26 nm) and 5% ($101 \pm$

3.2 nm) MFGM content ($p < 0.05$). Therefore, uncoated 10% liposomes produced much higher entrapment volume, as the entrapped volume is proportional to the radius to the third power. Meanwhile, the uncoated 10% MFGM dispersion also had much higher liposome concentration than that of either 5% or 3% MFGM dispersions. Hence, uncoated 10% MFGM liposomes gave the highest EE among the three uncoated tested formulations.

However, the EE was similar for the uncoated 3% ($43.0 \pm 5.0\%$) and 5% ($42.3 \pm 5.4\%$) liposomes, regardless of the difference in size ($p > 0.05$). The low EE for 5% liposomes may be caused by the failure to completely sediment all liposomes by ultracentrifugation. Some extremely small liposomes may still be present in the supernatant, which increases the unencapsulated SPH content, and reducing EE. The forces generated by ultracentrifugation may cause liposomal rupture or fusion causing encapsulated peptides to be released during this process. A similar effect was observed by Thompson (2005). In that study, $100,000 \times g$ was used for 8 h to remove hydrophilic material encapsulated in MFGM liposomes. He reported that 10% of liposomes failed to sediment completely even after 24 h at 100,000 g, and damage to sedimented liposomes was observed.

The use of ultracentrifugation is common for the removal of un-entrapped content from loaded-liposomes. Many of the liposome formulations contain significant levels of cholesterol (10-50 mol%) which has been shown to increase the rigidity and stability of the liposome bilayer (Colas *et al.*, 2007; Jain *et al.*, 2012; Liang *et al.*, 2004; Liu *et al.*, 2014; Muramatsu *et al.*, 1999; Wu *et al.*, 2004; Zalba *et al.*, 2012), thus allowing the liposomes to withstand the ultracentrifuge process without disruption of the liposomal membrane and the subsequent loss of encapsulated material. However, the cholesterol content of the Phospholac 700, an MFGM liposomal ingredient, was small (0.032%). Therefore, the loss of EE for MFGM liposomes was likely caused by damage during ultracentrifugation due to the fragile phospholipid membrane.

5.4 Effect of CH-Coating on Liposome Encapsulation Efficiency

The EE of SPH-containing CH-coated liposomes was examined using a range of CH concentrations (0.025 – 0.6% (w/v)). CH concentration exerted a remarkable influence on EE. The reason for the reduction of EE with the addition of CH below the “optimal” concentration was likely attributable to liposome collision and coalescence caused by bridging flocculation and charge neutralization as described in Section 5.2. Meanwhile, uncoated small liposomes may not be successfully removed or damaged by ultracentrifugation, leading to a decrease in EE measurements as described in Section 4.2.4. It has been proposed that a reduction in EE may occur as a result of the reduced association between SPH and the liposome surface (Garcia-Fuentes, Torres, & Alonso, 2005). Positively charged CH molecules and cationic SPH components both have a strong affinity for the liposome bilayer. Therefore, CH could displace the peptides, as a competitor for binding to the anionic lipid surface. This phenomenon has been reported by other authors with different core and liposome ingredients (González-Rodríguez *et al.*, 2007; Guo *et al.*, 2003). However, the displacement of the surface SPH could not be the main factor for the loss of EE because the EE was dramatically higher after the liposomes were fully saturated with CH (Figure 4-5).

5.5 *In Vitro* Release

Results showed that uncoated liposomes retained about 50% of entrapped SPH after 2 h of digestion in SGF, while only 10% of SPH was protected after a 4 h digestion in SIF. The trend was consistent with Liu *et al.* (2012), where 80% and 30% of core material was retained by MFGM liposomes after a 4 h digestion in SGF and SIF, respectively. The initial burst release within the first 30 min was likely due to desorption of the absorbed SPH from the liposome surface. The gradual release after 1 h of digestion was more likely due to the diffusion of the SPH through the coating layers via the hydrocarbon portion of the membrane and the pores within the membrane (Kuboi *et al.*, 2004). Generally, uncoated liposomes are relatively stable in acidic environments (Freund *et al.*,

2000). However, at $\text{pH} < 6.5$ the acidic environment could cause hydrolysis of saturated phospholipids and lead to destabilization of liposomes (Grit, Underberg, & Crommelin, 1993). In addition, Agrawal *et al.*, (2014) reported that the instability of uncoated liposomes was caused by adsorption of oppositely charged ions onto liposome surfaces, such as excess hydrogen ions, from the incubation media.

The poor stability of uncoated liposomes in SIF was mostly due to pancreatin, a proteolytic mixture containing the enzymes pancreatic lipase, phospholipase A_2 , and cholesterol esterase (Liu *et al.*, 2012). Pancreatic lipase catalyzes the hydrolysis of fatty acid ester linkage at the 1 and 3 positions, releasing fatty acids and 2-monoglycerides (Figure 5-2) (Johnson, 2003). Pancreatic lipase also hydrolyzes phospholipids at the 1 position, but at a low rate (De Haas *et al.*, 1965; Johnson, 2003). Moreover, phospholipase A_2 not only catalyzes the sn-2 ester bond hydrolysis of phospholipids to glycerophosphoric acids and 2-acyl lysophospholipids, but is also known for its ability to hydrolyze at the lipid-membrane interface (Vermehren *et al.*, 1998). In addition, cholesterol esterase has high activity of hydrolyzing cholesteryl esters, triacylglycerol, phospholipid, and lysophospholipid (Howles, Carter, & Hui, 1996). Therefore, liposomes were disrupted by hydrolysis, causing leakage of encapsulated SPH through holes formed on the lipid bilayer.

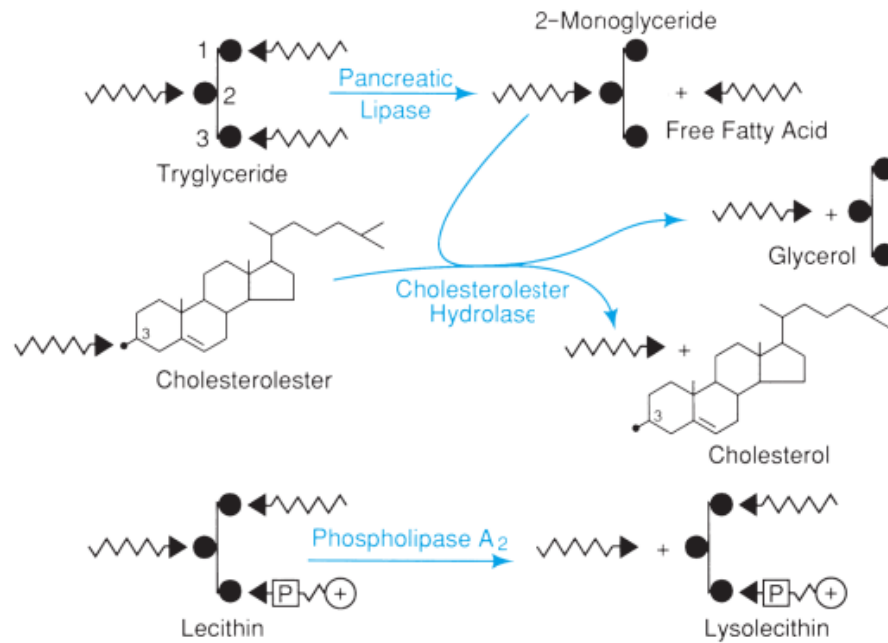


Figure 5-2. Fat digestion processes of three primary enzymes and the digestion products (Johnson, 2003).

CH-coated liposomes were found to be more stable in both SGF and SIF than uncoated liposomes, perhaps attributable to the formation of a robust protective coating layer by strong electrostatic attraction between the chitosan and the surfaces of the liposomes, preventing the exposure of liposomes to the external environment. The limited loss of SPH in SGF indicated that CH-coated liposomes could retain their integrity and protect against pepsin hydrolysis. Although the losses of SPH in the SGF were limited, those in SIF were significantly higher. CH is a weak base that has been observed to lose its charge in neutral and basic environments. When the pH is increased to 6.8, CH forms loops as the polyelectrolytes become less strongly charged. This increases the probability of aggregation due to bridging flocculation (Chen *et al.*, 2013; Claesson & Ninham, 1992; Henriksen *et al.*, 1994).

5.6 Physical Stability

5.6.1 FT and FD-RH Stability

Physical stability was evaluated by measuring the liposome size and SPH loss after perturbing the system by FT and FD-RH. The dramatic size change and SPH leakage from liposomes during freezing is believed to be caused by two main physicochemical processes, mechanical stress caused by ice formation and chemical destruction of liposomes due to a steep increase of solute (Nakhla, Marek, & Kovalcik, 2002). During freezing, ice crystals are formed in the bulk solution, which in turn forces liposomes closer together. Freezing gradually increases the liposome concentration in the non-freezing regions, thereby making them more prone to liposome coalescence and collision (Degner *et al.*, 2013; Thanasukarn, Pongsawatmanit, & McClements, 2004). Meanwhile, the formation of ice crystals may cause mechanical stress for the liposomes, as ice crystals may penetrate into the membrane of liposomes, leading to particle destabilization and leakage of core materials (Stark, Pabst, & Prassl, 2010).

Glycerol was added to the formulations as a cryoprotectant to postpone these aforementioned degradation mechanisms (Rudolph & Crowe, 1985). Glycerol (CH₂OH-CHOH-CH₂OH) has three OH groups, which are targets for H-bonding with the available oxygen atoms on the head of the phospholipids (Kundu, Majumde, & Preet, 2011). Therefore, glycerol will form a protective coating on the inner and outer membranes of the liposomes, which protects the liposomes from ice crystals (Figure 5-3).

Nevertheless, leakage of SPH was extremely high for uncoated liposomes after FT in this study. Harrigan *et al.* (1990) reported that the protection of liposome from FT by glycerol is a concentration-dependent process. Their study showed that when the glycerol concentration was about 15% (w/w), the freeze-thaw-induced leakage from egg phosphatidylcholine liposomes was significantly reduced. An optimal glycerol concentration is required, as both too little and too much glycerol leads to the destabilization of liposomes (Harrigan *et al.*, 1990).

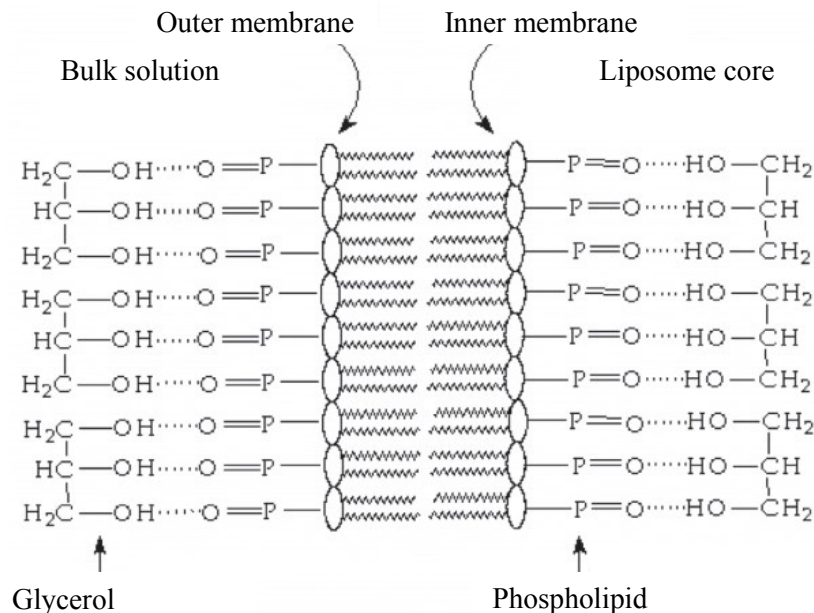


Figure 5-3. Schematic diagram of the interaction of glycerol with phospholipids by hydrogen bonding. The H-bond is formed between an oxygen atom of the phospholipid head group and an OH-group of glycerol. ‘.....’ lines represents H-bonding (Kundu *et al.*, 2011).

However, it was found that glycerol failed to stabilize the uncoated liposomes during FD-RH. After freeze-drying, the final product did not form a freeze-dried cake, as anhydrous glycerol is a liquid at room temperature. A similar effect was also observed by Stark *et al.* (2010). They reported that the freeze-dried liposomes appeared “gluey” and “smeary” and could not be re-suspended properly. Therefore, the proper use of “lyoprotectant”, substances that stabilize molecules during freeze-drying, still needs to be discussed. It has been reported that disaccharides such as sucrose and trehalose are the most effective lyoprotectants for liposomes (Hua *et al.*, 2003; Stark *et al.*, 2010). Immediately after drying, the disaccharides have very low molecular mobility and high viscosity, forming an amorphous glassy matrix, thereby preventing direct contact between liposome vesicles and helping improve stability (Rudolph, 1988). In addition, the sugar molecules may also stabilize the phospholipid membrane via hydrogen bonding (Crowe, Spargo, & Crowe, 1987). Crowe *et al.* (1987) explained this phenomenon through a *water replacement hypothesis*. The sugar molecules interact directly with the phospholipid hydrophilic head

groups, replacing the water molecules upon drying, and maintaining the space between the head groups.

The observation that an “optimal” CH coating concentration improves the stability of liposomes against severe physical stress during FT and FD-RH may be due to a number of different mechanisms. The CH interfacial layer provides a greater steric repulsion - provided by the long loops and tails of CH extending out into solution - between the particles than with the uncoated liposomes. Hence, the CH coating layer sterically stabilizes the suspension and prevents coalescence (Liang *et al.*, 2007). Furthermore, it is more difficult for ice crystals to penetrate through the thicker membrane during freezing (Ogawa, Decker, & McClements, 2003). Considering that CH can also form hydrogen bonds between the polymer and water molecules, it is expected to provide a similar protective mechanism as in the case of sugar molecules (Takeuchi *et al.*, 1998). A CH coating replaces the water hydrogen bonds forming a pseudo-hydration phase through their interaction with phospholipid head groups, which further improves the stability of the coated liposomes (Crowe *et al.*, 1988; Strauss *et al.*, 1986).

Nonetheless, when the CH concentration exceeds the “optimal” concentration, the stability of liposomes becomes impaired. Excess CH led to extensive aggregation during FT and increasing the particle size FD-RH (Figure 4-7A and Figure 4-8A). Therefore, excess CH could not further improve the ability to overcome physical stress during freezing or freeze-drying, as the high CH content causes particle aggregation due to depletion flocculation (Zhuang *et al.*, 2010). Depletion flocculation is similar to a mechanism found in emulsions (Guzey & McClements, 2006). When a non-absorbing polymer (excess CH) is added to a colloidal suspension, particles will restrict the presence of free moving polymers near to their surfaces because they will cause the loss of conformational entropy of the polymer chains (Fleer *et al.*, 1993). Therefore, depletion zones are formed around the surface of the particles, in which the free flowing polymers are redistributed away from the surface to avoid entropy loss (Fleer *et al.*, 1993). As a result, an osmotic pressure gradient is formed due to the polymer concentration between the surface of the colloid (the depletion zone) and the bulk. When depletion zones

overlap, a larger volume is available to the free polymers in the system, which increases the entropy of the free polymers. Therefore, the particles will finally aggregate with each other due to this entropy-driven attractive force (Jenkins & Snowden, 1996). Figure 5-4 illustrates the schematic illustration of depletion flocculation with high non-absorbing polymer chains around two colloids. In the present study, the excess free CH has perhaps created a gradient of osmotic pressure due to lower CH concentration near the CH-coated liposome surface than in the suspending medium. The particles flocculated when the repulsive interactions (electrostatic force, steric stabilization) between the coated droplets are not strong enough to balance the net attractive entropic force. Otake *et al.* (2006), González-Rodríguez *et al.* (2007), Bang *et al.* (2011), and Gibis *et al.* (2014) have also observed a similar phenomenon. However, Bang *et al.* (2011) also reported that there was a decrease in EE, because the excess CH destabilized the system and led to the release of encapsulated materials.

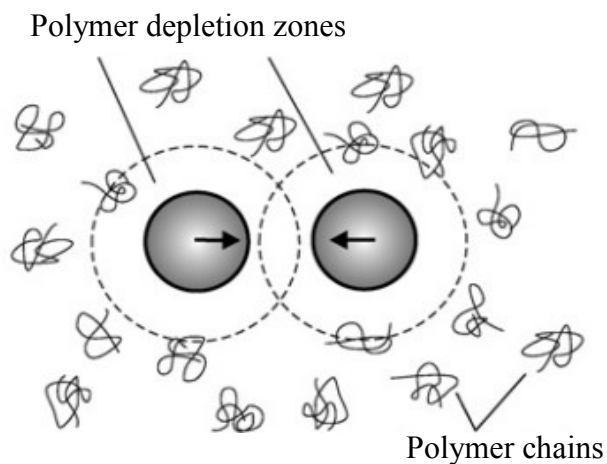


Figure 5-4 An illustration of depletion flocculation. The overlapping of depletion zones leads to a net attractive entropic force (black arrows) (Fan & Tuinier, 2010).

5.6.2 Long Term Storage

The stability of the liposome systems was determined by the change of particle diameter with time and the final SPH content. The aggregation and SPH loss at 4°C storage was less than at 20°C. The improvement of stability at lower temperatures may be due to the low permeability of the coating layers, the inhibition of aggregation (low molecular mobility), and the retardation of oxidative degradation of unsaturated fatty acids in the phospholipid bilayers (Gibis *et al.*, 2014; Zhao *et al.*, 2011).

Uncoated liposomes were significantly less stable than CH-coated liposomes during long-term storage at both storage temperatures. The fusion of liposomes is likely the main mechanism leading to SPH loss. Generally, liposomes are prone to aggregate and form larger vesicles over time. Liposome dispersions tend to move toward a minimum energy state, becoming more thermodynamically stable and involving a flat monolayer of lipid bilayer (Israelachvili, 2011). The observed increase in stability of CH-coated liposomes could be due to the electrostatic and steric repulsion as described in Section 5.2. The thicker membrane leads to slower diffusion of SPH through the coating layers, thereby increasing the SPH retention time. Meanwhile, MFGM phospholipid contains a higher percentage of saturated and mono-unsaturated fatty acids. Gibis *et al.* (2013) and Panya *et al.* (2010) reported that CH coating inhibited the oxidative degradation of phospholipids by forming a charged barrier to inhibit the contact of pro-oxidants, such as metals, with the phospholipid bilayers. In addition, since the overall surface charge of MFGM liposomes is negative, this may lead to electrostatic attraction of pro-oxidant metals, thereby increasing the chance of metal–lipid interactions and accelerating oxidative reactions of unsaturated phospholipids. Therefore, electrostatic deposition of CH-coating onto MFGM liposomes may prevent lipid oxidation by charge repulsion of metal ions, thus minimizing metal–lipid interactions (Gibis *et al.*, 2013; Shaw *et al.*, 2007).

CHAPTER 6 CONCLUSIONS

Overall, this work demonstrated that it is possible to encapsulate SPH in loaded MFGM phospholipids using high-pressure homogenization. The liposome size and encapsulation efficiencies increased with increasing MFGM phospholipid concentration. CH coatings were successfully attached to liposome surfaces by electrostatic interactions. The coating concentration had a great impact on zeta potential of coated particles, increasing with increasing levels of CH, and plateaued beyond “optimal” CH concentration. Stable CH-coated liposomes were formed when the CH concentration was close to the “optimal” concentration, achieved when the zeta potential reached a relatively constant value. “Optimal” CH concentration played a unique role with regard to particle size and encapsulation efficiencies of CH-coated MFGM phospholipid liposomes. Below the “optimal” CH concentrations, liposome collided with each other forming large aggregates by charge neutralization and bridging flocculation. The collision process in turn led to leakage of entrapped SPH.

CH coating also helped to retain encapsulated SPH during *in vitro* digestion in simulated gastrointestinal fluids compared to uncoated MFGM liposomes. Only 13.2% of SPH was released from 0.4% CH-coated MFGM liposomes in acidic SGF in 2 h, but 47.9% of SPH was released after 4 h in SIF. Therefore, CH-coated MFGM technology has the potential to be used for pH responsive oral delivery of SPH.

The stability of uncoated liposomes was greatly compromised after 4 weeks of storage at -30°C or freeze-drying. There was extensive loss of encapsulated SPH for uncoated liposomes after FT and FD-RH, indicating diffusion of SPH through the phospholipid bilayers. Uncoated liposomes experienced a relatively small size change during FT. Because glycerol was used as a cryoprotectant, it stabilized the size of uncoated liposomes during FT possibly by forming H-bonding with the head of the phospholipids. However, extensive size change was seen during FD-RH of uncoated liposomes, because uncoated liposomes could not form a dried cake after freeze-drying, since anhydrous glycerol is in its liquid form at room temperature.

CH coating improved the stability of liposomes during FT and FD-RH. The coating layer prevented aggregation and fusion of the liposomes by adding thickness to the membrane, increasing steric repulsion between particles, and forming a pseudo-hydration phase. Only 24% and 15% SPH was lost during FT and FD-RH. Excess CH coating resulted in a size increase after FT, which might be caused by depletion flocculation. Excess CH did not have an effect on SPH leakage.

Long-term storage results showed that 4°C was a better storage condition for both coated and uncoated liposomes. CH coated liposomes showed better ability to retain encapsulated SPH during storage. Liposomes with “optimal” coating concentration experienced minimal SPH loss and size change. Excess CH led to a dramatic size increase during 20°C storage and a bigger loss of SPH under both storage conditions.

CHAPTER 7 FUTURE WORK

Future work should be focused on improving the stability of the coated liposomes during FT and FD-RH. The type and concentration of cryoprotectants and lyoprotectants on SPH retention in CH-coated liposomes should be studied. Also, the retention of bioactivity of encapsulated SPH should be determined, as the encapsulation process may alter the properties of SPH. Meanwhile, It is recommended to test different loadings of SPH with constant MFGM level in order to get the maximum and the optimal SPH encapsulation ability of MFGM.

Because the CH-coated MFGM liposome delivery system for SPH was designed with oral administration in mind, it would be necessary to evaluate the mucoadhesive properties of CH-coated liposomes *in vitro* and *in vivo*. Meanwhile, although it has been shown that SPH was successfully encapsulated and the release in acidic pH was retarded by CH coatings in the liposomes, this does not confirm whether the SPH was still active. Hence, the *in vivo* behavior of the CH-coated liposomes after entering systemic circulation should also be monitored by measuring the bioactivity related to improvement of insulin sensitivity, possibly in animal models and clinical trials.

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