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LIPOSOMES AS CARRIERS FOR SYSTEMIC DELIVERY OF SALMON CALCITONIN BY INHALATION

By

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Under the direction and supervision of

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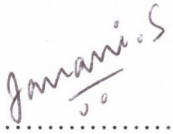
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Janani Swaminathan

Dedicated to my

beloved parents

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SUMMARY

The concept of delivering proteins and peptides to the systemic circulation via the lungs has been extensively investigated over the last 20 years. Main drivers for the approach are the lungs' high surface area, presumed low activity of metabolising enzymes, quick onset of action and improved patient compliance due to the ability of non-invasive delivery. In addition, less frequent dosing might be possible, if a sustained release delivery system was developed. One of the candidate peptides that have been studied for pulmonary delivery is salmon calcitonin (sCT). Liposomes are carrier systems that have been used for more than 40 years for both drug and protein/peptide delivery, with commercially successful products of, e.g., doxorubicin and amphotericin B. Whilst liposomes containing sCT for parenteral, oral and intranasal delivery have already been reported, liposomal systems for pulmonary sCT delivery have not been reported to date.

Liposomes were prepared by lipid film hydration method. Preliminary studies were performed to develop the best possible method for removal of non-encapsulated drug among - gel filtration using different exclusion limits of gels, centrifugation at 3000 rpm, ultracentrifugation at 52,000 rpm and filtration. Gel filtration using pre-packed columns was found to work best in my hands. Following this, a formulation development study was carried out to assess the impact of pH values of hydration buffer (pH 3.5, 5.0. 7.0, 8.5 and 10.0), surface charge (varied between neutral, negative and positive) and membrane rigidity (rigid and fluid lipids were used) on encapsulation efficiency and stability of sCT. High encapsulation efficiency and good stability of sCT was noticed when the pH value of hydration buffer was 3.5 with liposomes having a rigid membrane and positive surface charge. Percentage of positively charged lipid (Stearyl amine, SA) was varied between 10, 20 and 30% to report 10 and 20% SA based formulations were an optimal compromise between high encapsulation efficiency and acceptable surface charge (less than +25 mV which could otherwise lead to toxicity *in vitro* and *in vivo*). Next, formulations with 10 and 20% SA were substituted with 5 mol% DSPE-PEG. Following PEGylation, there was a significant reduction in zeta-potential and encapsulation efficiencies of DPPC:Chol:SA:DSPE-PEG_{2000} (55:20:20:5) and their non-charged PEG counterparts, DPPC:Chol:DSPE-PEG_{2000} (75:20:5), were higher than the other formulations studied. Formulations were then subjected to nebulisation in an Aeroneb Pro vibrating mesh nebuliser. This resulted in encapsulation efficiencies of PEGylated formulations being lesser compared to their non-PEG counterparts. In parallel, the impact of lyophilisation on the encapsulation efficiency was studied. Four formulations investigated were PEGylated liposomes [DPPC:Chol:DSPE-PEG_{2000} (75:20:5) and (65:30:5)] and their non-PEGylated counterparts [DPPC:Chol (80:20) and

(66.7:33.3)]. Variants for each formulation were: a batch that was not lyophilised, a batch that was lyophilised without cryoprotectant (trehalose-di-hydrate) and 3 batches that were lyophilised with varying ratios of lipid:cryoprotectant [(1:1), (1:5) and (1:10)]. Post lyophilisation, PEGylated formulations showed higher encapsulation efficiency even in the absence of trehalose-di-hydrate. Non-PEGylated formulations did not show an increase in encapsulation efficiency. This was investigated further by performing ^{31}P-NMR spectroscopy which revealed the presence of micelles and liposomes in the PEGylated formulations while the micelles were absent in the non-PEGylated formulations. Micelle formation occurred in PEGylated liposomes due to ineffective mixing of the base lipid (DPPC) and PEG lipid (DSPE-PEG) due to unequal chain lengths that could have led to migration of PEG lipid into solution which formed micelles that brought about an increase in surface area and consequent encapsulation of sCT.

In vitro release of sCT was demonstrated in Krebs-Ringer buffer of pH value 7.4, at 37°C and 100 rpm. Liposomes were present in the donor compartment that comprised the Float-A-lyzer unit. At lower sCT concentration, release of sCT from PEGylated liposomes [DPPC:Chol:DSPE-PEG_{2000} (75:20:5)] was significantly retarded which might be due to steric hindrance of PEG groups compared to their non-PEG counterpart [DPPC:Chol (80:20)]. No mass balance was noticed at the end of the study in either case due to instability of sCT at mentioned study conditions. The bioactivity of liposomal sCT was tested using a T47D cell-based cAMP release assay. Liposomes thus investigated were DPPC:Chol:DSPE-PEG_{2000} (75:20:5) and DPPC:Chol (80:20). Regardless of the formulation, the bioactivity was always approximately 50%. *In vivo* studies were then performed using male Wistar rats. The two liposomal formulations tested were DPPC:Chol:SA:DSPE-PEG_{2000} (55:20:20:5) and DPPC:Chol:DSPE-PEG_{2000} (75:20:5). The former was nebulised and the latter was intravenously administered at a concentration of 50 μg/kg of sCT. sCT solution administered at the same concentration and by the same route served as control. Following pulmonary administration, the plasma concentrations of sCT obtained with liposomes were lower than that obtained with the solutions. This might be attributed to the sustained release of sCT from the liposomes and clearance by lung macrophages or degradation by lung metabolising enzymes. Following intravenous administration, plasma levels of sCT liposomes were significantly higher than that obtained with sCT solution. Though liposomal formulations that were stable to nebulisation that showed prolonged sCT release kinetics *in vitro* and excellent encapsulation efficiency of sCT following lyophilisation were prepared, they proved suboptimal following pulmonary administration in an experimental animal model.

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1. INTRODUCTION

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1.1 PULMONARY DELIVERY OF PROTEINS AND PEPTIDES

1.1.1 Why pulmonary delivery of proteins/peptides?

The past 30 years of biotechnology discoveries have unleashed a wave of therapeutic proteins most of which are administered via injection to overcome degradation in the gastrointestinal tract. In addition IV methods are not patient compliant due to the pain and expense associated while pulmonary delivery offers a patient-friendly, non-invasive alternative to injections. Targeted protein delivery to the lungs has evolved to be one of the most widely investigated systemic or local protein delivery approaches over the recent decade. (1) Advantages of pulmonary delivery include, large surface area of the lung, good vascularisation providing immense capacity for solute exchange, ultra-thinness of the alveolar epithelium and avoidance of hepatic first pass. Moreover, no non- invasive route of delivery provides the speed of action that an inhaled drug can provide. (2, 3, 4)

1.1.2 Downsides of pulmonary delivery of proteins and peptides

Downsides of pulmonary delivery of proteins and peptides can be approached from both micro and macro levels. At micro or cell level, drawbacks include the mucociliary clearance (mucociliary escalator, coughing and alveolar clearance), presence of alveolar macrophages, enzyme activity, compatibility of protein with lung tissue and the small dose size. (1) Figure 1.1 illustrates the barriers that need to be crossed by proteins and peptides before they are absorbed into systemic circulation. (5) At a macro level, drawbacks would focus on comprehensive factors from bench (formulation related) to bedside (patient related). Figure 1.2 illustrates the collective problems associated with formulation and delivery of protein/peptide biopharmaceutical. (6-11)

1.1.3 Proteins and peptides that are under investigation for pulmonary delivery

Suitability for pulmonary delivery of various proteins and peptides has been investigated. Some of the proteins and peptides under investigation are mentioned in table 1.1. (12)

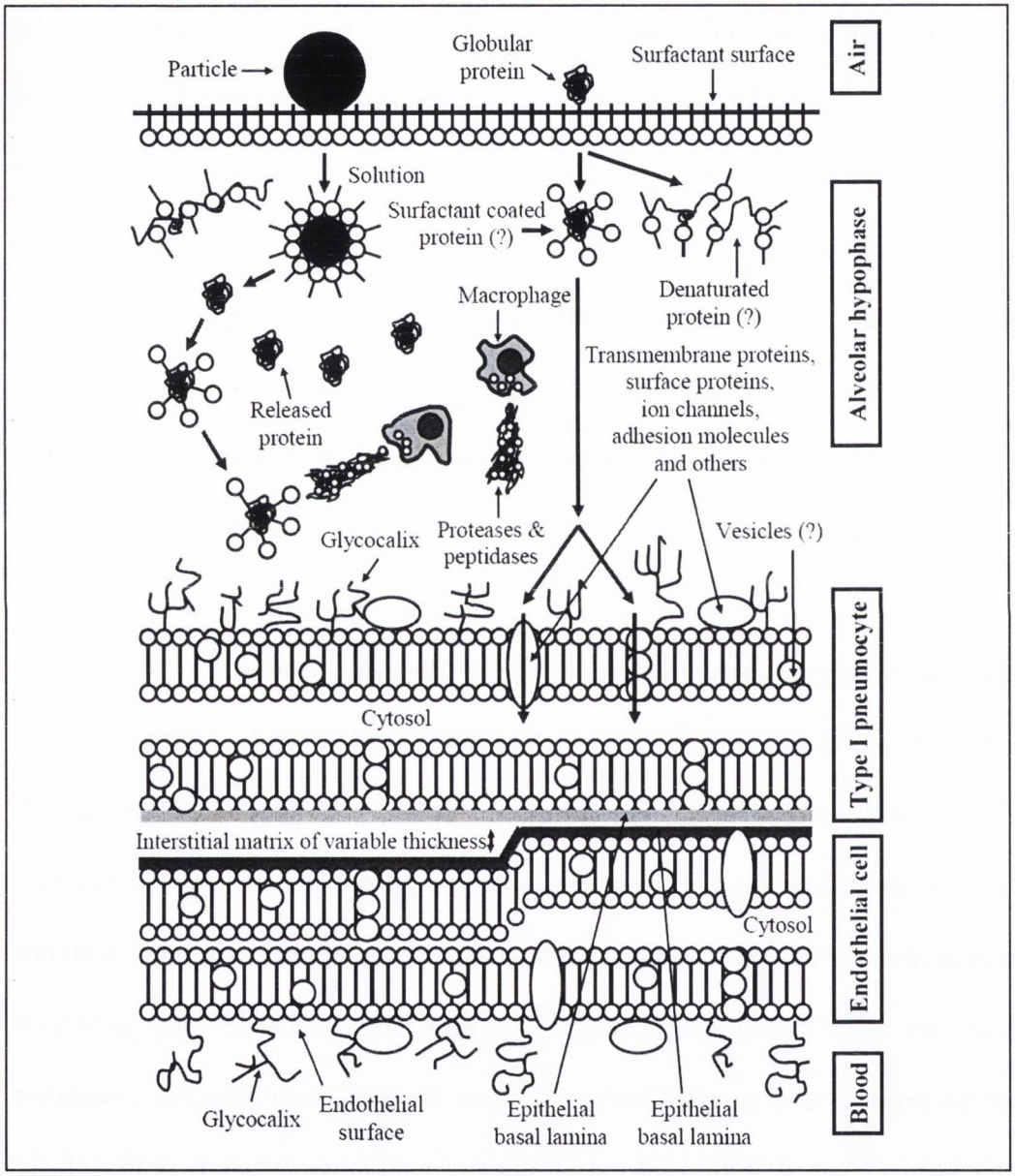


Figure 1.1: Barriers for absorption of peptides and proteins after peripheral/alveolar deposition (taken from reference 5). This figure illustrates the barriers a particle needs to cross once deposited in the lung including factors like

interaction with lung surfactant, lung macrophages and metabolising enzymes in addition to barriers offered by endothelial cells and pneumocytes.

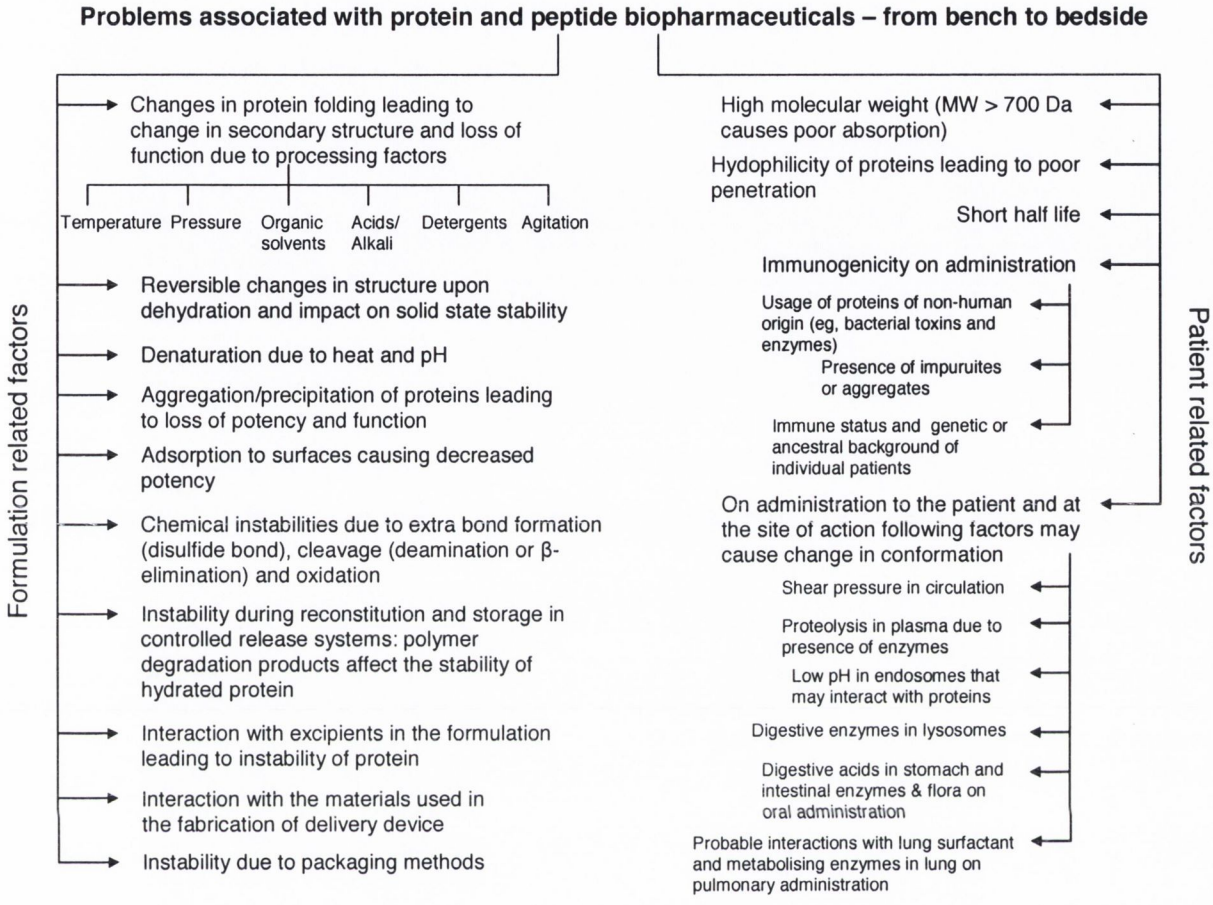


Figure 1.2. Problems associated with protein and peptide biopharmaceuticals – bench to bedside. (Compiled from references 6-11)

Table 1.1. Examples of proteins and peptides for inhalation (taken from reference 12)

Disease state	**Peptide / Protein**
Local	
Adult respiratory distress syndrome	Surfactant proteins (approved)
Cystic fibrosis	DNase (approved)
Emphysema/Cystic fibrosis	Alpha-1-antitrypsin and secretary leukoprotease inhibitor
Lung transplant	Cyclosporin A

Cancer/Pneumocystis carnii	Interferon-γ and interleukin-2
Alpha-1-antitrypsin deficiency	Alpha1 proteinase inhibitor
Asthma	IL-1R and Anti-IgE mAb
Anti-TB vaccine	Muramyl dipeptide
Oxidative stress	Catalase and superoxide dismutase
Systemic	
Osteoporosis	Calcitonin and parathyroid hormone
Growth deficiency	Human growth hormone
Multiple sclerosis	Interferon-β
Diabetes	Insulin (approved)
Cancer	LH-RH analogs
Viral infections	Ribavirin and interferon-α
Neutropenia	rhG-CSF
Anaemia	Erythropoietin
Anticoagulation	Heparin
Diabetes insipidus	dDAVP (1-deaminocysteine-8-D-arginine vasopressin)

1.2 LIPOSOMES AS CARRIERS FOR PROTEINS AND PEPTIDES

1.2.1 Liposomes

Liposomes are concentric bilayered vesicles in which an aqueous volume is entirely enclosed by a membranous lipid bilayer composed mainly of natural or synthetic phospholipids. The structure of a liposome vesicle is shown in Figure 1.3. Liposomes are the commonly used lipid-based drug delivery systems. Other lipid carriers being solid lipid nanoparticles (SLN), oily suspensions, submicron lipid emulsions, lipid implants, lipid microtubules, and lipid microspheres. (13)

Sterically stabilised or Stealth liposomes are those in which addition of a hydrophilic polymer or glycolipid such as PEG (Poly ethylene Glycol) or GM1

(Monosialoganglioside) causes an increase in their circulation time. The prolonged circulation time is made possible by attachment of the macromolecule (PEG/GM1) immediately adjacent to the liposomal surface (known as periliposomal layer) as shown in Figure 1.4, thus preventing the binding of plasma proteins to the liposomal surface and consequently monophagocytic system (MPS) or macrophage interactions. This is known as steric hindrance. (14, 15)

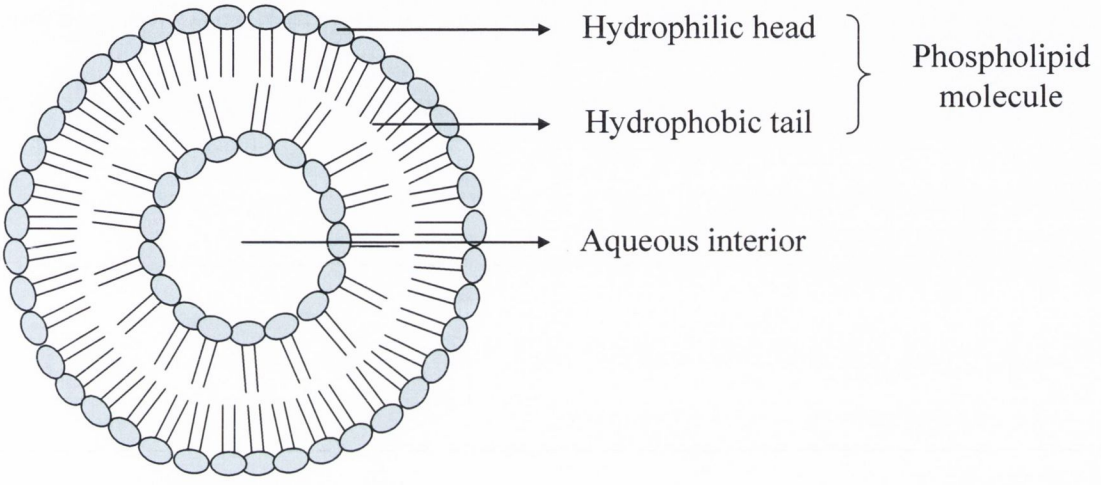


Figure 1.3. Structure of an unilamellar liposome

In addition to their advantages of strongly reduced MPS uptake and prolonged circulation times, stealth liposomes are able to extravasate at body sites where the permeability of vascular wall is increased which happens during an infection or inflammation and in case of tumours. From a formulation stability standpoint, the macromolecules (PEG/GM1) prevent instant dissolution of the liposomes in lung fluid, which predominantly consists of lecithin. (14, 15)

PEG is a linear polyether diol with good solubility in aqueous and organic media, biocompatibility, and lack of toxicity, low immunogenicity and antigenicity. Surface modification can be achieved by three ways: physical adsorption of the polymer onto the vesicle surface, incorporation of PEG-lipid conjugate during liposome preparation

or by covalent attachment of reactive groups onto surface of preformed liposomes. The most commonly used and preferred method is to anchor the polymer to the lipososmal membrane via a cross linked lipid, for eg- PEG-DSPE (Di-Stearoyl Phosphatidyl Ethanolamine). (14, 15)

GM1 is a brain tissue derived monosialoganglioside which when incorporated on the liposome surface prolongs circulation time. GM1 liposomes with diameter 90-200nm have longer blood retention and consequent accumulation in tissues, whereas those larger than 300nm preferentially accumulate in spleen and those less than 40nm probably penetrate the interstitial spaces of liver. MPS avoidance is reversed by removal of sialic acid moiety. (14, 15)

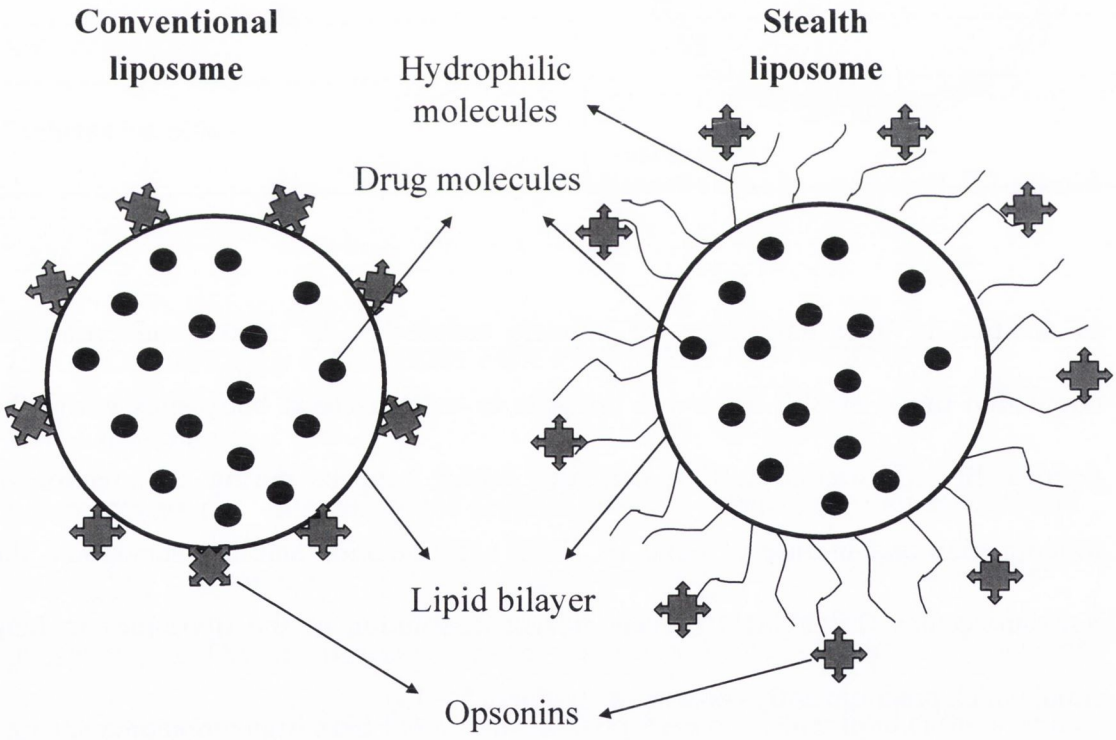


Figure 1.4. Stealth liposome

1.2.2 Classification of liposomes

Liposomes can be classified based on various parameters as shown in table 1.2

I) According to their size and lamellarity (16)

(Liposome classification based on size and lamellarity is represented in Figure 1.5.)

II) According to the method of their preparation (16-19)

(Liposome classification based on method of preparation is illustrated in Table 1.2

III) According to their surface properties (14, 15, 20, 21)

(e.g., conventional liposomes, sterically stabilised liposomes or immuno-liposomes and cationic liposomes)

IV) According to their application (22, 23)

(e.g., diagnostic liposomes or therapeutic liposomes)

Table 1.2. Classification of liposomes

I) According to size and lamellarity 1. Unilamellar vesicles 2. Oligolamellar vesicles 3. Multilamellar vesicles 4. Multivesicular vesicles
II) According to method of preparation 1. Liposomes prepared by solvent free procedures a) Dry film or thin film hydration: A dry lipid film obtained after evaporation of organic solvent is hydrated with a buffer containing the pay load b) Lipid partitioning of hydrophobic peptides: In this method, a pre-existing solution of unilamellar liposomes is used to dissolve the protein by allowing it to be solubilised within the lipid membrane itself

c) Lipid surfactants: In this method, a solution of detergent is mixed with lipids and protein and the combined solution is subject to chromatography to remove the detergent and convert mixed micelles to liposomes

d) Polymorphic lipid: This approach involves admixing of a protein with a polymorphic lipid (capable of undergoing induced structural rearrangement from micellar state to bilayer configuration) to form liposomes

e) Freeze-thaw liposomes: In this method, freezing and thawing of either small unilamellar vesicles or concentric bilayer liposomes in the presence of pay load leads to breakage and re-formation of bilayers with subsequent encapsulation of pay load

f) Freeze-dehydration liposomes: Here pay load is freeze dried or lyophilised in the presence of liposomes that brings about its encapsulation

g) Osmotically dried vesicles: In this procedure, internal osmotic pressure in the aqueous interior is used as the driving force to achieve disruption and reformation of membrane and encapsulation of pay load

2. Liposomes prepared by solvent containing procedures

a) Reverse phase evaporation technique: In this approach an aqueous buffer containing the pay load is introduced into a mixture of phospholipid and organic solvent and the organic solvent is removed by evaporation under pressure.

b) Stable plurilamellar vesicle procedure: Lipid film as obtained in thin film hydration method or MLV's is used as the primary source. To this an organic solvent and buffer are added and sonicated till organic solvent evaporated and liposome suspension is obtained.

c) Ethanol injection technique: In this technique, due to immediate dilution of ethanol (containing lipids) in aqueous phase lipid molecules precipitate and form bilayer fragments and subsequently liposomes encapsulating the aqueous phase

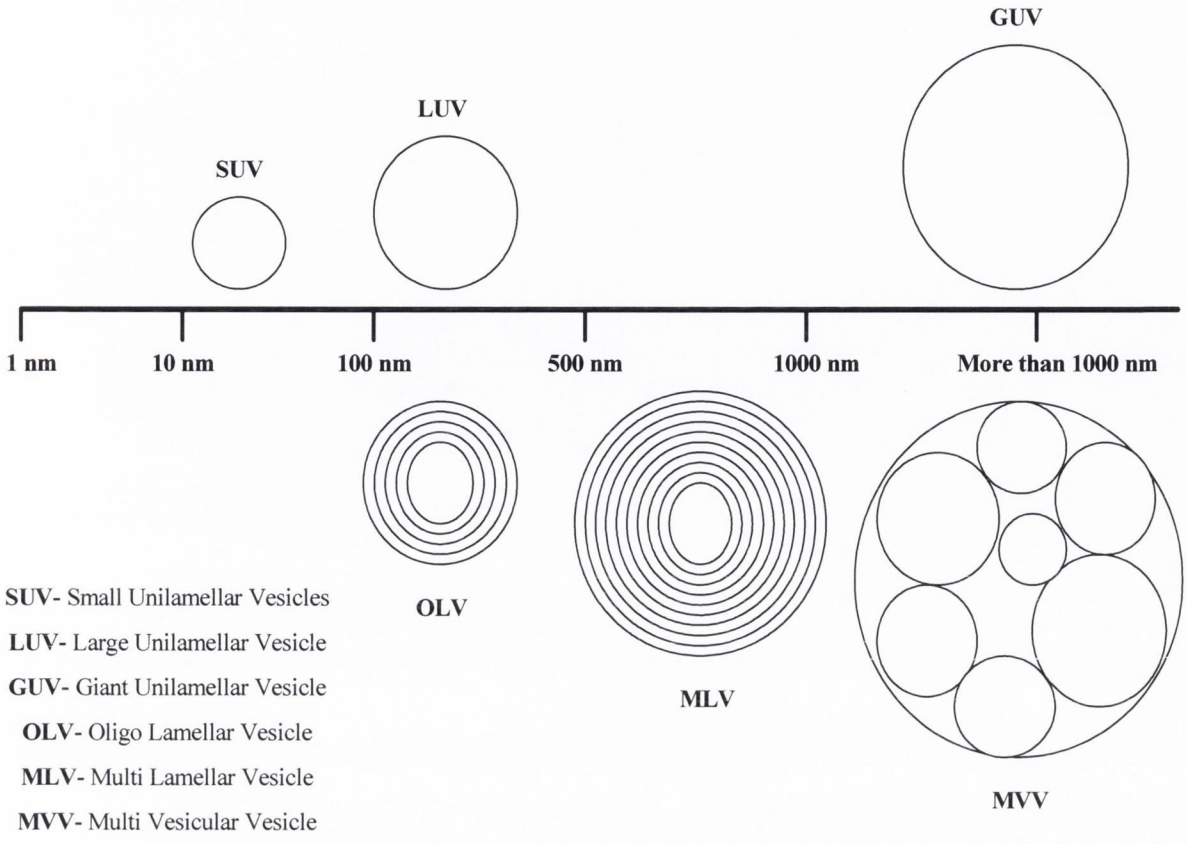


Figure 1.5. Classification of liposomes according to size and lamellarity

1.2.3 Routes by which liposomes can be administered

Various routes by which liposomes containing biopharmaceuticals can be administered, including the nature of formulation and device used for administration are schematically represented in Figure 1.6.

The four widely investigated liposome encapsulated peptides for various routes of administration are insulin, salmon calcitonin, parathyroid hormone and interleukins. (24)

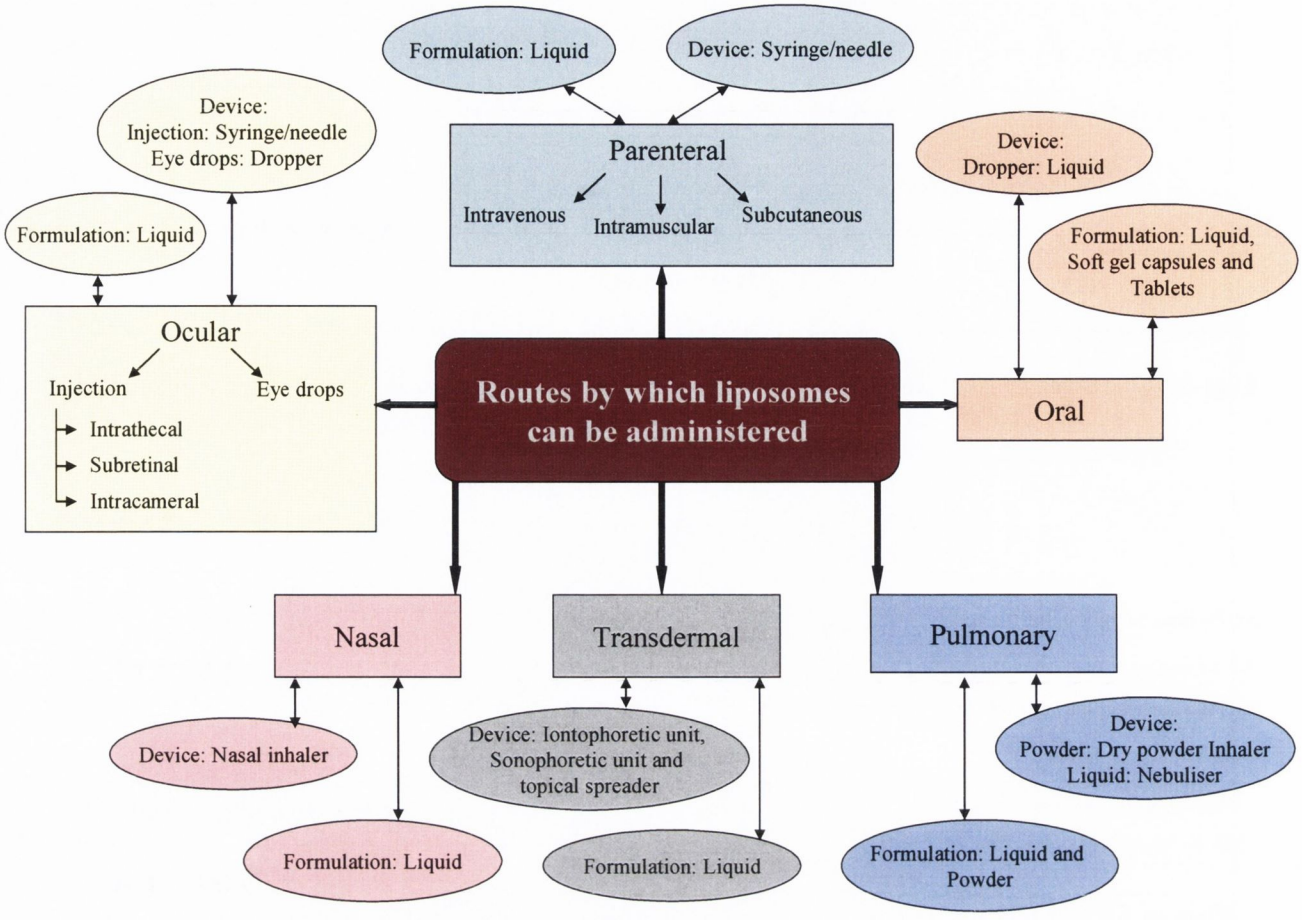


Figure 1.6. Routes of administration of liposomes

1.2.4 Liposomes for pulmonary peptide delivery: Suitability as carriers, early studies and recent in vitro/in vivo studies

1.2.4.1 Suitability of liposomes as carriers for pulmonary delivery of protein/peptide

In spite of availability of other advanced drug delivery systems like microparticles and nanoparticles, liposomes are considered to be promising candidates for pulmonary drug delivery due to the following reasons: (25)

- Ability to solubilise poorly soluble drugs
- Capacity to provide a reservoir for sustained release, prolonging local and systemic therapeutic levels

- Facilitation of intracellular delivery of drugs especially to alveolar macrophages
- Avoidance of local irritation of lung tissue
- Ability to target specific cell populations using surface bound ligands or antibodies
- Potential to be absorbed across the epithelium intact to reach the systemic circulation

Another reason for their choice is the chemical similarity of liposome formulations with lung surfactant. Human pulmonary surfactant is a mixture of lipids and proteins, of which the lipids account for nearly 20% of total tissue dry weight. The lipid pool comprises about 80% phospholipids, principally Dipalmitoylphosphatidylcholine (DPPC); and 8% neutral lipids (Cholesterol (Chol) and free fatty acids). Other phospholipid components of surfactant are Phosphatidyl Glycerol (PG) and Phosphatidyl Inositol (PI). The four Surfactant Proteins (SPs), SP-A, SP-B, SP-C and SP-D, account for 12% of the surfactant content. Of these SP-A and SP-D are large, glycosylated water soluble molecules, whereas SP-B and SP-C are highly hydrophobic small peptides.

1.2.4.2 Early in vivo studies on liposomes

Some early studies performed by various scientists suggest the safety and efficacy of liposomes as pulmonary drug delivery systems. Some of the previous studies performed reported the following: Geiger *et al.* (1975) reported the rapid uptake of radiolabelled DPPC liposomes in phosphate buffered saline by alveolar type I and II cells and slower uptake of the same by macrophages. Oyarzun *et al.* (1980); Oyarzun and Clements (1977) and Pre *et al.* (1983) reported on the safety of radiolabelled

liposomes composed of D- and L-isomers of DPPC and DPPC:DPPG instilled into rabbit lungs. No untoward pulmonary responses (i.e., arterial blood gases, pH, lung appearance, lavage fluid lactate dehydrogenase activity, protein content and cell consistency) were observed following liposome instillation which suggested safety of liposomes as drug carriers. It was clear from the results of the studies that liposomes comprising DPPC or DPPC:DPPG when delivered to the lung, associate rapidly with the alveolar surfactant and enter the surfactant pool without disturbing normal metabolic activity or stimulating alveolar macrophage activity. (26, 27)

Juliano and Mc Cullough (1980) demonstrated that the half-life of cytosine arabinoside encapsulated in liposomes was twelve times longer than the same compound administered in solution. Mizushima *et al.* (1983) successfully prepared aerosols of lipid-prostaglandin E1, a potent bronchodilator with irritating properties which precludes its use in man. He also demonstrated the effectiveness of the aerosol in protecting guinea pigs from histamine-induced dyspnoea. (26)

1.2.4.3 Recent in vitro/in vivo studies

Pulmonary liposomes have been used for both local (delivery to the lung) and systemic delivery of biopharmaceuticals like proteins and peptides. Locally their efficacy in the treatment of pulmonary infections like pulmonary tuberculosis and pulmonary aspergillosis has been investigated. In addition their role in nucleotide delivery and its efficacy in management of cystic fibrosis, asthma and lung cancer have been studied. Systemically focus was laid on demonstrating the efficacy of liposome encapsulated anti-cancer drugs and interleukins for first line and adjuvant cancer therapies. Additionally the advantage of liposomal carriers for macrophage

targeting and systemic delivery of peptides (eg: insulin in diabetes mellitus) were also established. (28)

1.2.5 Factors to be considered in formulating liposomes for pulmonary delivery

1.2.5.1 Particle size and surface charge

One of the most important formulation parameter for pulmonary delivery is the particle size. Due to the well defined branching architecture of the tracheo-bronchial tree leading to progressive narrowing of its airways at succeeding bifurcations, size of liposomal vesicle becomes a critical property in formulation development. Larger particles (4-7 μm) tend to deposit in the airways while particles between 1-3 μm and those in submicron range (<1 μm) reach the lower airways and deeper lung. When delivering aerosolised liposomes, the liposomal particles become a part of the aerosol droplet and pulmonary deposition is considered a function of aerosol droplet size rather than of liposomal vesicle size, which was elegantly demonstrated by Farr *et al.* (1985). (26, 29)

Surface charge refers to the net charge of the liposomal surface (ζ-potential). The ζ-potential of liposomes can be modified by incorporating charged lipids (positive and negative) in the formulation. Liposomes bearing net positive or negative charge are known to accumulate to a greater extent in the lung in comparison to uncharged/neutral vesicles. (26) However, even liposomes prepared from neutral lipids possess non-zero ζ-potentials over a wide range of ionic strengths. This is due to the fact that zwitterionic head group of neutral lipids absorbed anions or cations leading to slightly negative or positive ζ-potentials. (26, 30)

1.2.5.2 Rigidity and stability in bronchoalveolar lavage fluid (BAL)

The rigidity of liposomal membranes constitutes yet another important formulation property, especially, during nebulisation of liposomes or assessment of liposomal stability in BAL. Rigidity can be brought about by the use of rigid phospholipids or incorporation of cholesterol. The factor is also known to have an influence on the leakage of encapsulated drugs, particularly when the temperatures during nebulisation are higher than the phase transition temperature (T_g) of the lipid mixture. Stability in BAL is also enhanced with increasing transition temperature of the lipid mixture.

It was observed by Niven and co-workers that nebulisation of liposomes above T_g of the phospholipids resulted in increased release of encapsulated carboxyfluorescein. Bridges *et al.* (1998) studied the influence of rigidity using air jet and ultrasonic nebulisers. They concluded a greater disruption of fluid egg-PC liposomes and increased resistance of DPPC:Chol liposomes with both nebuliser types. (31) Zaru *et al.* (2007) reported better encapsulation and retention of rifampicin after nebulisation with rigid liposomes in comparison to fluid ones. Moreover, the retention was found better with increasing rigidity or higher T_g. In other words DSPC (distearoylphosphatidylcholine):Chol liposomes were more resistant to nebulisation compared to DPPC:Chol and PC:Chol liposomes. (32) Shek *et al.* (1994) demonstrated increased stability of liposomes in BAL with increasing T_g in the order (dimyristoylphosphatidylcholine) DMPC<DPPC<DSPC encapsulating glutathione. Incorporation of cholesterol had a positive effect towards membrane stability in DSPC and DMPC liposomes, whereas it caused membrane fluidisation and increased glutathione release from DPPC liposomes. (29)

1.2.5.3 Liposomal dry powder inhalers (DPI)

There are three approaches for pulmonary delivery of pay loads – by formulating them as pressurised Metered Dose Inhalers (pMDI's), by nebulising the formulation using different types of nebulisers or by formulation of a DPI. Conventional DPIs are formulated either as loose agglomerates of micronised drug particles with particle size less than 5 μm or as carrier-drug mixtures of micronised drug particles adhered onto surface of large lactose carriers. A brief principle of DPI preparation is illustrated in Figure 1.7.

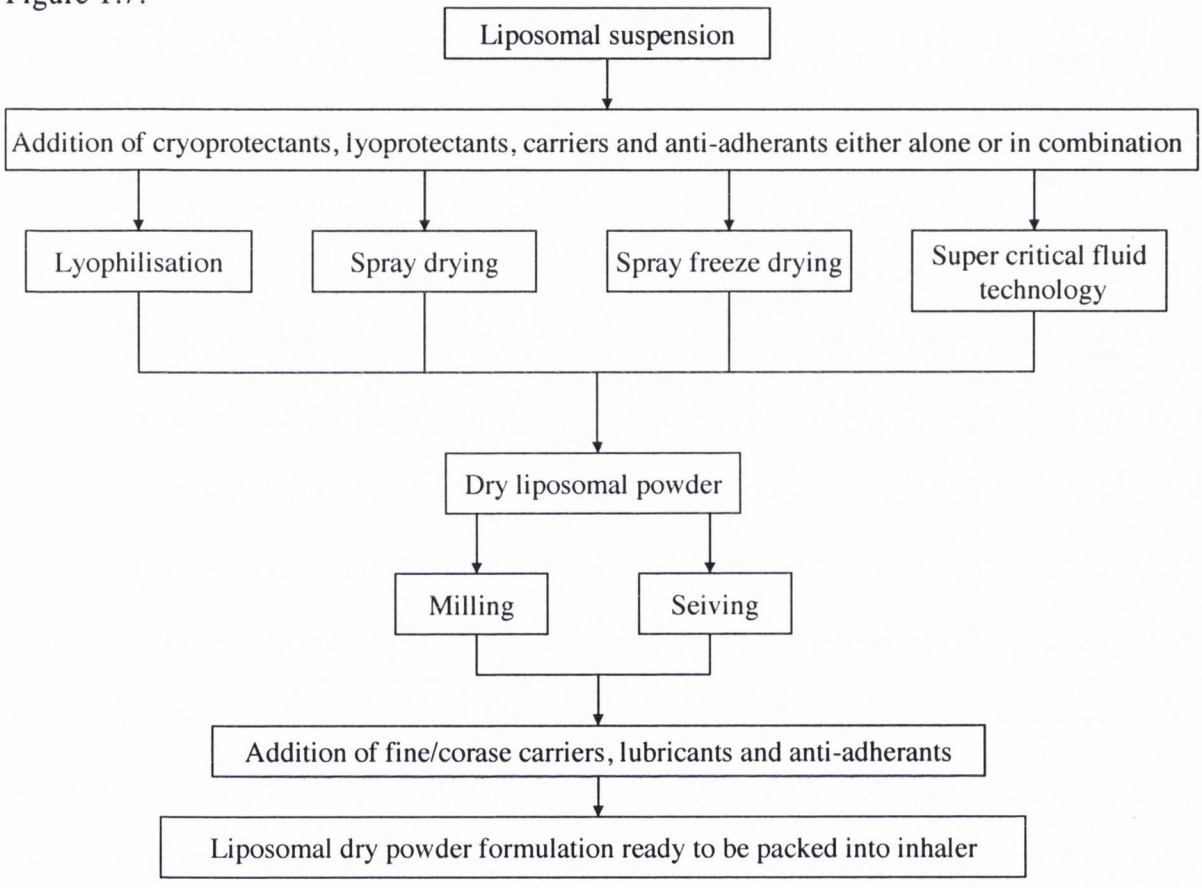


Figure 1.7. Principle of liposomal DPI formulation

However, current trends in DPI formulation include the preparation of liposomal suspensions, which are then converted into dry liposomal powders after the addition of cryoprotectants, carriers, additives and anti-adherents (alone or in combination) by

lyophilisation, spray drying, spray freeze drying or supercritical fluid technology. To the liposomal dry powder obtained coarse carriers, lubricants and anti-adherents are added and sieved to obtain the final liposomal dry powder formulation.

Recent studies suggest the success of DPI technology for pulmonary delivery of liposomal drugs. Bi *et al.* (2008) demonstrated the hypoglycaemic ability of spray-freeze-dried insulin-loaded liposomes in rats delivered by DPI. Insulin liposomes were prepared by reverse phase evaporation technique and the liposomal suspension thus obtained was spray freeze dried. On administration to rats by intratracheal instillation, insulin-loaded liposomes reduced the blood glucose level in rats. This effect lasted markedly longer in comparison to that obtained by treatment with insulin solution. (33)

Shahiwala *et al.* (2003) reported the effectiveness of therapy with levonorgesterol in comparison to oral administration of the same. Liposomes of levonorgesterol were prepared by reversed phase evaporation technique and the liposomal suspension was subjected to freeze-drying. On intratracheal instillation, the bioavailability of the formulations decreased in the following order: liposomal formulation of the drug > physical mixture of drug and liposomal constituents > plain drug formulation > oral administration of the drug. Moreover, the orally administered formulation showed a high initial (maximum plasma concentration) C_{max} followed by sub therapeutic concentrations, whereas formulations after pulmonary administration showed a lesser initial C_{max} followed by maintenance of effective plasma concentration up to 60 hours. (34) The biggest disadvantage of liposomal dry powder formulation is leakage of encapsulated drug during lyophilisation and jet milling. Desai *et al.* (2002) suggested a novel approach to overcome these deleterious effects. Using lactose as cryoprotectant during lyophilisation, they proved 90% drug retention in liposomes

after reconstitution, which was reduced to 40% on jet milling. They then tried preparation of a phospholipid based dry powder formulation which does not require a lyophilisation step. This was made possible by blending phospholipids, drug and cryoprotectant in powder form and micronising them. The micronised powder was stored at room temperature (in contrast to liposomal suspensions that need to be refrigerated) which on reconstitution gave MLVs. (35)

1.2.5.4 Nebulisation of liposomes

Nebulisation is most common approach in pulmonary delivery of liposomes. Figure 1.8 classifies the available nebulisers in the market on the basis of principle behind nebulisation.

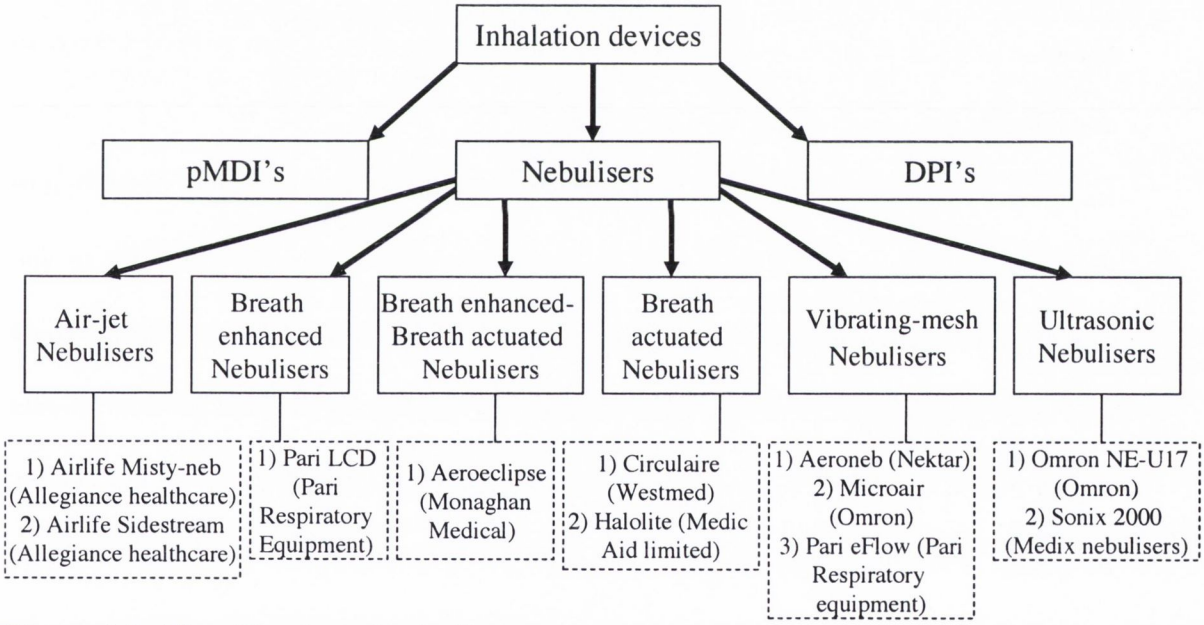


Figure 1.8. Classification of nebulisers

Several studies have been performed on the mentioned nebulisers to assess their efficiency and compatibility in liposomal drug delivery. Factors to be considered during nebulisation include the aerosol droplet size, rate of aerosol output,

temperature of the drug solution reservoir, viscosity of the drug solution, lipid concentration, rigidity of liposome composition and PEGylation related effects.

Steckel *et al.* (2003) reported the factors affecting aerosol performance during nebulisation with jet (Pariboy) and ultrasonic (Multisonic) nebulisers. They published the series of changes occurring to the drug solution in the reservoir over 10 minutes of nebulisation time. The Pariboy device was characterised by an initial drastic decrease in temperature leading to decreased saturated vapour pressure, increased viscosity and aerosol droplet size. Later the particle size decreased due to increased solute concentration in the reservoir and decrease in surface tension. In the Multisonic nebuliser they observed a drastic increase in temperature initially leading to increase in saturated vapour pressure, decrease in viscosity, increased solute concentration, reduced surface tension and reduced aerosol droplet size. Later with decrease in temperature the particle size increased. (36)

Bridges *et al.* (1998 and 2000) demonstrated the factors influencing jet nebulisation of liposomes using three different nebulisers. An increase in aerosol droplet size and viscosity was noted with increase in lipid concentration from 5 to 80 mg/ml, which was more pronounced with Pari (Pari LC) and Cirrus, but not Sidestream. The aerosol output in general decreased with increasing lipid concentrations, which was, however, the least with the Sidestream. They reported a relationship between the mean liposome size and residual lipid concentration in the nebulisation reservoir. An increase in mean liposome size beyond 2.5 μm led to increased retention of the same in the chamber due to reduced ability to be included in the aerosol. This group also reported a direct relationship between the size of air jet orifice and aerosol droplet size, which may be explained as increase in size of air jet orifice leading to reduced

pressure of driving gas and shear energy for droplet formulation contributing to increased droplet size. (31, 37)

Elhissi *et al.* (2007) compared liposome nebulisation via jet (Pari LC plus) and vibrating mesh (Aeroneb Pro) nebulisers. Size of liposomes reduced following nebulisation with both nebulisers, however, the reduction was more pronounced with the Pari which is thought to damage the liposomes to a greater extent. Entrapped drug losses accounted for 88% with the Pari and 44% for the Aeroneb device. It was also concluded that extrusion of liposomes through 1 μm membrane filter enhanced stability to nebulisation and reduced drug loss. The Aeroneb device had shorter nebulisation time and produced higher mass output rates compared to the Pari, whereas the latter one produced smaller droplets, higher mass and drug outputs. (38)

Leung *et al.* (1996) studied the stability of liposomes of different composition (Egg PC and Egg PC:Chol 1:1) to ultrasonic nebulisation using a Medix ultrasonic nebuliser. They published a linear relationship between lipid concentration and viscosity. They also reported less marked size reduction of liposomal vesicles in the nebuliser reservoir on inclusion of 50mol% Chol. Cholesterol imparts rigidity to the bilayer making it more resistant to disruptive forces during nebulisation. (39)

Recently, Gaspar *et al.* (2009) assessed the potential of a vibrating mesh nebuliser (Aeroneb Pro) and an intratracheal nebulisation catheter (AeroProbe) to aerosolise liposomal suspensions. A small size reduction of non-extruded liposomes following nebulisation was reported which was not observed with extruded formulations. These data confirm Elhissi's results (2007). (38) Other physicochemical parameters like ζ-potential and phospholipid content did not change significantly following nebulisation. Hence, both nebulisers were deemed suitable for pulmonary delivery of

liposomal formulations with the additional advantage of AeroProbe that allows targeted delivery into select lung regions. (40)

Rau *et al.* (2004) compared the performance of five nebuliser models of three types- constant output (Misty-Neb and Sidestream), breath-enhanced (Pari LCD) and breath-actuated (Circulaire and AeroEclipse) in terms of inhaled drug, exhaled drug, drug lost to deposition in the apparatus and drug left in dose bottle. Drug inhaled or deposited in the breathing simulator was highest with the AeroEclipse (38.7±1.3%) and lowest with the Pari LCD (15.2±4.2%). Drug exhaled or lost to ambient to air was least with the AeroEclipse (6.6±3.3%) and most with the Misty-Neb (26.8±0.7%). Percentage of drug lost due to deposition in the apparatus was highest with the Circulaire (75.8±0.5%) and lowest with the AeroEclipse (51±2.1%). The duration of nebulisation was shortest with the Circulaire and longest with the AeroEclipse. (41)

Leung *et al.* (2004) investigated the rate, consistency and efficiency of drug delivery with breath-enhanced (Pari LC Star), breath-actuated (Halolite) and breath-enhanced breath-actuated (AeroEclipse) nebulisers. The nebulisers were tested for their *in vitro* performance and the respiratory pattern of seven cystic fibrosis patients. The rate of drug deposition was highest for the Pari LC star and lowest for the Halolite device. The rate of deposition was independent of the respiratory pattern for the Pari LC Star and the AeroEclipse, but was dependant on respiratory rate for the Halolite. The AeroEclipse had least amount of drug wastage. They finally concluded that the Pari LC Star would be the nebuliser of choice to achieve minimum treatment time, the AeroEclipse would be preferred to minimise drug wastage and the Halolite would be the best to accurately deliver a specific drug dose. (42)

Nerbrink *et al.* (2003) demonstrated the effect of humidity in entrained air on constant output (Micro-Neb) and breath-enhanced (Pari LC+) nebulisers. Their results

indicated an increase in aerosol size with increasing (relative humidity) rH with the Micro-Neb, whereas the Pari LC+ did not show any change in aerosol droplet size. The results could be explained as follows: In the Micro-Neb, the entrained air mixes with small amount of cool high humidity aerosol in the T-tube, during which the temperature of entrained air decreases and that of aerosol-laden air increases. Evaporation is thought to occur, the extent of which depends on temperature difference between entrained air and aerosol laden air. If the rH of the ambient air is high, indicating a low water holding capacity, the aerosol droplets absorb the humidity and grow in size and vice versa. In contrast, the Pari LC+ does not have a T-tube allowing the mixing of entrained air and aerosol. The entrained air, however, is brought in contact with aerosol at the aerosol generation point, where humidification occurs. No change in aerosol droplet size is noticed even with low rH of entrained air due to vast surface area and massive production of droplets which at any time ensures a high water vapour potential. (43)

Addition of PEG molecules provides stealth effect to liposomes prolonging their circulation time in lung surfactant or plasma. (44) When considering pulmonary delivery, stability of PEGylated liposomes to nebulisation becomes an important assessment factor. Anabousi *et al.* (2006) demonstrated the effect of nebulisation on liposomes and their stability in lung surfactant. PEGylated and non-PEGylated liposomes of different rigidity (DSPC and DPPC) were used. On nebulisation through air-jet (Pari LC star) and ultrasonic (Aeroneb Pro) nebulisers, PEGylated liposomes showed marginal size and zeta potential changes, in comparison to non-PEGylated liposomes. When stability was assessed in commercial lung surfactant (Alveofact), PEGylated preparations (DSPC > DPPC) were more stable than non-PEGylated ones.

The effect of temperature on stability in Alveofact was also determined and the stability was better at 4°C than at 37°C, based on drug retention data. (45)

In a related study Kleemann *et al.* (2007) reported the stability of rigid PEGylated and non-PEGylated to nebulisation using three different nebulisers: vibrating mesh (Aeroneb Pro), ultrasonic (Optineb) and air-jet (Pari LC). A significant decrease in hydrodynamic diameter was observed for those nebulised through the Pari LC and Optineb, whereas no change was observed on nebulisation through the Aeroneb suggesting less damage caused by the nebuliser. Drug retention post nebulisation, was about 70% for non-PEGylated preparation and between 39 and 47% for the PEGylated liposomes. (46)

1.3 OSTEOPOROSIS AND SALMON CALCITONIN

1.3.1 Osteoporosis

Osteoporosis is a skeletal disease characterised by low bone mass and microarchitectural deterioration of bone tissue resulting in compromised bone strength and increased susceptibility to fracture. Bone mass during adult life is a measure of the amount of bone accumulated from prenatal stages until maturity along with that lost during aging. The disease could be classified into 2 basic forms: primary and secondary. Primary osteoporosis results from cumulative bone loss as people age and undergo sex hormone changes. Secondary osteoporosis results from a variety of medical conditions, diseases and use of certain medications like glucocorticoids. (47)

First line of treatment is the use of oral bisphosphonates like alendronate and risedronate or intravenous pamidronate infusion in patients who cannot tolerate oral bisphosphonates. Hormones like calcitonin, teriparatide (recombinant human parathyroid hormone), estrogen and certain estrogen receptor modulators have also

been used. External hip protectors and exercise training have been the physical treatment methods adopted. (48)

1.3.2 Salmon calcitonin

Calcitonin is a 32-amino-acid peptide secreted by the C cells of the thyroid in mammals. The hormone was discovered by Copp *et al.* in 1961. It has a molecular weight of 3431 Da. Calcitonin acts in association with parathyroid hormone and 1,25-dihydroxycholecalciferol to mediate and "fine tune" short-term calcium homoeostasis. (49) This is brought about by limiting osteocytic osteolysis and increasing osteoblast activity. (50) Isolated calcitonin sources include porcine, salmon, ovine and eel of which salmon calcitonin (sCT) is most widely used as its intrinsic potency is 40-50 times higher than that of human calcitonin. (49, 50, 51) sCT is illustrated in Figure 1.9. (52)

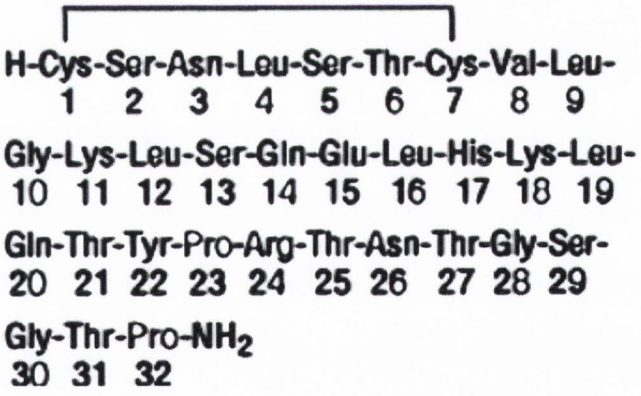


Figure 1.9. Salmon calcitonin

sCT is rapidly inactivated when given orally. Following injection, sCT is quickly metabolised, primarily in the kidneys leading to a bioavailability of about 70%; plasma protein binding is about 30 to 40%. sCT is known to have a good absorption profile via the nasal mucosa. About 3% of an intranasal dose of sCT is reported to be

bioavailable compared with the same dose given by intramuscular injection which is good for intranasal application. (53)

Marketed products of sCT include nasal sprays (Miacalcin and Fortical) and a subcutaneous injection (Calcimar) for the treatment of osteoporosis, Paget's disease of the bone and hypercalcaemia. (54, 55, 56)

1.3.3 Liposomes encapsulating salmon calcitonin for parenteral, oral and intranasal administrations

1.3.3.1 Parenteral delivery of salmon calcitonin liposomes

Fukunaga *et al.* (1984) performed an elaborate study on calcitonin liposomes for parenteral delivery. Variables in the study were: payload {salmon calcitonin (sCT) and human calcitonin (hCT)}, liposome composition (Egg PC with and without equimolar quantities of Chol), vesicle size (MLV and SUV) and route of administration (intravenous and intramuscular). In general, liposome entrapment enhanced hypocalcaemic potency of parenterally administered sCT and hCT in rats. Following i.v. administration, MLV showed better activity than SUV and cholesterol inclusion in MLV prolonged hypocalcaemia. After i.m. administration, cholesterol free liposomes were more potent compared to their counterparts regardless of size. (57)

1.3.3.2 Oral delivery of salmon calcitonin liposomes

Arien *et al.* (1995) worked on cholate-induced disruption of salmon calcitonin loaded liposomes to obtain a better understanding of the reason behind hypocalcaemic effect on oral administration of sCT liposomes while liposomes in general are disrupted in gastrointestinal tract. Calcein and sCT liposomes in the presence of cholate solution

were studied. Gel filtration of cholate treated liposomes loaded with calcein showed separation of calcein from lipid fraction. On the contrary, 50% sCT was re-encapsulated in the liposomes following cholate disruption, suggesting the formation of lipid-sCT complexes that retain most of sCT initially embedded in the membrane. (58) Song *et al.* (2002) for the first time prepared proliposomes using microporous sorbital particles as core and phosphatidylcholine as wall former containing sCT. On contact with water a liposomal dispersion was obtained in which a certain concentration of sCT was entrapped in liposomes. Permeability of sCT across Caco-2 cell monolayers was enhanced using liposome as carriers. (59)

Takeuchi *et al.* (2003) evaluated Carbopol (CP-lip) and chitosan (CS-lip) coated multilamellar vesicles (MLV) formulated with DPPC:Chol 40:1 mole ratio. They were compared with positively (P-lip) and negatively (N-lip) charged non-coated liposomes. The mucoadhesive property decreased in the order CS-lip $\geq$ CP-lip > P-lip > N-lip. On administration of CS-lip and CP-lip to rats, enhanced and prolonged reduction in blood calcium level was observed. The overall pharmacological effect measured in terms of area under the curve was 2.4 and 2.8 times higher for CP-lip and CS-lip compared to non coated liposomes. (60) Yamabe *et al.* (2003) compared the bioavailability (BA) of double liposomes (DL) containing four different types of inner liposomes – neutral (NL) and cationic (CL) liposomes prepared using coatsome method and neutral (VET) and cationic (c-VET) liposomes prepared using a mechanochemical method. DL in general produced an increase in BA in all groups compared to control following oral administration in rats. BA of VET-DL was highest and was 6.8-fold higher than sCT solution taken orally. (61) Ebato *et al.* (2003) published a similar work on DL where again the DL containing a cationic inner liposome produced a hypocalcaemic efficacy in rats measured by 6.47% increase in

relative bioavailability on comparison with subcutaneous administration of sCT solution at a dose of 1 μg/kg in rats. This was the highest value obtained among others investigated while it was an insignificant increase in relative terms . (62)

Song *et al.* (2005) reported on enhanced intestinal absorption of sCT from proliposomes containing bile salts using rats and Caco-2 cell monolayers. Seventeen surfactants were examined and sodium taurodeoxycholate (NaTDC) appeared to be the most advantageous based on the balance between permeation enhancement (10.8-fold) and reduced damage to cells (3.6-fold decrease in TEER value). Proliposomes for *in vivo* studies prepared contained 0.75% w/w sCT and 2.5% w/w NaTDC on sorbitol core and phosphatidylcholine wall former. Administration of reconstituted proliposomes to rats duodenally resulted in a 7.1-fold increase in bioavailability. (63)

Thongborisute *et al.* (2006) investigated the mucoadhesive properties of chitosan coated (CS) and dodecylated chitosan coated (DC) sCT liposomes with mucin in small intestine of rats. Zeta potential of DC-lip showed positive values in both neutral and negatively charged liposomes while CS-lip seemed to be less effective in coating as seen by lower positive values. CS provided solely electrostatic forces for coating liposomes while DC provided electrostatic forces and hydrophobic interactions due to insertion of it into lipid bilayer. On oral administration to rats, DC-lip showed greater potential in reducing blood calcium concentration in the first 12 h compared to CS-lip. So DC-lip was concluded to be the best for oral administration of sCT. (64)

1.3.3.3 Nasal delivery of salmon calcitonin liposomes

Law *et al.* (2001) performed an elaborate investigation on formulation development parameters of sCT liposomes like liposomal charge, concentration of charge inducing agent and pH of loading medium on encapsulation efficiency (EE), leakage behaviour

and chemical stability of sCT. Loading efficiency of sCT in liposomes followed the order: negatively charged liposomes > neutral liposomes > positively charged liposomes with leakage following exactly the opposite order. Increase in concentration of phosphatidylserine lead to increase in EE while increase in percentage of stearylamine lead to decrease in EE. Salmon calcitonin EE was better at pH 7.4 compared to pH 4.3 with leakage kinetics vice versa. Chemical stability of sCT demonstrated a pseudo first order kinetics. (65) Law *et al.* (2001) evaluated the hypocalcaemic effects following intranasal administration of sCT containing liposomes in rabbits. The bioavailability of sCT marked by decrease in blood calcium levels followed the order: positively charged liposome > negatively charged liposome > sCT solution. Increase in bioavailability due to positive liposomes was because of accumulation of these on the negatively charged nasal mucosal surface resulting in increased residence time and consequently absorption. (66)

Chen *et al.* (2009) demonstrated the potential of positively charged (P-UFL) and negatively charged (N-UFL) ultraflexible liposomes as carriers in improving the absorption of sCT post intranasal administration in rats. UFL performed significantly better than sCT solution on intranasal administration while there was no significant difference amongst the UFL's. Moreover, decrease in serum calcium levels following subcutaneous injection of sCT solution was similar to that of UFL. Results of toxicity study revealed UFL to be slightly toxic to nasal mucosa; however, an overall evaluation regarded them as potential sCT carriers for nasal administration. (67)

Plessis *et al.* (2010) formulated a novel lipid based colloidal delivery system called Pheroid vesicles (liposome like bilayer vesicles) for improving the absorption of nasally administered sCT at a dose of 10IU/kg body weight. The absorption enhancement with Pheroid vesicles was compared with that obtained with N-trimethyl

chitosan chloride (TMC). With inclusion of 0.5%w/v TMC, the C_{max} of sCT increased from 72.6±6.1 pg/ml to 478.5±6.1 pg/ml after nasal administration. With Pheroid vesicles the C_{max} was 262.64±17.1 pg/ml and the T_{max} decreased from 35 to 14 min proving the need for further investigation of this delivery system. (68)

The above mentioned examples clearly show the benefit following the encapsulation of sCT in liposomes and also suggest them to be good carriers for the cargo in all routes of administration.

1.3.3.4 Pulmonary delivery of salmon calcitonin liposomes

We, to the best of our knowledge, were the first to investigate salmon calcitonin liposomes for pulmonary delivery.

1.4 AIMS OF PHD PROJECT

The aims of my PhD project were

- To optimise liposomal formulations for pulmonary delivery of a peptide drug, salmon calcitonin (sCT).
- To characterise these liposomes *in vitro* in terms of encapsulation efficiency (check and compare the change in encapsulation efficiency following PEGylation and/or lyophilisation of liposomes in the presence and absence of a cryoprotectant), ability to withstand nebulisation stresses (following nebulisation with Aeroneb Pro vibrating mesh nebuliser), bioactivity (on T47D cell model) and controlled release properties (assessment of release of sCT from premade dialysis bags in KRB of pH value 7.4).
- To investigate the pharmacokinetic behaviour of these liposomes after intratracheal delivery to an experimental model of healthy male Wistar rats.

2. sCT LIPOSOMES: FORMULATION DEVELOPMENT, CHARACTERISATION AND NEBULISATION

Parts of this chapter have been submitted as:

J. Swaminathan, C. Ehrhardt. Formulation and *in vitro* characterisation of liposomal systems for systemic delivery of salmon calcitonin (sCT) by oral inhalation. *Eur J Pharm Biopharm*. Under review.

2.1 ABSTRACT

Purpose: Salmon calcitonin is currently marketed for the treatment of osteoporosis, Paget's disease and hypercalcaemia, with intravenous and intranasal formulations being available. Pulmonary delivery, however, might further improve the drug's efficacy. Here, a variety of liposomal systems of sCT were prepared and characterised, with the intention to develop a controlled release formulation for administration via the pulmonary route.

Methods: Primarily four different methods (gel filtration, centrifugation, ultracentrifugation and filtration) were checked for their efficacy and reproducibility in separation of non-encapsulated sCT following liposome preparation. Consequently ability of sCT to form dimers under the working conditions was also analysed. With the streamlined methods in place, the influence of pH of the sCT loading buffer, charge of the vesicle surface and varying degrees of membrane rigidity on particle size, ζ-potential and sCT encapsulation efficiency was studied. The most promising formulations from these preliminary studies were further investigated with regards to their performance during nebulisation using Aeroneb Pro vibrating mesh nebuliser. Simultaneously, the impact of addition of 5mol% DSPE-PEG$_{-2000}$ to the lipid composition was studied.

Results: Gel filtration using P-6 gel (exclusion limit of 6,000 Da) was the most suitable method for separation of non-encapsulated sCT. Laboratory working conditions did not facilitate in sCT dimerisation. Formulation development studies revealed that liposomes with acidic (i.e., pH 3.5) loading buffer and comprising rigid lipid membranes showed a good compromise between small particle size, high encapsulation efficiency and sCT stability. Whilst positive surface charge was preferable for plain liposomes, this effect was diminished in PEGylated vesicles.

PEG-liposomes generally had higher encapsulation efficiencies and all liposomes were stable to nebulisation.

Conclusions: PEGylated liposomes hold promise as delivery systems for systemic delivery of aerosolised salmon calcitonin via the lungs. These systems were able to encapsulate high amounts of the peptide and withstand nebulisation stresses. These optimised formulations will be tested *in vitro* on T47D cell model and *in vivo* on male Wistar rats.

2.2 INTRODUCTION

Osteoporosis is one of the most common metabolic bone disorders marked by reduced bone strength, decreased bone mass and microarchitectural deterioration of bone tissue, ultimately resulting in increased susceptibility to fracture. (48) Salmon calcitonin, a 32-amino-acid peptide secreted by the C cells of the thyroid, is used in the clinic for the treatment of osteoporosis, Paget's disease and hypercalcaemia, and is currently marketed as injectable solutions (Calcimar®, Miacalcin®) as well as nasal sprays (Fortical®, Miacalcin®). (49, 69 & 6) Injections are obviously very inconvenient for the patient and the nasal formulations suffer from a wide range of bioavailability ranging from 0.3 to 30%. (50) Hence, it is believed that the full market potential of sCT products has not yet been realised. (70, 71) Some of the limitations such as poor bioavailability after oral and nasal administration can potentially be avoided by using the pulmonary route. Relative bioavailabilities of approximately 10-18% and fast appearance in the bloodstream were previously reported from pre-clinical studies in healthy volunteers after oral inhalation of different sCT formulations. (72, 73) Although sCT bioavailability post-inhalation is already higher than can be achieved by any other non-invasive application route, it is desirable to

further increase the drug's bioavailability and to better control its release, in order to reduce the dose and the risk of potential side effect, and ultimately, the costs associated with the therapy.

One potential solution to achieve this goal might be the use of liposomal formulations. These can be prepared from endogenous constituents of lung surfactant, such as DPPC. (74) Due to their versatility and biocompatibility, liposomes are promising candidates for pulmonary drug delivery that enable the delivery of compounds with formulation issues (e.g., lipophilic drugs and sensitive biopharmaceuticals) and to create a drug depot in the lung for sustained release. (25, 40) Liposomes encapsulating sCT for parenteral delivery have been first published as early as 1984. (57) Subsequent studies with sCT liposomes aimed at oral (59, 61-63) and intranasal delivery (65-67), however, pulmonary administration of liposomal sCT has not been reported to date (25).

The aim of this work was to formulate liposomes containing salmon calcitonin and to characterise them *in vitro* in terms of their suitability for pulmonary delivery. When formulating sCT liposomes, factors such as sCT concentration, temperature of processing, pH of the medium used for liposome preparation, techniques used for separation of non-encapsulated drug (gel filtration, centrifugation, ultracentrifugation and filtration) and probability of dimer formation need to be considered. Dimerisation is a common degradation pathway for proteins and peptides. (80) Dimerisation could happen when the ambient working temperatures are high, exposure to high salt concentrations or when high concentrations of proteins/peptides are being worked with. (80, 81 & 82) Andreotti *et al.* reported the formation of head to tail dimer in sCT whose basic unit was a α-helix in the region Leucine^{12} to Tyrosine^{22} which inhibits fibril maturation in sCT. (81) After establishing these preliminary parameters, the

impact of surface charge, membrane rigidity and pH of loading buffer had to be determined to streamline the best liposomal formulation in terms of small particle size and PdI and high encapsulation efficiency with good nebulisation stability.

2.3 MATERIALS AND METHODS

2.3.1 Materials

Dipalmitoylphosphatidylcholine (DPPC) and egg PC were purchased from Avanti Polar Lipids (Alabaster, AL, USA) while Chol, Stearyl amine (SA), glucose, HEPES, calcium chloride dihydrate, sodium dihydrogen phosphate, iron (III) chloride hexahydrate, ammonium thiocyanate, chloroform, acetonitirile and magnesium sulphate heptahydrate were procured from Sigma-Aldrich (Dublin, Ireland). Sodium chloride, potassium chloride and sodium bicarbonate were purchased from Fisher Scientific (Dublin, Ireland). Ultra pure water was used in all experiments and generated by a Millipore Synergy unit (Cork, Ireland). A Rotavapor R-210 rotary evaporator was used for lipid film preparation (Büchi, Flawil, Switzerland). The Lipex thermobarrel extruder and the polycarbonate filters used in the extrusion step were bought from Northern Lipids (Burnaby, BC, Canada) and Whatman (Maidstone, UK), respectively. Econo-pac pre-packed columns with an exclusion limit of 6,000 Da and the Bio-gel for 10,000 Da self-packed columns used for gel filtration were purchased from Alpha Technologies (Wicklow, Ireland). GE columns with an exclusion limit of 5,000 Da were procured from GE Healthcare Life sciences (Buckinghamshire, UK). Syringe driven filter units of pore size 0.45 and 0.22 μm and Centriplus centrifugal filter device with an exclusion of 50,000 Da were purchased from Millipore (Cork, Ireland). Centrifugation was performed in a Hettich centrifuge (Beverly, MA, USA) and ultracentrifugation was accomplished using a Beckman

ulltracentifuge (Beckman Coulter, Galway, Ireland). Particle size, polydispersity index (PdI) and ζ-potential were measured using a Zetasizer Nanoseries ZS (Malvern Instruments, Malvern, UK). The concentration of sCT was determined using a Shimadzu HPLC system (Kyoto, Japan) comprising a LC-10AT pump, auto sampler SIL-10AD, degasser DGU-14A, UV-Vis detector SPD-10A and system controller SCL-10A. Jones chromatography (Cardiff, UK) reverse phase C-18 columns, 150×4.6 mm, with 4-μm packing were used. In some cases sCT concentration was determined using a BCA protein assay kit (Thermo scientific, Rockford, Illinois, USA).

2.3.2 Methods

2.3.2.1 Liposome preparation

Liposomes were prepared by the lipid film hydration method. Briefly, lipids were dissolved in chloroform and subsequently, the solvent was removed using a rotary evaporator to obtain a dry lipid film. This film was then hydrated with bicarbonated Krebs-Ringer buffer (KRB) of relevant pH value (3.5, 5.0, 7.0, 8.5 and 10.0), which contained sCT at a concentration of 0.2 mg/ml, to a final lipid concentration of 10 μmol/ml. The multi lamellar vesicles thus obtained were subjected to size reduction using a thermobarrel extruder. Polycarbonate filters of pore sizes ranging from 100 to 400 nm were used to generate small unilamellar vesicles. Hydration and extrusion were carried out either at room temperature or 42-43°C, depending on the T_g of the lipids used in the formulation. Several methods were tested to streamline the best possible method for removal of non-encapsulated sCT.

2.3.2.2 Preliminary experiments – Removal of non-encapsulated sCT

The following methods were tested for removal of non-encapsulated sCT.

1. Gel filtration using sephadex columns with exclusion limits of 5,000 Da (P-5), 6,000 Da (P-6) or 10,000 Da (P-10).
2. Centrifugation at 3000 rpm
3. Ultracentrifugation
4. Filtration using a syringe driven filter unit

Gel filtration using Sephadex columns

P-5 and P-6 pre-packed columns were washed with KRB at a pH value of 7.4 while the P-10 self-packed columns were packed initially according to manufacturers instructions following which they were also equilibrated with KRB at pH value of 7.4 before elution of liposomes. The maximum volumes of liposomal suspension that could be added to the columns for elution were: 2.5 ml for P-5 and 3.0 ml for P-6 and P-10 columns. Gel filtration was performed twice on liposome samples. Extruded liposomes formed the feed for first gel filtration and the eluate obtained from first filtration was the feed for the second gel filtration. The formulation tested in all cases was DPPC:Chol:SA (70:20:10) which was a model formulation. Parameters determined were particle size, poly dispersity index (PdI), ζ-potential and encapsulation efficiency. Each experiment was performed in singlet (n = 3 for particle size, PdI, ζ-potential and n = 2 for encapsulation efficiency (EE))

Centrifugation at 3000 rpm

Two different centrifugation experiments were performed at 3000 rpm. In Method 1: 4 ml of extruded liposome was added to a Centriplus centrifugal filter device. The unit was centrifuged at 3000 rpm for 4 h. At every hour, 1 ml of the liposome sample in

donor compartment was withdrawn and was replaced with KRB at pH value of 7.4. The sample in receiver compartment was collected as such. Encapsulation efficiency was determined in donor and sCT content in receiver compartment hourly samples while particle size, PdI and ζ-potential were determined only on the donor compartment sample at the end of 4 hours. Mass balance of sCT was also determined. In Method 2: 4 ml of extruded liposomes was added to a Centriplus centrifugal filter device containing 4 ml of KRB at pH value of 7.4. The unit was centrifuged for 1 h at 3000 rpm and characterisation tests performed were exactly the same as mentioned earlier for the donor and receiver compartment samples. The experiments were performed in duplicate and the formulation tested was DPPC:Chol:SA (70:20:10) which was a model formulation and $n = 6$ for particle size, PdI and ζ-potential and $n = 4$ for EE.

Ultracentrifugation

Ultracentrifugation was performed on liposomal suspension diluted 1:10 with KRB at pH value 7.4 spun at 52,000 rpm (3,00,000 g) for 2 h at 17°C. The liposome samples were divided into two parts: one part was centrifuged immediately after extrusion and the other after twenty four hours. The encapsulation efficiency results were compared with liposome samples that were gel filtered through P-6 pre-packed columns at both intervals. Characterisation tests performed were particle size, PdI, ζ-potential and encapsulation efficiency. This experiment was carried out in singlet on DPPC:Chol:SA (60:20:20), a model formulation ($n = 3$ for particle size, PdI, ζ-potential and $n = 2$ for EE).

Filtration using a syringe driven filter unit

Liposomes post extrusion were filtered through the syringe driven filtration unit to check if this method was feasible to separate non-encapsulated sCT without affecting the integrity of liposomal membrane. Briefly, liposome extruded through 400 nm filter was filtered through 0.45 μm and 0.22 μm filter unit while 200 nm and 100 nm extruded liposomes were filtered through 0.22 μm filter unit. Control for this experiment was KRB at a pH value of 7.4. Characterisation tests performed were physical appearance, particle size and PdI on filtered samples. The formulation tested was DPPC:Chol (66.7:33.3), a model formulation and this experiment was performed in singlet (n = 3 for particle size and PdI).

2.3.2.3 Preliminary characterisation – sCT dimerisation

These experiments were performed to check for dimer formation in sCT under conditions where the practical experiments were performed. Three millilitres of 0.2 mg/ml sCT solution in KRB of five different pH values (3.5, 5.0, 7.0, 8.5 and 10.0) were prepared, gel filtered through P-6 column and ten 1 ml aliquots were collected upon elution with KRB of pH 7.4. These aliquots were tested for sCT content by both reverse phase high performance liquid chromatography (RP-HPLC) and bicinchonic assay (BCA). The experiment was performed in singlet for RP-HPLC estimation (n = 2) and again in singlet for estimation of sCT concentration using BCA assay (n = 3).

2.3.2.4 Liposome formulation and performance

In proof-of-concept studies, liposomes with neutral, negative and positive surface charges were prepared using either DPPC:Chol (66.7:33.3), DPPC:Chol:DPPG (70:20:10) or DPPC:Chol:SA (70:20:10). The pH values of the hydration buffers used

were 3.5, 5.0, 7.0, 8.5 and 10.0. In all subsequent studies, hydration of the lipid film was performed only at pH 3.5, as data became available in the meantime that sCT stability was optimal at this pH. (75) To determine the impact of increasing positive vesicle surface charge on encapsulation efficiency of sCT, formulations containing 20% and 30% SA were also prepared. Finally, it was investigated if the addition of polyethylene glycol (i.e., 5mol% DSPE-PEG_{2000}) had an impact on physiochemical parameters and EE of the vesicles. PEGylated formulations are preferred for *in vivo* studies since they circumvent opsonisation by forming a stealth layer around liposome vesicle which is hydrophilic in nature. (15)

2.3.2.5 Physicochemical characterisation

All formulations were analysed for the particle size, PdI and the particle surface charge (ζ-potential). Disposable low volume cuvettes were used for particle size determination, whereas disposable capillary cells were used for ζ-potential measurements. Samples for physicochemical characterisation were prepared by diluting liposomal suspensions with KRB (pH 7.4). The measurements were based on dynamic light scattering at 173° backscatter detection. Each experiment was repeated in triplicates from 3 independent batches ($n = 9$).

2.3.2.6 HPLC method for determination of sCT content

The concentration of salmon calcitonin was determined by RP-HPLC. An isocratic elution method was used with a flow rate of 1 ml/min. The mobile phase was prepared by mixing 660 ml ultra pure water, 340 ml acetonitrile, 1.16 g sodium chloride and 320 µl trifluoroacetic acid. UV absorption of sCT was detected from 190 to 340 nm, and the actual spectrophotometric measurement carried out at 215 nm. Each HPLC

result was the average of 2 independent measurements from 3 liposome batches (n = 6).

2.3.2.7 Encapsulation efficiency

Samples were prepared by addition of Triton X-100 to liposomal suspensions such that the final concentration of the detergent in the sample was 1%. The samples were then incubated at 37°C for 30 min, before being subjected to HPLC analysis. The phospholipid content was determined using Stewart's assay. (76) Each value of Stewart's assay was the average of 3 individual measurements from 3 independent liposome batches (n = 9).

Encapsulation efficiency following gel filtration and following nebulisation was calculated using the following equations

$$\frac{(\mu g\ sCT/\mu mol\ total\ lipid)\ after\ gel\ filtration \cdot 100}{(\mu g\ sCT/\mu mol\ total\ lipid)\ before\ gel\ filtration} \qquad \text{(Eq. 1)}$$

$$\frac{(\mu g\ sCT/\mu mol\ total\ lipid)\ after\ nebulisation \cdot 100}{(\mu g\ sCT/\mu mol\ total\ lipid)\ nebulisation\ control} \qquad \text{(Eq. 2)}$$

Encapsulation efficiency was the average of 2 individual determinations from three independent batches (n = 6).

2.3.2.8 Aerosolisation studies

An Aeroneb Pro vibrating mesh nebuliser, kindly donated by Aerogen Ltd. (Dangan, Ireland), was used to study the effect of aerosolisation stresses on the different liposomal systems. Three millilitres of freshly prepared liposome suspension was nebulised into a 50 ml volumetric cylinder. The collected condensate was then gel filtered to remove sCT which was released during the manoeuvre. Control samples were treated identically minus the nebulisation step. Aerosolisation was carried out

for formulations containing 10% and 20% SA, with and without 5mol% DSPE-PEG_{2000}. In addition, formulations, one without SA and DSPE-PEG_{2000} and one without SA, but including DSPE-PEG_{2000}, were analysed. Nebulisation experiments were performed on formulations from 3 different batches (n = 9 for particle size, PdI and ζ-potential and n = 6 for EE).

2.3.2.9 Statistical analysis

Data are presented as mean ± standard deviation. Differences among more than two group means were determined by one-way analysis of variance (ANOVA) with Student-Newman-Keuls post-hoc tests. $P < 0.05$ was considered statistically significant.

2.4 RESULTS

2.4.1 Preliminary experiments – Removal of non-encapsulated sCT

Gel filtration using Sephadex columns

Liposome encapsulated sCT following gel filtration via P-5, P-6 and P-10 gel columns are compared in Figure 2.1. On comparing the figures, liposome encapsulated sCT decreases in the order P–5 gel > P–6 gel > P–10 gel. Physicochemical characters of these formulations are shown in Table 2.1. No significant differences ($^*P \leq 0.5$) in any of the physiochemical parameters were observed between consequent gel filtrations through the same gel column and among all exclusion limits tested.

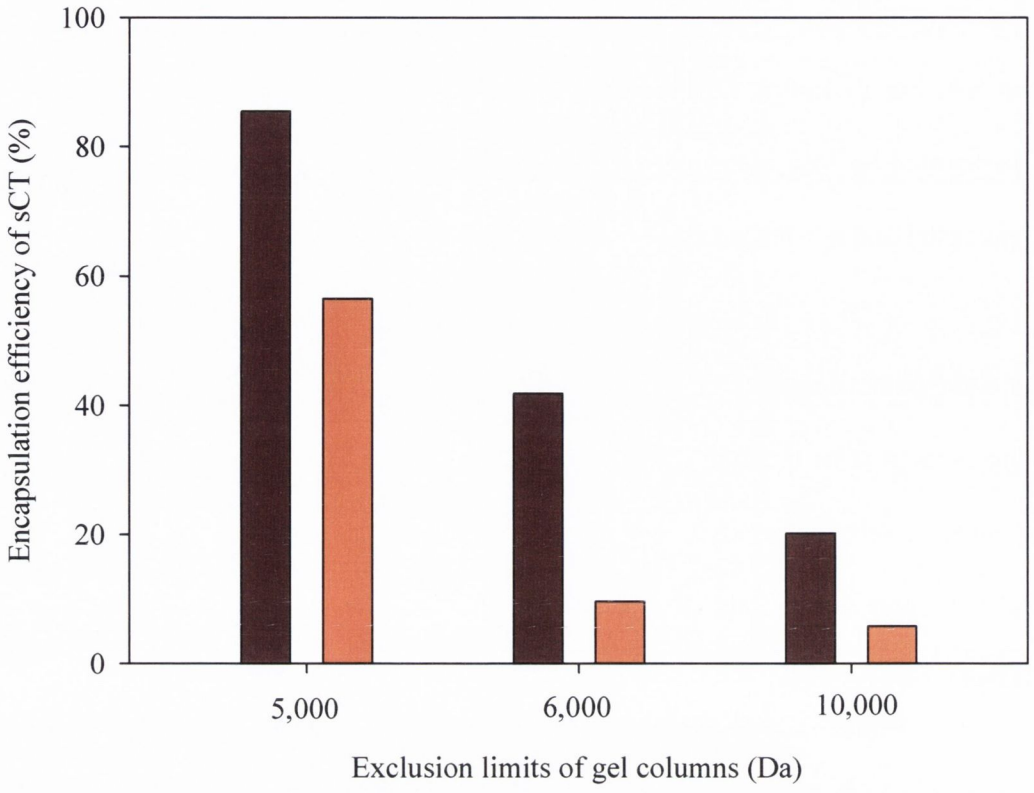


Figure 2.1. Comparison of liposome associated sCT following gel filtration through P-6 and P-5 Sephadex columns. Cocoa brown and orange bars represent liposome-sCT association following first and second gel filtrations. The formulation tested was DPPC:Chol:SA (70:20:10) and the results are an average of two independent EE values.

Table 2.1. Physicochemical parameters for gel filtration experiments. The formulation tested was DPPC:Chol:SA (70:20:10) in singlet and the results tabulated are an average of 3 independent measurements.

Parameter	Particle size (nm)	PdI	ζ-potential (mV)
P – 5 gel parameters			
After 1st gel filtration	150.4 ± 0.5	0.032 ± 0.02	11.8 ± 0.4
After 2nd gel filtration	155.6 ± 8.9	0.045 ± 0.02	12.1 ± 0.65
P – 6 gel parameters			

After 1st gel filtration	148.5 ± 0.96	0.083 ± 0.01	10.4 ± 1.16
After 2nd gel filtration	145.2 ± 0.74	0.052 ± 0.01	12.4 ± 0.31
P – 10 gel parameters			
After 1st gel filtration	145.4 ± 0.39	0.051 ± 0.02	12.8 ± 0.27
After 2nd gel filtration	144.6 ± 2.32	0.083 ± 0.01	11.9 ± 0.65

Centrifugation at 3000 rpm

The results of physicochemical parameters and encapsulation efficiency (Table 2.2) following centrifugation at 3000 rpm suggested method 1 to be more accurate in terms of being comparative to the results obtained with gel filtration through P-6 Sephadex column. Method 2 showed very high EE similar to that obtained with P-5 Sephadex column. The decrease in sCT encapsulation efficiency with time in method 1 is shown in Figure 2.3.

Table 2.2. Physicochemical parameters and encapsulation efficiency following centrifugation of liposomes at 3000 rpm. The results were obtained on donor compartment samples after 4 h in method 1 and after one hour in method 2. The experiments were performed in duplicate and the formulation tested was DPPC:Chol:SA (70:20:10) such that $n = 6$ for particle size, polydispersity index (PdI), zeta potential (ζ-potential) and Stewart's assay and $n = 4$ for sCT content by RP-HPLC. Method 1 was more accurate since the results obtained by this method were comparable with that obtained on gel filtration through P-6 Sephadex column.

Parameter	Method 1	Method 2
Particle size (nm)	157.5 ± 11.9	170.5 ± 1.6
PDI	0.14 ± 0.08	0.28 ± 0.01
Zeta potential (mV)	17.9 ± 1.5	12.2 ± 0.8

Encapsulation efficiency (%)	31.8 ± 1.2	81.6 ± 1.1

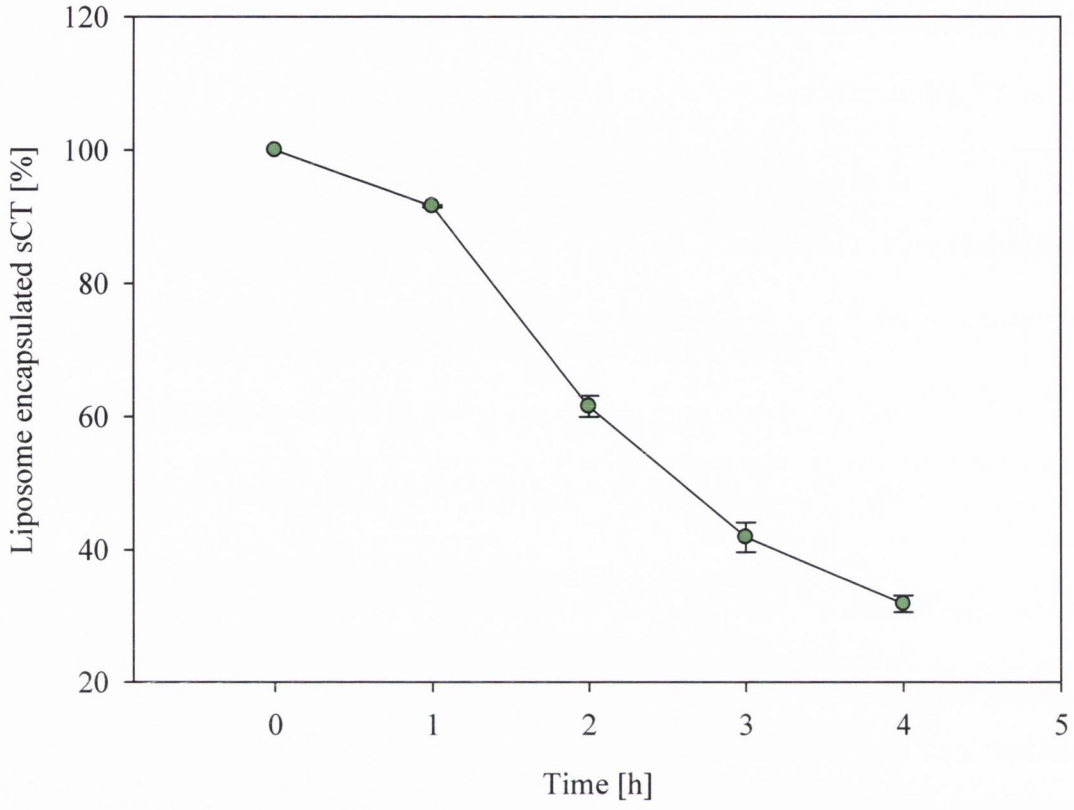


Figure 2.2. Encapsulation efficiency of sCT following centrifugation at 3000 rpm – Method 1. Encapsulation efficiency of sCT decreases with time and at the end of 4 hours reaches a value similar to that obtained following first gel filtration through Sephadex P-6 gel. The formulation tested was DPPC:Chol:SA (70:20:10) in duplicate and the results are an average of 4 independent encapsulation efficiency values.

Ultracentrifugation

Encapsulation efficiency of sCT in liposome following gel filtration through P-6 gel and ultracentrifugation is shown in Figure 2.4. Ultracentrifugation lead to significantly ($*P \leq 0.5$) higher sCT loss compared to gel filtration when performed immediately after extrusion and 24 hours after extrusion.

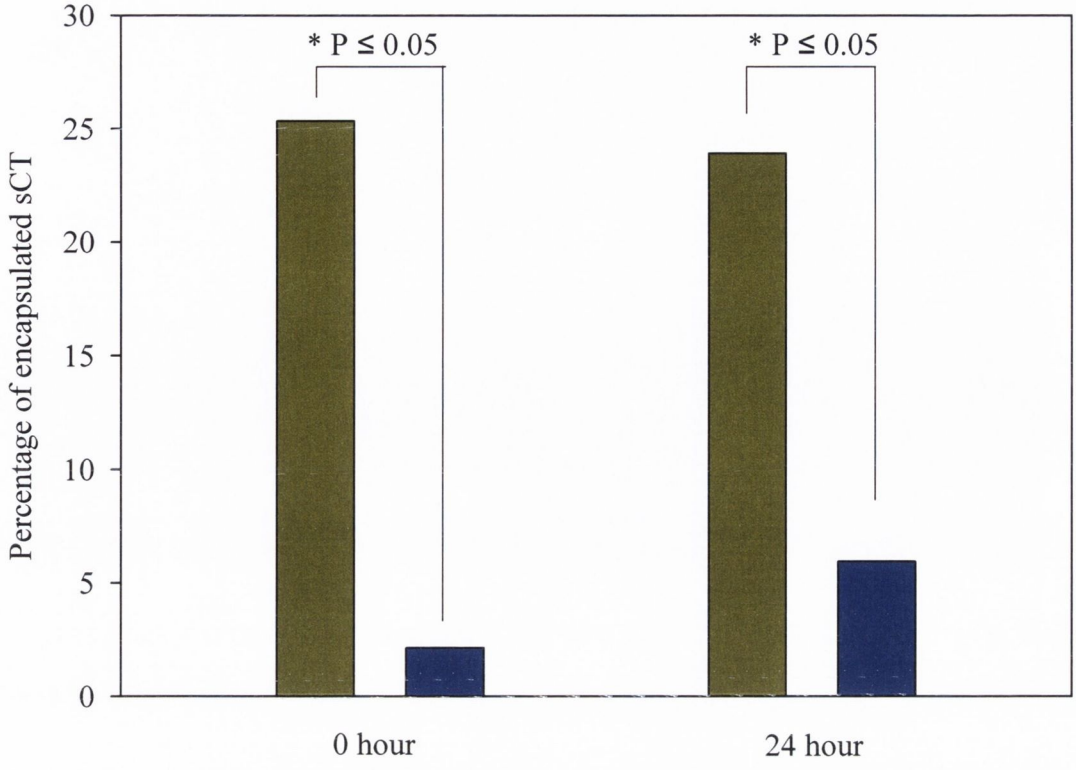


Figure 2.3. Gel filtration versus ultracentrifugation. Comparison of encapsulation efficiency of sCT following gel filtration through P-6 Sephadex column (algae green bars) and ultracentrifugation (ink blue bars) at 52,000 rpm for 3 hours immediately after extrusion and 24 hours post extrusion. The formulation tested was DPPC:Chol:SA (60:20:20) in singlet. The results are an average of 2 independent measurements.

Filtration using a syringe driven filter unit

Particle size, PdI and physical appearance of liposomes extruded (through 400, 200 and 100 nm filters) and filtered (through 0.45 and 0.22 μm filters) are summarised in Table 2.3. Particle size is very high for all batches except those extruded through 100 nm filters and PdI is not within specifications (0.02 – 0.2) for any of the batches.

Table 2.3. Separation of non-encapsulated sCT using syringe driven filtration units. Control (pH 7.4 KRB) and liposomes extruded through 400, 200 and 100 nm filters were filtered through 0.45 and 0.22 μm filters. The formulation tested was DPPC:Chol (66.7:33.3) in singlet. (n = 3 for particle size and PdI)

Parameter	Physical appearance	Particle size (nm)	PdI
Control – pH 7.4 KRB			
KRB – unprocessed	Clear	2451 ± 601.5	1
KRB – 0.45 μm filtered	Clear	1577 ± 1097	0.786 ± 0.174
KRB – 0.22 μm filtered	Clear	1843 ± 283.8	0.976 ± 0.042
400 nm extruded liposomes			
400 nm extruded	Translucent	6213 ± 1145	0.831 ± 0.156
400 nm extruded + 0.45 μm filtered	Translucent	2975 ± 584	0.672 ± 0.021
400 nm extruded + 0.22 μm filtered	Clear	513.2 ± 48.02	0.868 ± 0.116
200 nm extruded liposomes			
200 nm extruded	Translucent	640.3 ± 158.2	0.897 ± 0.093
200 nm extruded + 0.22 μm filtered	Translucent	483.6 ± 171.6	0.877 ± 0.149
100 nm extruded liposomes			
100 nm extruded	Translucent	190.3 ± 1.2	0.423 ± 0.03
100 nm extruded + 0.22 μm filtered	Translucent	179.5 ± 1.7	0.344 ± 0.07

2.4.2 Preliminary characterisation – sCT dimerisation

Results for presence of sCT dimers in laboratory/working conditions like 0.2 mg/ml sCT concentration, different loading buffer pH values (3.5, 5.0, 7.0, 8.5 and 10.0) and temperature of processing suggested the absence of sCT dimers in all cases (Figures 2.4.A and 2.4.B). The plots in all pH values show the same sCT elution profile with a sCT mass balance in all cases indicating the absence of sCT dimers.

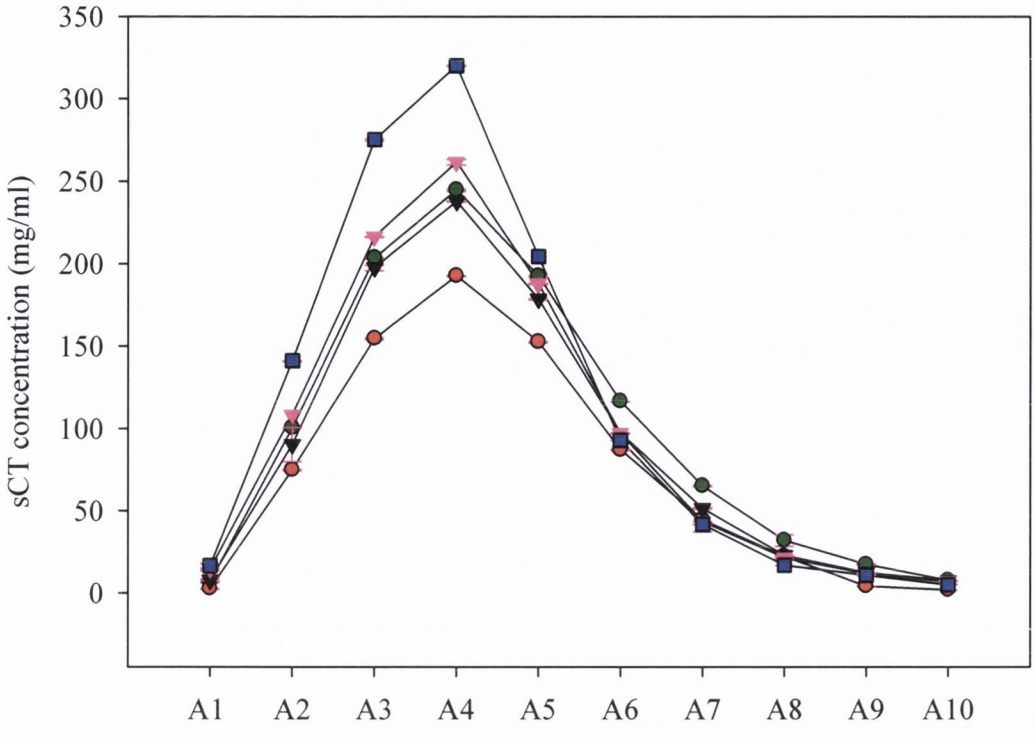


Figure 2.4.A. RP-HPLC results for sCT dimerisation check

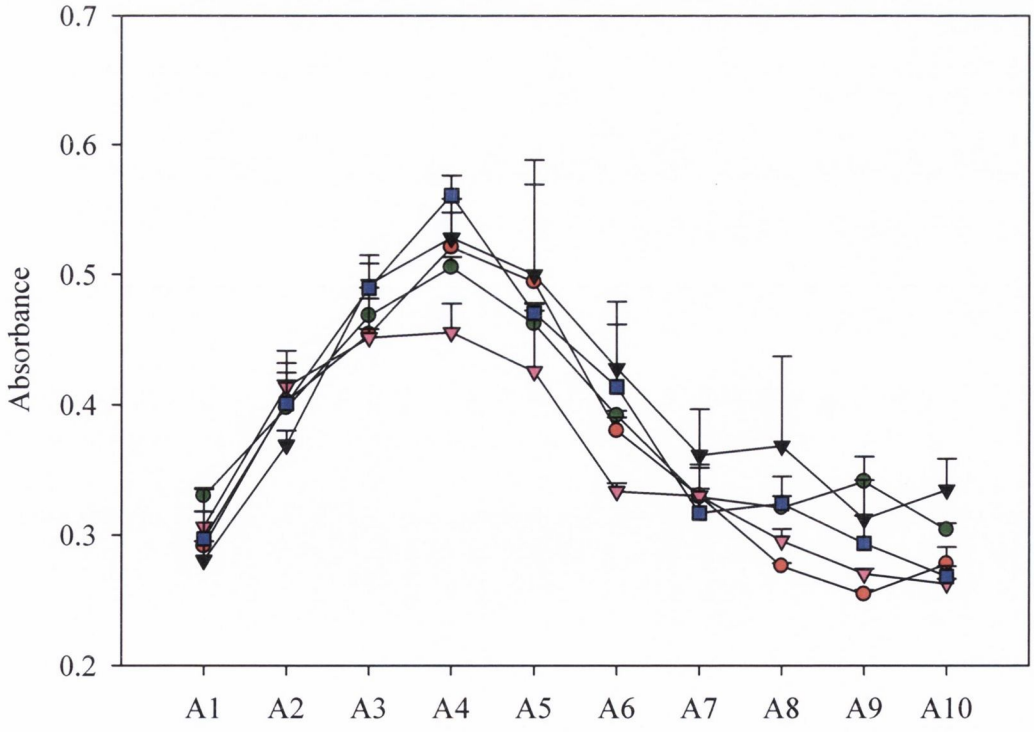


Figure 2.4.B. BCA assay results for sCT dimerisation check

Figures 2.4.A and 2.4.B Results for presence of sCT dimers following gel filtration through P-6 Sephadex column and estimation by RP-HPLC and BCA assay. Green circles represent pH value 3.5, red circles pH value 5.0, black triangle pH value 7.0, pink triangle pH value 8.5 and blue square pH value 10.0. A1 to A10 indicate the aliquot numbers. The experiment was performed in singlet for analysis via each method. ($n = 2$ for RP-HPLC and $n = 3$ for BCA assay).

2.4.3 Liposome formulation and physicochemical characterisation

Thirty different liposomal formulations were prepared with varying surface charges and membrane rigidities as well as different pH values of hydration buffer (i.e., pH 3.5, 5.0, 7.0, 8.5 and 10.0). These formulations were characterised physicochemically and with respect to their sCT encapsulation efficiency (Table 2.4, Appendix figures A.1 to A.12). The particle size and PdI of all formulations ranged from 100 to 200 nm and 0.07 to 0.44, respectively, with the non-charged vesicles being generally larger and more polydisperse compared with the ones with net positive or negative membrane charge. Encapsulation efficiency of sCT was increased in liposomes with rigid membranes (i.e., using DPPC; phase transition temperature 41°C) in comparison to those prepared from more fluid lipids (i.e., using PC; phase transition temperature -36°C). Moreover, liposomes with positive surface charge showed overall higher encapsulation of sCT. Accordingly, the amount of SA was increased from 10mol% to 20mol% and 30mol%, to test the hypothesis that increased positive ζ-potential was accompanied by higher sCT payload. At 20mol% SA, however, no further increase in encapsulation efficiency was observed (Table 2.5).

Table 2.4. Physicochemical parameters and encapsulation efficiency of liposomal preparations intended for pulmonary delivery of salmon calcitonin. Particle size,

PdI and ζ-potential were measured using a zeta-sizer. Encapsulation efficiency was determined my RP-HPLC and Stewart's assay for sCT content and lipid concentration, respectively. Data are presented as mean ± S.D. (n = 9 for particle size, PdI and ζ-potential and n = 6 for EE).

pH 3.5				
Formulation	Particle size (nm)	PdI	ζ-potential (mV)	Encapsulation efficiency (%)
PC:Chol 66.7:33.3	178.53 ± 7.75	0.27 ± 0.06	0.36 ± 1.34	17.78 ± 3.92
PC:Chol:PG 70:20:10	131.77 ± 5.17	0.10 ± 0.01	-8.83 ± 0.93	15.74 ± 4.79
PC:Chol:SA 70:20:10	132.03 ± 1.04	0.10 ± 0.04	11.15 ± 0.90	17.90 ± 3.05
DPPC:Chol 66.7:33.3	185.36 ± 10.28	0.37 ± 0.01	2.27 ± 1.30	40.31 ± 7.04
DPPC:Chol:DPPG 70:20:10	134.00 ± 13.13	0.11 ± 0.07	-4.07 ± 1.02	25.46 ± 2.63
DPPC:Chol:SA 70:20:10	141.55 ± 18.27	0.07 ± 0.03	12.52 ± 1.30	41.44 ± 0.88
pH 5.0				
PC:Chol 66.7:33.3	161.00 ± 18.08	0.22 ± 0.03	-1.72 ± 1.59	14.53 ± 4.15
PC:Chol:PG 70:20:10	141.65 ± 7.48	0.11 ± 0.04	-8.73 ± 1.90	11.36 ± 1.83
PC:Chol:SA 70:20:10	127.40 ± 3.02	0.12 ± 0.01	10.80 ± 0.81	17.50 ± 2.58
DPPC:Chol 66.7:33.3	197.03 ± 17.60	0.44 ± 0.02	1.24 ± 1.74	16.82 ± 3.25
DPPC:Chol:DPPG 70:20:10	163.93 ± 18.18	0.27 ± 0.08	-2.15 ± 0.99	17.42 ± 10.51
DPPC:Chol:SA 70:20:10	139.38 ± 7.20	0.07 ± 0.03	19.02 ± 8.50	31.02 ± 4.17
pH 7.0				
PC:Chol 66.7:33.3	179.47 ± 1.28	0.25 ± 0.02	-0.51 ± 0.43	23.43 ± 3.12
PC:Chol:PG 70:20:10	134.18 ± 7.13	0.09 ± 0.02	-10.38 ± 1.46	15.73 ± 1.06
PC:Chol:SA 70:20:10	134.10 ± 8.49	0.10 ± 0.02	15.12 ± 4.46	20.46 ± 0.99
DPPC:Chol 66.7:33.3	171.25 ± 4.03	0.38 ± 0.03	0.38 ± 0.93	30.38 ± 0.68

DPPC:Chol:DPPG 70:20:10	141.40 ± 2.94	0.12 ± 0.01	-1.75 ± 0.76	28.92 ± 7.67
DPPC:Chol:SA 70:20:10	154.15 ± 4.35	0.09 ± 0.02	24.73 ± 1.95	25.90 ± 0.34
pH 8.5				
PC:Chol 66.7:33.3	176.72 ± 1.21	0.26 ± 0.02	1.29 ± 1.67	21.74 ± 2.81
PC:Chol:PG 70:20:10	134.52 ± 8.26	0.10 ± 0.01	-7.75 ± 0.73	25.29 ± 2.68
PC:Chol:SA 70:20:10	144.68 ± 19.82	0.14 ± 0.02	13.27 ± 0.66	39.05 ± 7.60
DPPC:Chol 66.7:33.3	163.32 ± 13.69	0.37 ± 0.07	0.62 ± 0.87	23.41 ± 2.43
DPPC:Chol:DPPG 70:20:10	169.55 ± 15.63	0.33 ± 0.03	-4.62 ± 1.32	29.39 ± 4.12
DPPC:Chol:SA 70:20:10	131.87 ± 12.65	0.20 ± 0.05	22.27 ± 2.18	39.19 ± 3.39
pH 10.0				
PC:Chol 66.7:33.3	111.73 ± 1.63	0.22 ± 0.02	-1.00 ± 0.37	36.02 ± 5.06
PC:Chol:PG 70:20:10	100.85 ± 1.60	0.13 ± 0.02	-8.78 ± 2.75	36.85 ± 3.85
PC:Chol:SA 70:20:10	105.68 ± 3.69	0.11 ± 0.01	5.64 ± 3.86	30.57 ± 4.42
DPPC:Chol 66.7:33.3	146.73 ± 19.21	0.32 ± 0.03	-0.30 ± 1.37	24.53 ± 2.17
DPPC:Chol:DPPG 70:20:10	132.83 ± 35.27	0.29 ± 0.21	-4.56 ± 2.90	43.86 ± 13.55
DPPC:Chol:SA 70:20:10	119.33 ± 1.27	0.18 ± 0.02	10.66 ± 10.47	74.23 ± 26.27

Table 2.5. Physicochemical parameters and encapsulation efficiency of liposomes containing increasing amounts of SA. Particle size, polydispersity index (PdI) and ζ-potential were measured using a zeta-sizer. Encapsulation efficiency was determined using HPLC and Stewart's assay for sCT content and lipid concentration, respectively. Data are presented as mean ± SD (n = 9 for PdI and ζ-potential; n = 6 for encapsulation efficiency).

Formulation	Hydrodynamic (nm)	PdI	ζ-potential (mV)	Encapsulation efficiency (%)
DPPC:Chol:SA 70:20:10	141.55 ± 18.27	0.04 ± 0.03	12.52 ± 1.30	41.44 ± 0.88

DPPC:Chol:SA 60:20:20	138.83 ± 1.05	0.06 ± 0.03	24.57 ± 1.26	40.24 ± 3.41
DPPC:Chol:SA 50:20:30	141.41 ± 2.99	0.06 ± 0.01	32.62 ± 2.13	36.88 ± 3.23

2.4.4 Nebulisation of liposomes

The six liposomal formulations that underwent nebulisation were: DPPC:Chol:SA (70:20:10), DPPC:Chol:SA:DSPE-PEG_{2000} (65:20:10:5), DPPC:Chol:SA (60:20:20), DPPC:Chol:SA:DSPE-PEG_{2000} (55:20:20:5), DPPC:Chol (66.7:33.3) and DPPC:Chol:DSPE-PEG_{2000} (75:20:5). Neither the particle size nor the ζ-potential changed significantly following aerosolisation (Figure 2.5; Table 2.6). Of note, the polydispersity increased; in some cases significantly, indicating that liposome size distribution was affected by the aerosolisation process (Table 2.6). From, Figure 2.6, no significant ($*P \leq 0.05$) change in encapsulation efficiency was observed between PEGylated formulations and their non-PEG counterparts post nebulisation, with the exception of DPPC:Chol (66.7:33.3) and DPPC:Chol:DSPE-PEG_{2000} (75:20:5) where there was a significant increase in encapsulation efficiency of the PEG formulation pre and post nebulisation.

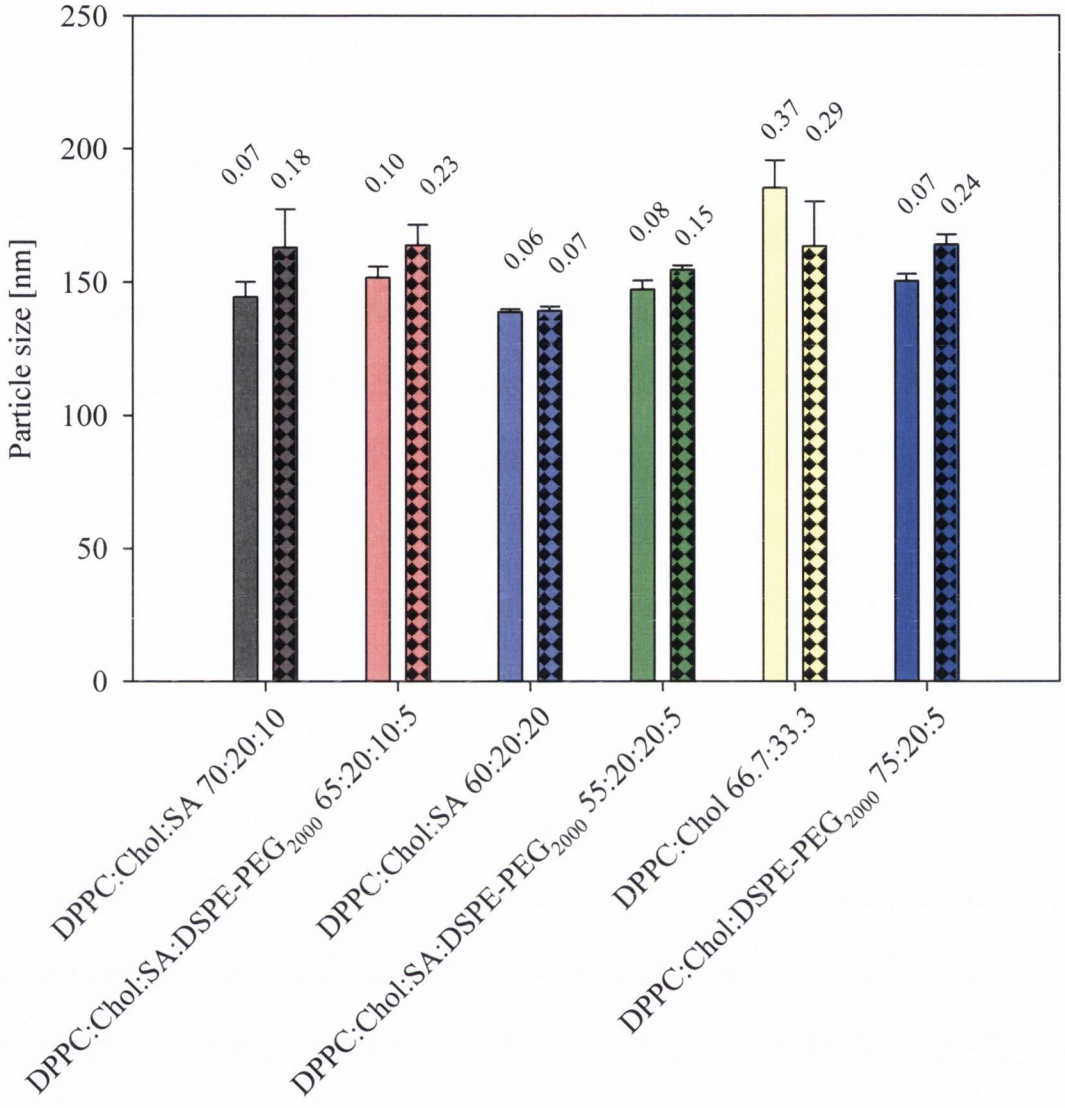


Figure 2.5. Effect of nebulisation on the particle size of sCT-containing liposomes. Hydrodynamic particle diameter of PEGylated and non-PEGylated liposomes before (plain coloured bars) and after nebulisation (diagonally checked coloured bars) is shown. Data were obtained by aerosolisation of 3 ml liposome aliquots for 15 min in an Aeroneb Pro vibrating mesh nebuliser. No significant ($P \leq 0.05$) changes were observed. Data are presented as mean ± SD ($n = 9$).

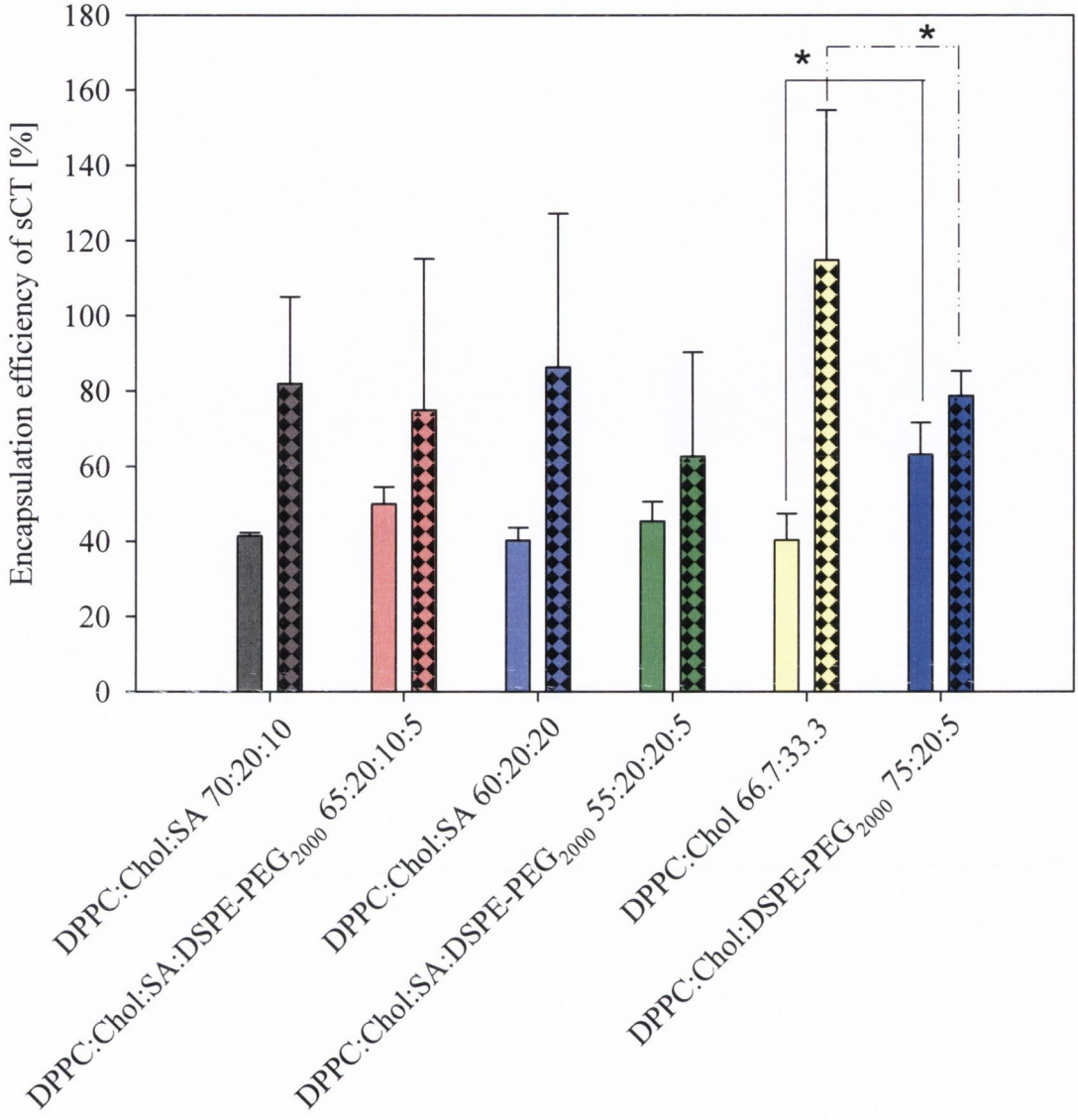


Figure 2.6. Effect of nebulisation on the encapsulation efficiency of sCT in liposomes. Encapsulation of sCT before (plain coloured bars) and after nebulisation (diagonally checked coloured bars) is shown. Three millilitres of liposome aliquots were aerosolised for 15 min in an Aeroneb Pro vibrating mesh nebuliser. Subsequently, HPLC analysis for sCT and Stewart's assay for phospholipid content were carried out. Data are presented as mean ± SD (n = 6).

Table 2.6. Polydispersity index (PdI) and ζ-potential of PEGylated and non-PEGylated liposomal formulations for pulmonary delivery of salmon calcitonin, pre- and post nebulisation using an Aeroneb Pro vibrating mesh nebuliser. Data are presented as mean ± SD (n = 9).

Formulation	PdI		ζ-potential (mV)	
	Pre nebulisation	Post nebulisation	Pre nebulisation	Post nebulisation
DPPC:Chol:SA 70:20:10	0.07 ± 0.03	0.18 ± 0.05	12.52 ± 1.30	12.66 ± 1.57
DPPC:Chol:SA:DSPE-PEG_{2000} 65:20:10:5	0.10 ± 0.07	0.23 ± 0.07	0.25 ± 0.39	0.21 ± 1.43
DPPC:Chol:SA 60:20:20	0.06 ± 0.03	0.07 ± 0.02	24.57 ± 1.26	24.40 ± 1.99
DPPC:Chol:SA:DSPE-PEG_{2000} 55:20:20:5	0.08 ± 0.03	0.15 ± 0.02	0.95 ± 1.35	0.47 ± 1.33
DPPC:Chol 66.7:33.3	0.37 ± 0.01	0.29 ± 0.06	2.27 ± 1.30	1.70 ± 0.51
DPPC:Chol:DSPE-PEG_{2000} 75:20:5	0.07 ± 0.03	0.24 ± 0.04	-0.48 ± 0.84	-0.97 ± 0.50

2.5 DISCUSSION

The withdrawal of Exubera® (the Pfizer/Nektar inhalable insulin) from the market, resulted in increased scepticism towards pulmonary macromolecule delivery. (71) Afrezza® (MannKind's insulin product), however, has started a new wave of interest in inhalable peptide drugs for systemic action, and it seems to have overcome some of the drawbacks that led to the failure of Exubera®. (77) This work set out to determine, whether suitable liposomal formulations could be prepared for the pulmonary delivery of salmon calcitonin. Initially the motive was to select the best method among the most commonly used methods for separation of non-encapsulated sCT from liposomes post preparation and also to establish reproducibility of the selected

method. The methods investigated were gel filtration, centrifugation, ultracentrifugation and filtration.

Comparison of encapsulation efficiency following gel filtration via P-5, P-6 and P-10 gels suggested P-5 and P-10 gels to be unsuitable. The small exclusion limit of P-5 gel did not provide an effective means of non-encapsulated sCT separation considering the molecular weight of sCT being 3431 g/mol. On the other hand, P-10 gel removed excess payload due to its large exclusion limit. This was due to the amphiphilicity of sCT that caused its association with both lipid vesicle and aqueous interior. (78 & 79). Exclusion limit of 6,000 Da provided by P-6 gel was most optimum for separation of non-encapsulated sCT and was also reproducible. This was contradictory to the results published by Chen *et al* who used a Sephadex G-25 column with an exclusion limit of 1,000 to 5,000 Da and obtained encapsulation efficiencies between 22 and 55% depending on the formulation. (67) The decrease in encapsulation efficiency following second gel filtration further confirmed the amphiphilic nature of sCT. This could have caused the establishment of a dynamic equilibrium of sCT between the inside and outside of liposome vesicle following removal of sCT after first gel filtration. The sCT that managed to penetrate the liposome vesicle to the outside was removed during second gel filtration.

Centrifugation at 3000 rpm by method 1 lead to similar encapsulation efficiency as obtained with P-6 sephadex column. However, the time taken for achieving the same was enormous suggesting this method to be more tedious and less suitable. Method 2 did not accomplish complete separation of non-encapsulated sCT that suggested method 1 to be superior to method 2. Centrifugation methods were not suitable for separation of non-encapsulated sCT. Ultracentrifugation brought about a significant reduction in sCT encapsulation in liposome. This could have been due to the

amphiphilic nature of sCT and spinning enhancing its release from within the liposome to exterior. Ultracentrifugation was also not suitable for separation of non-encapsulated sCT and this was contradictory to the results published by Law *et al* and Song *et al*. Law *et al* used 280,000 g for 20 minutes at 4°C and washed the pellet thrice with buffer at the same speed and duration to bring about separation of non-encapsulated sCT. They reported encapsulation efficiencies ranging from 10 to 50% depending on the formulation. (65 & 66) Song *et al* on the other hand used 300,000 g for 90 minutes to separate non-encapsulated sCT and determined the sCT in supernatant to determine sCT leaked during the process. (63) Since we got reproducible and explanatory results with P-6 gel, further experiments were designed using this as the means of non-encapsulated drug separation.

Absence of sCT dimers could be suggested from the elution profiles of sCT following gel filtration through P-6 sephadex column. Mass balance of sCT in all pH value buffers confirms the absence of same. Windisch *et al* reported the formation of sCT dimers following exposure of sCT solutions at 60°C for 5 days. (80) Andreotti *et al* published the existence of sCT dimers at pH value 3.3 (20mM acetate and 100mM sodium chloride buffer) and pH value 7.4 (20mM phosphate and 100mM sodium chloride buffer) while human calcitonin was reported to be a monomer at pH 3.3 and a monomer-dimer at pH 7.4. (81) Stevenson *et al* concluded the presence of sCT dimers at high sCT concentrations of 50 mg/ml and 100 mg/ml and consequently suggested the best stability of sCT in water at pH value of 3.3. (82)

Liposomes encapsulating sCT and formulated by using varying compositions of PC, DPPC, Chol, SA, DPPG and DSPE-PEG_{2000}, were characterised regarding their particle size PdI and ζ-potential. The liposomal sCT formulations proved to be stable

against the stresses of aerosolisation with an Aeroneb Pro nebuliser and they also showed sustained release of the peptide drug *in vitro*.

Our studies ascertained that liposomes comprising more rigid membranes (due to the use of DPPC) and a positive surface charge were an expedient compromise between acceptable physicochemical characteristics, high encapsulation efficiency and acceptable stability of salmon calcitonin. Non-charged formulations (comprising both rigid and fluid phospholipids) showed a higher degree of fusion immediately after preparation, leading to higher particle size and PdI, compared to charged vesicles. The amphiphilic nature of sCT might be responsible for a higher degree of membrane permeation and consequently, lower encapsulation efficacies in the case of the liposomal formulations based on more fluid phospholipids. (78 & 79) Liposomes with positive surface charge showed increased encapsulation efficiency compared to its neutral and negative counterparts for the net positively charged sCT (isoelectric point of 10.4 (83)) in half of the cases. Though the reason for this is not clear, it could be due to charge repulsion at the membrane. Negatively charged lipids, on the other hand, might have a certain amount of surface-adsorbed sCT, which is removed during gel filtration. An increase in concentration of SA from 10 to 30mol%, however, did not result in a significant change of encapsulation efficiency. Moreover, the ζ-potential was very high in 30% SA formulations, resulting in exclusion of these vesicles from further studies.

Other groups have previously reported EE values between 45% and 55% for various non-PEGylated liposomal systems for the delivery of sCT. (67, 65) Our results are somewhat in disagreement with data published by Law *et al.* (65) who observed higher encapsulation of sCT into liposomes with negative surface charges and increased leakage of the peptide from positively charged vesicles. Of note, their

vesicles were based on 1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) with an almost 50% higher cholesterol content compared to our formulations. Moreover, these systems were significantly larger, non-extruded liposomes. It can hence be hypothesised that the interactions between lipid membranes and amphiphilic peptides strongly depend on the fatty acid composition, the cholesterol content and the vesicles size. (84)

Of note, PEGylation of liposomes abolished the effects of surface charge on encapsulation efficiency due to Born hydration repulsion caused by the establishment of a non-diffusive water layer around the liposomes, in addition to steric repulsion caused by the PEG-chains. (15 & 45) Interestingly, this also resulted in the highest encapsulation efficiency of approx 63%, which was achieved with the DPPC:Chol:DSPE-PEG_{2000} (75:20:5) composition.

The increases in PdI observed for some formulations after nebulisation might be attributed to the physical stresses that led to vesicles fusing. Nebulisation also resulted in a relatively higher loss of lipids compared to the controls, whereas the loss of sCT in both samples was similar. As a consequence, a higher (sCT:lipid) *after nebulisation* / (sCT:lipid) *nebulisation control* ratio with an apparent increase in encapsulation efficiency of sCT was observed. Interestingly, this effect was less pronounced in the PEGylated formulations. PEG in the membrane during nebulisation might cause a less ordered state of the lipids, leading to increased vesicle disruption and faster sCT release. (46) Once the systems have settled following aerosolisation, the shielding effect of the PEG, however, might prevent re-uptake of peptide lost during nebulisation.

2.6 CONCLUSIONS

Among the methods investigated for separation of non-encapsulated sCT, gel filtration using prepacked columns with an exclusion limit of 6,000 Da was selected as the accurate cut-off and reproducible method. Absence of dimers in working conditions was also established. Liposomal formulations with a rigid membrane, positive surface charge and pH value of hydration buffer 3.5 was chosen as the optimal compromise between high encapsulation efficiency of sCT and small particle size. On PEGylation of these formulations, surface charge was abolished. PEGylated liposomes hold promise as delivery systems as they were able to encapsulate high amounts of the peptide and withstand nebulisation stresses. In order to further increase stability and encapsulation efficiency of the pay load, lyophilisation of PEGylated liposomes will be investigated. In addition, ongoing *in vitro* and *in vivo* studies will ascertain the bioactivity and pharmacokinetics of the sCT containing liposomal formulations.

3. sCT LIPOSOMES: LYOPHILISATION AND ^{31}P-NMR SPECTROSCOPY

Parts of this chapter will be submitted as:

J. Swaminathan, C. Ehrhardt. sCT liposomes: Lyophilisation and ^{31}P-NMR spectroscopy. *J Liposome Res*. Under preparation.

3.1 ABSTRACT

Purpose: The objective of this part of my PhD project was to assess the impact of lyophilisation (with and without the cryoprotectant, trehalose-di-hydrate) on encapsulation efficiency of salmon calcitonin (sCT) in liposomes.

Methods: The four formulations investigated were DPPC:Chol:DSPE-PEG_{2000} (75:20:5) and (65:30:5) and DPPC:Chol (80:20) and (66.7:33.3). Lipid films were prepared and hydrated with loading buffer containing sCT and different concentrations of cryoprotectant amounting to 20 different formulations altogether. The liposomes were lyophilised, reconstituted and extruded to obtain small unilamellar vesicles. Non-encapsulated sCT was separated by gel filtration using pre-packed Sephadex columns. The controls of each formulation were a non-lyophilisation batch (formulations that were not lyophilised) and a lyophilisation batch (formulations that were lyophilised without the cryoprotectant). The formulations were analysed for particle size, polydispersity index, ζ-potential and encapsulation efficiency. ^{31}P-NMR (Phosphorous Nuclear Magnetic Resonance spectroscopy) was performed on selected formulations.

Results: Post lyophilisation, no significant change in particle size and ζ-potential was noted regardless of the presence or absence of cryoprotectant. Encapsulation efficiency, on the other hand, increased following lyophilisation in both PEGylated (lyophilisation control batch) and non-PEGylated liposomes (cryoprotectant batches only). ^{31}P-NMR revealed the presence of two distinct vesicle populations - liposomes and micelles - in PEGylated formulation. The presence of micelles might be responsible for the observed encapsulation enhancement of sCT in the PEGylated formulation.

Conclusions: Lyophilisation resulted in an additional increase in encapsulation efficiency of sCT in PEGylated liposomes, even in the absence of a cryoprotectant, due to presence of micellar vesicles.

3.2 INTRODUCTION

Salmon calcitonin (sCT), a 32-amino-acid peptide secreted by the C cells of the thyroid, is used in the clinic for the treatment of osteoporosis, Paget's disease and hypercalcaemia and is currently marketed as injectable solutions (Calcimar®, Miacalcin®) as well as nasal sprays (Fortical®, Miacalcin®). (49, 69 & 6) Various research groups have been investigating the suitability of liposomal carriers for delivery of sCT via parenteral (57), intranasal (65-67) and oral (59, 61-63) routes. The biggest issue with the stability of liposomal formulation arises due to the fact that sCT is amphiphilic and the liquid formulation cannot be expected to provide a long shelf-life. (78 & 79)

Lyophilisation is one of the most promising approaches to ensure long-term stability of biopharmaceuticals as well as advanced drug delivery systems, such as liposomes. (85) In addition, increased permeability of payload across the bilayer upon rehydration following lyophilisation in the presence of a lyoprotectant has been reported, resulting in increased encapsulation efficiency. (86) The freeze-drying process is very versatile allowing the use of different concentrations of cryo/lyoprotectants, temperatures and duration of lyophilisation and drying cycles, making it suitable for the most sensitive payloads. (87 & 88) During lyophilisation, there occurs a change in gel to liquid crystalline phase transition when the pay load is lost and to overcome this, lyoprotectants are added which avoids the phase transition. Sugars as lyoprotectants act by protecting membrane integrity and preventing pay

load leakage due to their high T_g. (85) Lyophilisation of insulin and VIP-containing liposomes has been explored, whilst freeze-drying of salmon calcitonin (sCT) liposomes has not been reported to date. (89 & 90)

This work was set out to determine the impact of lyophilisation on encapsulation efficiency of the optimal formulations from my previous work. Subsequently, the impact of a small increase in percentage of cholesterol on lyophilisation was analysed. Trehalose-di-hydrate was used as cryoprotectant of choice, due to its reported benefits. (85, 87, 91-93) Different concentrations of trehalose were studied in the external medium alone and in internal and external compartment together. The observed increase in encapsulation efficiency in the case of PEGylated liposomes was investigated using ^{31}P-NMR spectroscopy.

3.3 MATERIALS AND METHODS

3.3.1 Materials

DPPC and DSPE-PEG_{2000} were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA) while Chol, trehalose-di-hydrate, glucose, HEPES, calcium chloride dihydrate, sodium dihydrogen phosphate, iron (III) chloride hexahydrate, ammonium thiocyanate, chloroform, acetonitirile and magnesium sulphate heptahydrate were procured from Sigma-Aldrich (Dublin, Ireland). Sodium chloride, potassium chloride and sodium bicarbonate were purchased from Fisher Scientific (Dublin, Ireland). Ultra pure water was used in all experiments and generated by a Millipore Synergy unit (Cork, Ireland). A Rotavapor R-210 rotary evaporator was used for lipid film preparation (Büchi, Flawil, Switzerland). A Virtis freeze-dryer (Ipswich, UK) was used to lyophilise the liposome samples following hydration. A Lipex thermobarrel extruder and the poly carbonate filters used in the extrusion step were bought from

Northern Lipids (Burnaby, British Columbia, Canada) and Whatman (Maidstone, UK), respectively. Econo-pac columns with an exclusion limit of 6,000 Da used for gel filtration were purchased from Alpha Technologies (Wicklow, Ireland). Particle size, polydispersity index (PdI) and ζ-potential were measured using a Zetasizer Nanoseries ZS (Malvern Instruments, Malvern, UK). The concentration of sCT was determined using a Shimadzu HPLC (Kyoto, Japan), comprising a LC-10AT pump, auto sampler SIL-10AD, degasser DGU-14A, UV-Vis detector SPD-10A and system controller SCL-10A. Jones chromatography (Cardiff, UK) reverse phase C-18 column, 150×4.6 mm, with 4-μm packing was used. A Bruker AD3 spectrometer (Billerica, MA, USA) with 400 MHz spectral width was used to obtain ^{31}P-NMR spectra.

3.3.2 Methods

3.3.2.1 Liposome preparation: non-lyophilisation batches

Liposomes were prepared by the lipid film hydration method. Briefly, lipids were dissolved in chloroform and subsequently, the solvent was removed using a rotary evaporator to obtain a dry lipid film. This film was then hydrated at 42-43°C with KRB of 3.5 pH value, which contained sCT at a concentration of 0.2 mg/ml, to get a final lipid concentration of 10 μmol/ml. The samples were then subjected to size reduction at 42-43°C using an extruder fitted with polycarbonate filters of pore sizes ranging from 100 to 400 nm. Non-encapsulated sCT was separated by gel filtration on pre-packed columns using KRB of pH value 7.4 as elution buffer. The four formulations investigated were DPPC:Chol:DSPE-PEG$_{2000}$ (75:20:5) and DPPC:Chol:DSPE-PEG$_{2000}$ (65:30:5), their non-PEGylated counterparts DPPC:Chol

(80:20) and DPPC:Chol (66.7:33.3). Both lyophilisation and non-lyophilisation batches were prepared in triplicates.

3.3.2.2 Preliminary study for determination of cryoprotectant addition

The lipid film was prepared as described above. This was and then hydrated with two different combinations of loading buffer as illustrated in Figure 3.1.

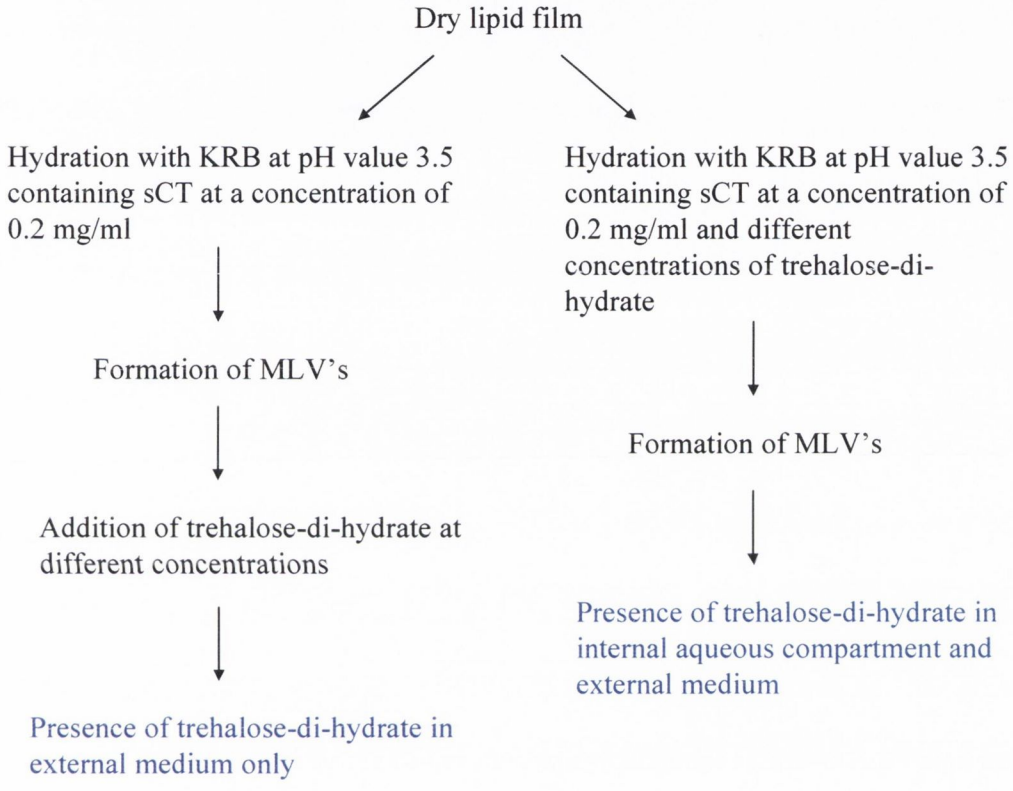


Figure 3.1. Illustration of two different methods of lipid film treatment pre lyophilisation

Trehalose-di-hydrate, the cryoprotectant was either added post hydration of the lipid film with KRB (pH 3.5) containing sCT at a concentration of 0.2 mg/ml (i.e., trehalose was in the external medium only) or it was a part of the loading buffer as well (i.e., trehalose was in the inner aqueous compartment and the external medium). A liposomal formulation without cryoprotectant served as control in this case. This was deemed necessary to check if the presence of cryoprotectant was required only in

the external medium or in both (internal and external) compartments. This was studied only in DPPC:Chol (66.7:33.33) liposomes and only as a single experiment.

3.3.2.3 Liposome preparation: lyophilisation batches with and without cryoprotectant

Liposomal formulations intended for freeze-drying were prepared exactly in the same manner as the non-lyophilisation batches until the hydration step. In this case, trehalose di-hydrate of varying concentrations (1:1, 1:5 and 1:10 ratios of lipid:trehalose) was added to the hydration solution that constituted KRB (pH 3.5) containing sCT at a concentration of 0.2 mg/ml to get a final lipid concentration of 10 μmol/ml. In the case of the lyophilisation control batches the hydration solution comprised all ingredients except trehalose di-hydrate. Temperature was maintained at 42-43°C during hydration. The four formulations studied were identical to the ones described in the non-lyophilised formulations section. Each formulation in turn had four variants with a lyophilisation control batch (no cryoprotectant) and three different concentrations of cryoprotectant. Lyophilisation batches with and without cryoprotectant were prepared in triplicates.

3.3.2.4 Lyophilisation of liposomal formulations

The multilamellar vesicles (lyophilisation control batches and lyophilisation batches with cryoprotectant) obtained following hydration were quick frozen using liquid nitrogen. The frozen samples were lyophilised for 48 h. Temperature was -60 to -80°C and pressure was 20 to 30 millitorr during the drying process. After 48 h, samples were collected and stored at 4°C until further processing.

3.3.2.5 Reconstitution and extrusion of the samples

Following lyophilisation, samples were reconstituted with KRB (pH 3.5) and left in the water bath maintained at 42-43°C for 5 min. The samples were then extruded and gel filtered as explained above.

3.3.2.6 Preliminary experiment characterisation

The liposome formulations were characterised at 4 different stages of preparation (illustrated in Figure 3.2). Content of sCT and lipid (n = 2) were determined at all 4 stages, while physicochemical parameters, i.e., particle size and PdI (n = 3) were studied at stages 1, 2 and 4.

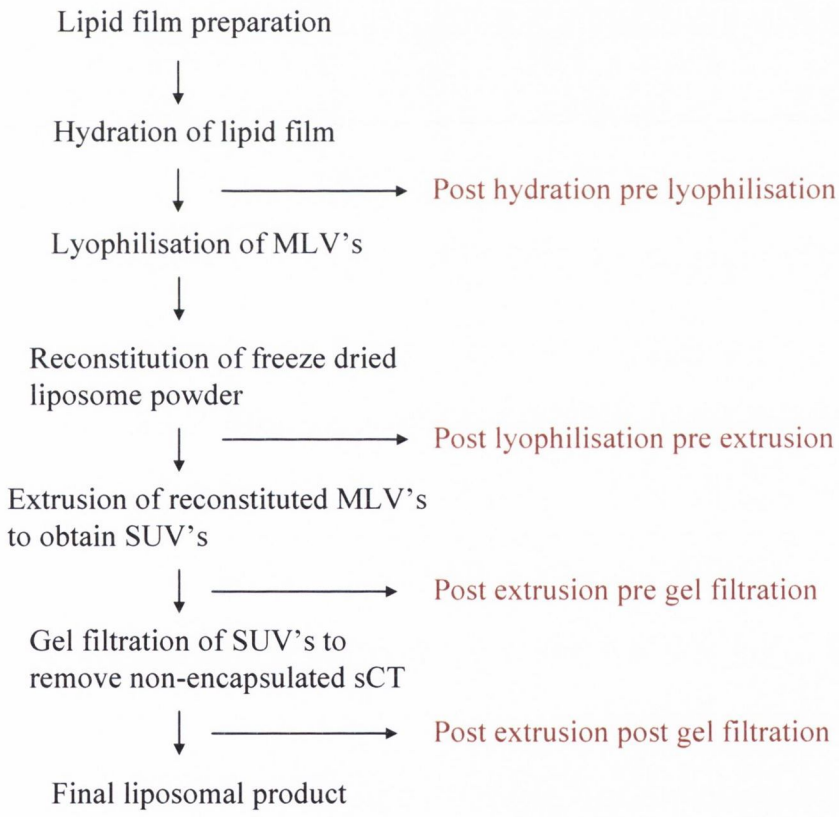


Figure 3.2. Characterisation stages in preliminary lyophilisation study

3.3.2.7 Physicochemical characterisation

All formulations were analysed for their particle size, PdI and the particle surface charge (ζ-potential). Disposable low volume cuvettes were used for particle size estimation, whereas disposable capillary cells were used for ζ-potential determination. Samples for physicochemical characterisation were prepared by diluting liposomal suspensions with KRB (pH 7.4). The measurements were based on dynamic light scattering at 173° backscatter detection. Each experiment was repeated in triplicates from 3 independent batches (n = 9) for lyophilisation experiments and in triplicates form 2 independent batches (n = 6) for release study batches.

3.3.2.8 HPLC method for determination of sCT content

Concentration of sCT was determined by RP-HPLC. An isocratic elution method was used with a flow rate of 1 ml/min. The mobile phase was prepared by adding 660 ml ultra pure water, 340 ml acetonitrile, 1.16 g sodium chloride and 320 µl trifluoroacetic acid. UV absorption of sCT was measured from 190 to 340 nm, with the actual spectrophotometric detection carried out at 215 nm. Each HPLC result was the average of 2 independent measurements from 3 liposome batches (n = 6) for lyophilisation experiments and average of 2 measurements from 2 liposome batches (n = 4) for release study batches.

3.3.2.9 Encapsulation efficiency

Samples were prepared by adding Triton X-100 to liposomal suspensions such that the final concentration of the detergent in the sample was 1%. Samples were then incubated at 37°C for 30 min, before being subjected to HPLC analysis. The phospholipid content was determined using Stewart's assay (76). Data of the

Stewart's assay was the average of 3 individual measurements from 3 independent liposome batches ($n = 9$).

Encapsulation efficiency following gel filtration was calculated using the following equation

$$\frac{(\mu g \text{ sCT}/\mu mol \text{ total lipid}) \text{ after gel filtration} \times 100}{(\mu g \text{ sCT}/\mu mol \text{ total lipid}) \text{ before gel filtration}} \qquad \text{(eq. 1)}$$

Encapsulation efficiency was the average of 2 individual determinations from three independent batches ($n = 6$).

3.3.2.10 NMR spectroscopy

Static ^{31}P-NMR spectra were obtained with 40.7 kHz spectral width at 162 MHz ^{31}P resonance frequency. One-hundred thousand scans were acquired using the method described by Leal *et al.* (95). Empty liposome samples (without sCT) for NMR spectroscopy were prepared by hydration of lipid film with D_2O and extrusion of the obtained multilamellar vesicles as described above. The formulations studied were DPPC:Chol:DSPE-PEG_{2000} (75:20:5) and DPPC:Chol (80:20). Following extrusion, samples were incubated with varying concentrations of Triton-X 100 initially. A concentration of 0.00625% (w/v) Triton-X 100 was chosen to work with at later stages. Spectra were also obtained without Triton-X 100 in controls.

3.3.2.11 Statistical analysis

Data are presented as mean ± standard deviation. Differences among more than two group means were determined by one-way analysis of variance (ANOVA) with Student-Newman-Keuls post-hoc tests. $P < 0.05$ was considered statistically significant.

3.4 RESULTS

3.4.1 Preliminary study for determination of cryoprotectant addition

The results for all parameters (physicochemical and encapsulation) at all four experimental stages are tabulated in Tables 3.1 and 3.2 and Figure 3.3. Particle size confirmed the presence of MLV's in the first two stages and SUV's in the third stage.(Table 3.1) No significant difference was observed in particle size between two variants corresponding to trehalose-di-hydrate addition in any of the stages. High PdI was observed in the first 2 stages while in the third stage presence of trehalose-di-hydrate in the internal compartment and external medium caused a significant ($*P \leq 0.05$) reduction in PdI. Encapsulation efficiency (Table 3.2 and Figure 3.3) of sCT was higher on inclusion of a cryoprotectant (compared to control) during lyophilisation regardless of the compartment it was present. Among the batches containing cryprotectant, presence of trehalose-di-hydrate in the internal and external medium showed significantly ($*P \leq 0.05$) higher encapsulation of sCT in 1:5 and 1:10 lipid:trehalose-di-hydrate ratios compared to trehalose-di-hydrate in external medium only.

Table 3.1: Physicochemical parameters at different stages of liposome preparation. Particle size and PdI at 3 stages of liposome preparation. The formulation tested was DPPC:Chol (66.7:33.3). Data are presented as mean ± SD (n = 3)

Particle size (nm)			
Parameter	Post hydration pre lyophilisation	Post lyophilisation pre extrusion	Post extrusion post gel filtration
Control	7945 ± 1142	7974 ± 1648	193.3 ± 5.14
Trehalose- external only			
1:1 lipid:trehalose	7721 ± 2156	10110 ± 1052	161.1 ± 0.22

1:5 lipid:trehalose	8898 ± 490.5	9905 ± 231.7	177.2 ± 2.19
1:10 lipid:trehalose	4723 ± 2863	12910 ± 1626	168.5 ± 1.7
Trehalose – Internal and external			
1:1 lipid:trehalose	8680 ± 2036	6137 ± 712.8	149.9 ± 1.48
1:5 lipid:trehalose	7840 ± 228.3	7595 ± 1260	151.4 ± 0.57
1:10 lipid:trehalose	10400 ± 2515	8961 ± 1068	144.2 ± 0.27
Polydispersity index			
Parameter	Post hydration pre lyophilisation	Post lyophilisation pre extrusion	Post extrusion post gel filtration
Control	0.695 ± 0.124	0.287 ± 0.220	0.309 ± 0.022
Trehalose - external only			
1:1 lipid:trehalose	0.606 ± 0.102	0.275 ± 0.134	0.173 ± 0.023 *
1:5 lipid:trehalose	0.549 ± 0.171	0.287 ± 0.257	0.265 ± 0.022 *
1:10 lipid:trehalose	0.529 ± 0.132	0.244 ± 0.039	0.223 ± 0.020 *
Trehalose - internal and external			
1:1 lipid:trehalose	0.579 ± 0.301	0.613 ± 0.224	0.081 ± 0.026 *
1:5 lipid:trehalose	0.396 ± 0.284	0.422 ± 0.155	0.071 ± 0.018 *
1:10 lipid:trehalose	0.627 ± 0.175	0.481 ± 0.121	0.020 ± 0.004 *

Table 3.2: Encapsulation parameter (sCT:lipid ratio) in all stages of liposome preparation. The formulation tested was DPPC:Chol (66.7:33.3). Data presented as mean of two independent measurements ($n = 2$).

sCT:lipid ratio (µg/µmol)				
Parameters	Post hydration pre lyophilisation	Post lyophilisation pre extrusion	Post extrusion pre gel filtration	Post extrusion post gel filtration
Control	13.068	15.391	12.199	2.484

Trehalose - external only				
1:1 lipid:trehalose	18.288	15.261	13.415	6.543
1:5 lipid:trehalose	19.363	15.736	13.468	5.814
1:10 lipid:trehalose	17.720	16.303	15.267	5.437
Trehalose - internal and external				
1:1 lipid:trehalose	22.914	17.068	16.099	6.448
1:5 lipid:trehalose	23.673	13.137	11.180	6.904
1:10 lipid:trehalose	21.141	16.544	13.523	7.062

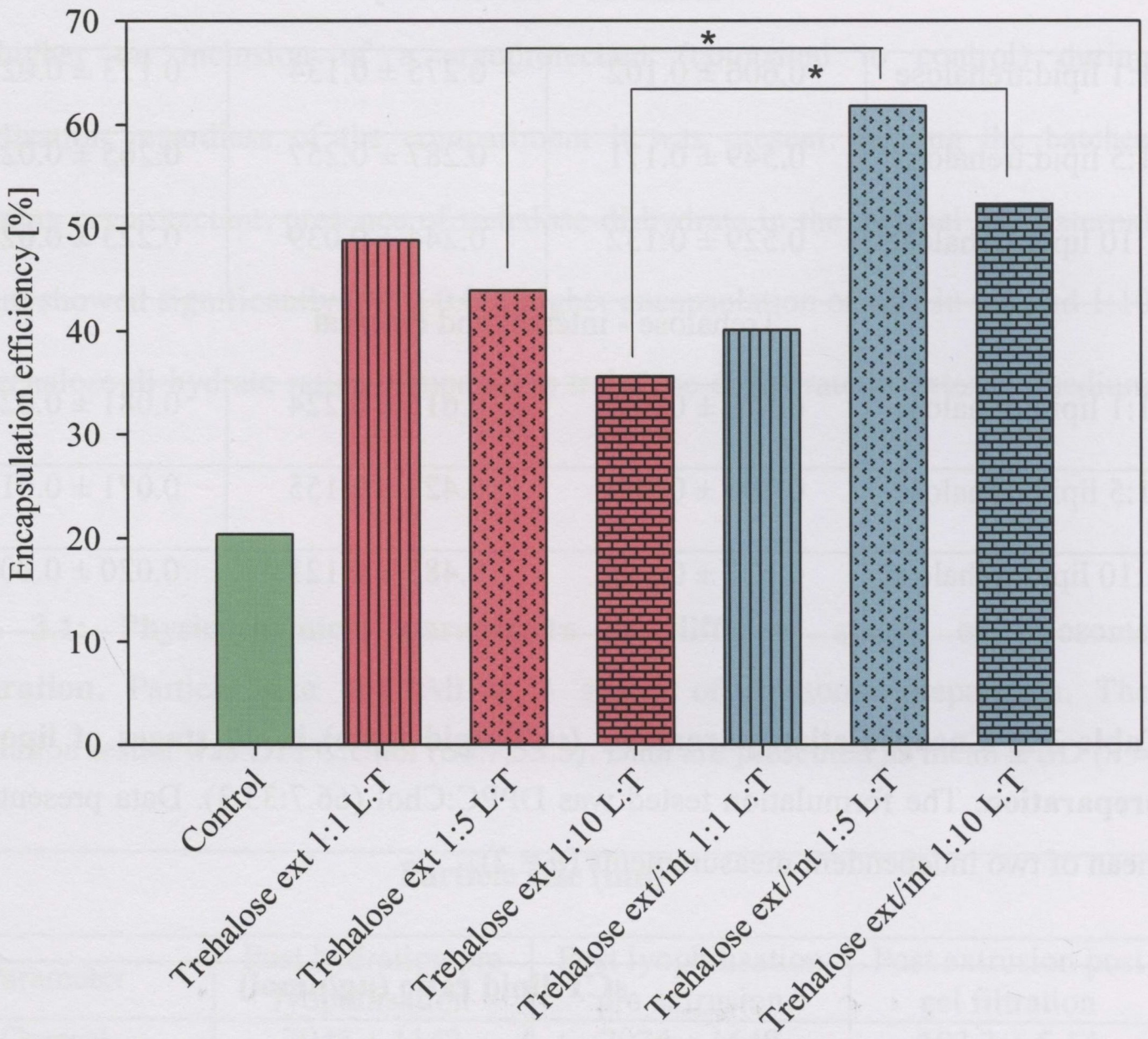


Figure 3.3. Comparison of encapsulation efficiency of sCT in SUV's (final liposomal preparation) containing trehalose-di-hydrate in external and

external/internal compartments. The formulation tested was DPPC:Chol (66.7:33.3). Data presented as mean of two independent measurements.

3.4.2 Physicochemical characteristics of non-lyophilised and lyophilised formulations

Twenty different liposomal formulations were prepared and characterised of which sixteen formulations underwent freeze-drying and four non-lyophilised formulations (non- lyophilisation batch) acted as controls for the freeze-drying process. On the other hand, four of the sixteen lyophilised formulations did not contain a cryoprotectant (lyophilisation control) and these acted as controls to assess the impact of cryoprotectant addition in the formulations. Particle size, PdI and ζ-potential of the formulations are presented in Figure 3.4 and Table 3.3.

Lyophilisation did not result in a significant increase in particle size in any of the studied formulations. (Figure 3.4) The particle sizes of non-PEGylated formulations [DPPC:Chol (80:20)] were slightly higher than their PEGylated counterparts [DPPC:Chol:DSPE-PEG_{2000} (75:20:5)] however this is not pronounced in the other group with higher cholesterol concentration. In general, there was an insignificant change in particle size between the non-lyo batch and the lyophilised batches in all four formulations studied. From Table 3.3, there was a significant ($*P \leq 0.05$) decrease in PdI in DPPC:Chol (66.7:33.3) following lyophilisation, while the other formulations showed an insignificant change. Zeta-potential was always in the neutral range for all formulations investigated.

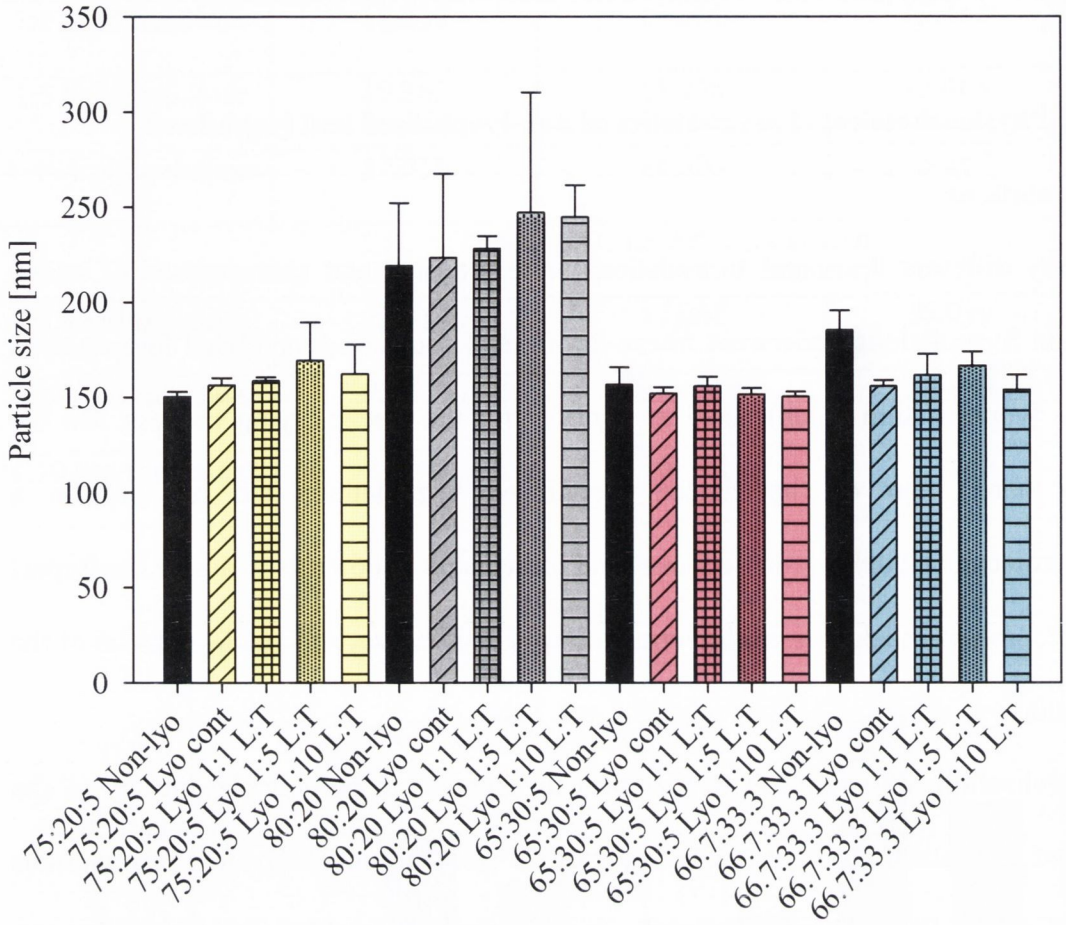


Figure 3.4. Particle size of non-lyophilised and lyophilised liposomal formulations. Particle size of formulations that were not freeze-dried (black bars), lyophilised DPPC:Chol:DSPE-PEG_{2000} (75:20:5, yellow bars), lyophilised DPPC:Chol (80:20, grey bars), lyophilised DPPC:Chol:DSPE-PEG_{2000} (65:30:5, pink bars) and lyophilised DPPC:Chol (66.7:33.3, light cyan bars). Data presented as mean±SD (n = 9)

Table 3.3: Polydispersity index (PdI) and ζ-potential of non-lyophilised and lyophilised formulations. Data presented as mean±SD (n = 9)

Parameter	PDI	ζ-potential (mV)
DPPC:Chol:DSPE-PEG_{2000} 75:20:5		

Non-lyo liposome	0.07 ± 0.03	-0.48 ± 0.84
Lyo control	0.06 ± 0.04	-0.30 ± 0.65
Lyo with 1:1 Lip:Treh	0.07 ± 0.02	-0.28 ± 0.53
Lyo with 1:5 Lip:Treh	0.13 ± 0.05	-0.61 ± 1.00
Lyo with 1:10 Lip:Treh	0.10 ± 0.07	-1.04 ± 0.31
DPPC:Chol 80:20		
Non-lyo liposome	0.43 ± 0.19	4.42 ± 0.68
Lyo control	0.37 ± 0.15	4.05 ± 0.73
Lyo with 1:1 Lip:Treh	0.42 ± 0.06	4.02 ± 0.83
Lyo with 1:5 Lip:Treh	0.43 ± 0.16	4.05 ± 0.75
Lyo with 1:10 Lip:Treh	0.43 ± 0.06	3.91 ± 0.59
DPPC:Chol:DSPE-PEG$_{2000}$ 65:30:5		
Non-lyo liposome	0.11 ± 0.06	0.25 ± 0.95
Lyo control	0.05 ± 0.02	0.07 ± 1.06
Lyo with 1:1 Lip:Treh	0.08 ± 0.02	0.69 ± 2.16
Lyo with 1:5 Lip:Treh	0.06 ± 0.01	0.17 ± 0.91
Lyo with 1:10 Lip:Treh	0.06 ± 0.01	-0.08 ± 1.18
DPPC:Chol 66.7:33.3		
Non-lyo liposome	0.37 ± 0.01 *	2.27 ± 1.30
Lyo control	0.09 ± 0.02 *	2.41 ± 0.26
Lyo with 1:1 Lip:Treh	0.14 ± 0.08 *	2.37 ± 0.36
Lyo with 1:5 Lip:Treh	0.16 ± 0.02 *	2.61 ± 0.75
Lyo with 1:10 Lip:Treh	0.07 ± 0.04 *	2.63 ± 1.16

3.4.3 Encapsulation efficiency of non-lyophilised and lyophilised formulations

There was an increase in encapsulation efficiency of sCT in most PEGylated formulations following lyophilisation (Figure 3.5). There was no correlation between the concentration of trehalose di-hydrate (cryoprotectant) and encapsulation efficiency of sCT, however, PEGylated formulations showed highest encapsulation efficiency even in the absence of a cryoprotectant. Non-PEG formulations showed only a small increase in encapsulation efficiency in the presence of trehalose-di-hydrate.

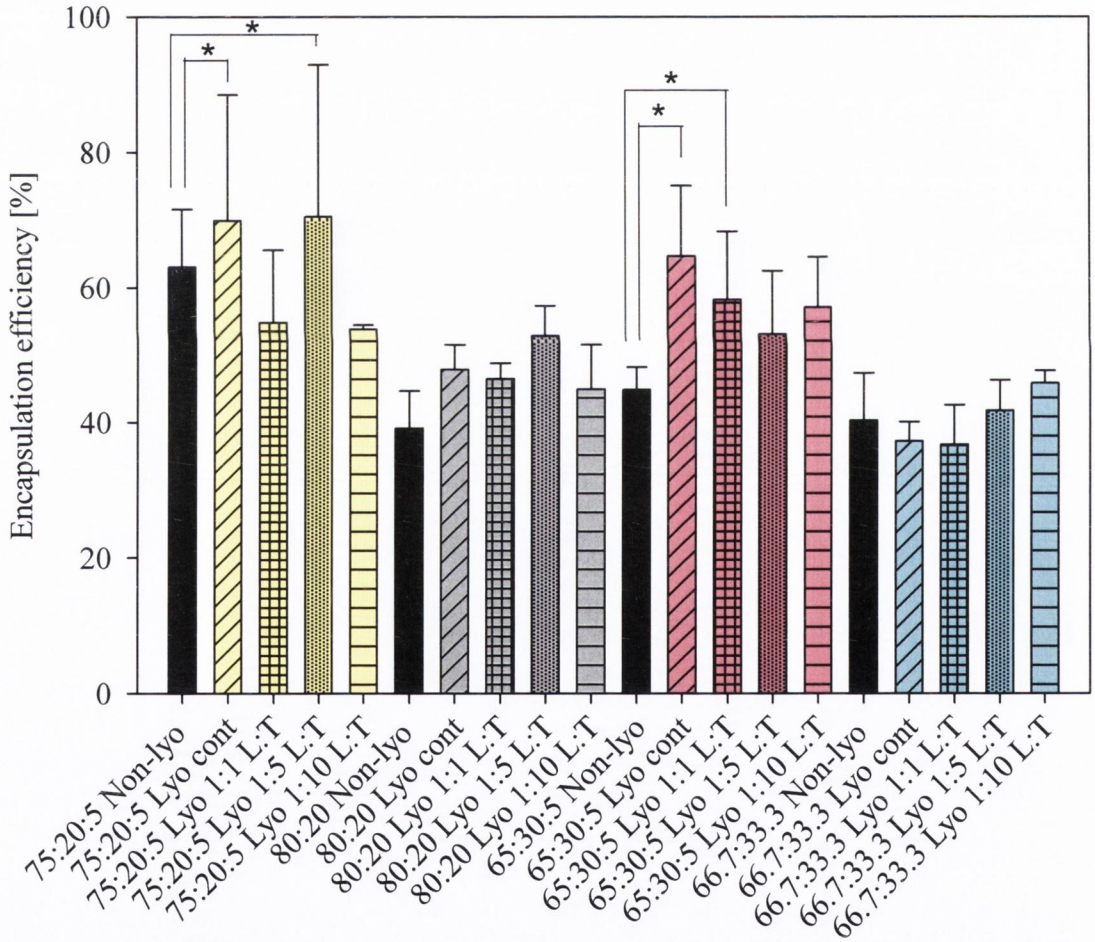


Figure 3.5. Encapsulation efficiency of non-lyophilised and lyophilised liposomal formulations. Encapsulation efficiency of sCT in formulations that did not undergo lyophilisation (black bars), lyophilised DPPC:Chol:DSPE-PEG_{2000} (75:20:5, yellow bars), lyophilised DPPC:Chol (80:20, grey bars), lyophilised DPPC:Chol:DSPE-

PEG_{2000} (65:30:5, pink bars) and lyophilised DPPC:Chol (66.7:33.3, light cyan bars).. Data presented as mean±SD ($n = 9$).

Freeze-drying with 1:5 lipid:trehalose di-hydrate and lyophilisation control showed the maximum encapsulation efficiency in DPPC:Chol:DSPE-PEG_{2000} (75:20:5) while 1:5 lipid:trehalose-di-hydrate formulation showed best encapsulation efficiency for DPPC:Chol (80:20) formulations. Lyophilisation control batch showed the highest encapsulation efficiency in DPPC:Chol:DSPE-PEG_{2000} (65:30:5) and lyophilised 1:10 lipid:trehalose di-hydrate showed the highest encapsulation efficiency among DPPC:Chol (66.7:33.3) formulations. PEGylated formulations showed significantly ($^*P \leq 0.05$) higher encapsulation efficiencies compared to their non-PEG counterparts in all cases, i.e., non lyophilisation batch, lyophilisation control batch and freeze-drying with all trehalose di-hydrate concentrations.

3.4.4 NMR spectroscopy

NMR spectra without Triton-X 100 revealed the presence of an additional sharp peak representing micelles in the PEGylated formulation [DPPC:Chol:DSPE-PEG_{2000} (75:20:5)] in addition to the broad peak in both formulations, suggesting the presence of liposomes. (Figure 3.6 and Appendix Figures A.13 and A.14) The sharp micelle peak was absent in the non-PEGylated formulation

On addition of increasing concentrations of Triton-X 100 to the PEGylated formulation, the liposome peak faded away and was very faintly observable at the highest concentration of Triton-X 100 studied (i.e., 2% w/v) (Figure 3.7 and Appendix Figure A.15).

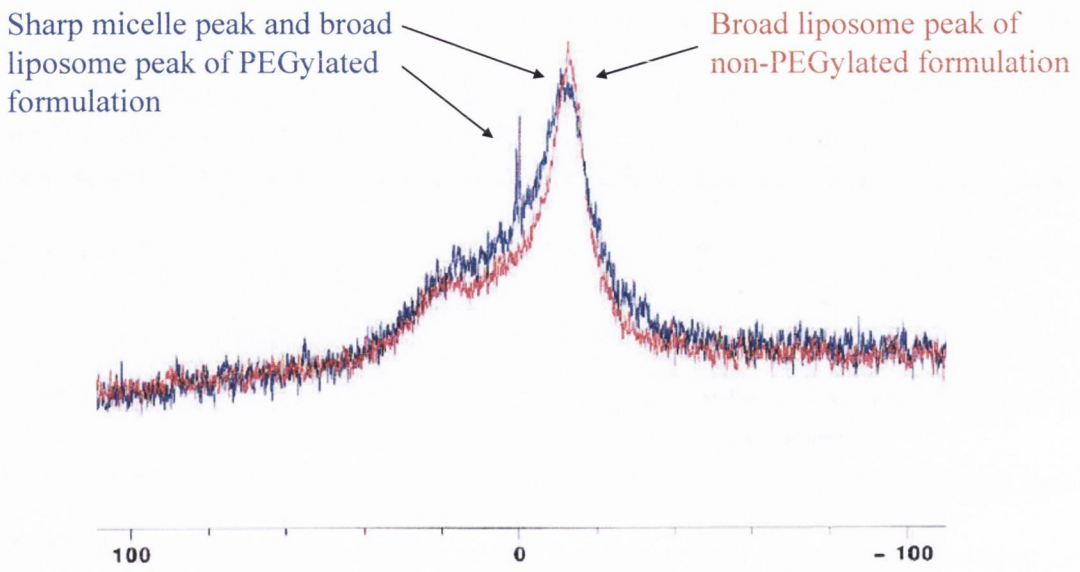


Figure 3.6. Representative ^{31}P-NMR spectra of PEGylated liposomes and the corresponding non-PEGylated formulations without Triton-X 100. The formulations investigated were DPPC:Chol:DSPE-PEG_{2000} (75:20:5) and DPPC:Chol (80:20).

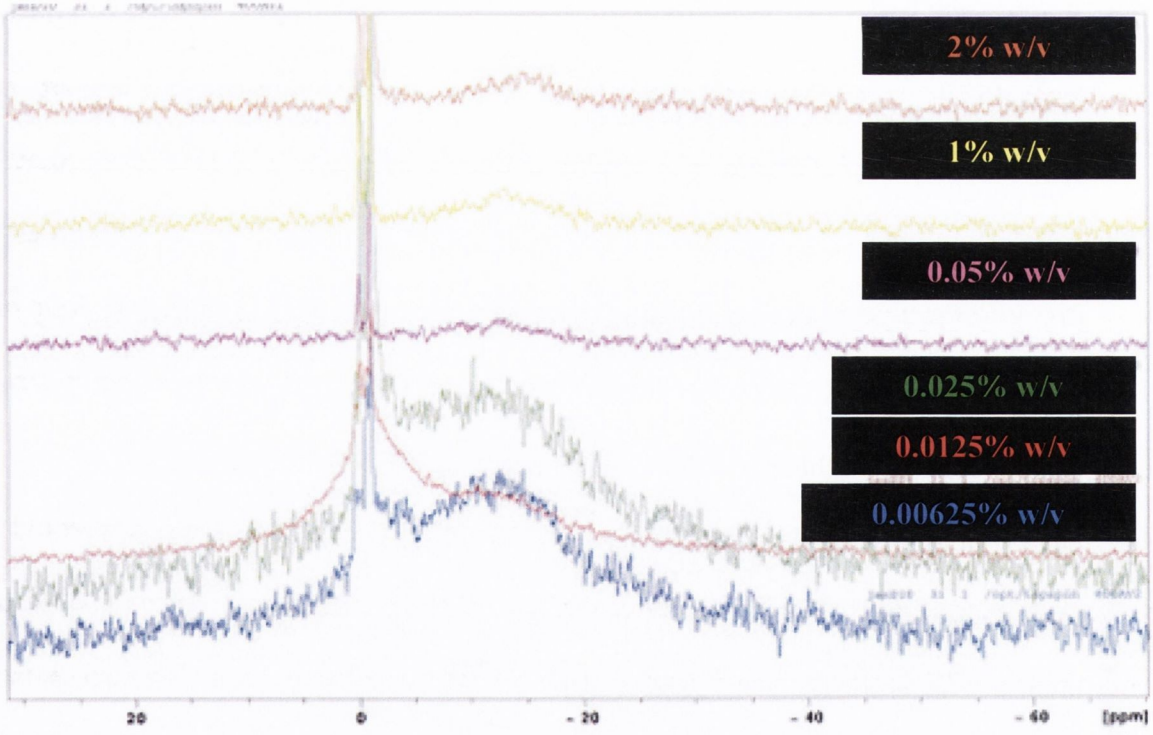


Figure 3.7. Representative ^{31}P-NMR spectra with varying concentrations of Triton-X 100. NMR spectra of DPPC:Chol:DSPE-PEG_{2000} (75:20:5) treated with varying concentrations of Triton-X 100

3.5 DISCUSSION

The purpose of our earlier work was to identify the optimal liposomal formulation for the delivery of salmon calcitonin. PEGylated liposomes were an optimal compromise between high encapsulation efficiency, sustained sCT release, and retained bioactivity of sCT.

Here, the objective was to assess if lyophilisation would be able to lead to an additional increase in the encapsulation efficiency of both PEGylated and non-PEGylated liposomes. In addition, the impact of different concentrations of trehalose di-hydrate and its presence in different compartments on encapsulation efficiency of sCT had to be investigated. Lastly, the reason behind the higher encapsulation efficiency observed for PEGylated liposomes in comparison to their plain counterparts had to be elucidated.

Preliminary studies suggested that the presence of trehalose di-hydrate in both internal compartment and external medium facilitated increased encapsulation of sCT especially in the SUV post gel filtration. This can be explained by a better protection afforded against the disruption of liposomal bilayer (in non-PEGylated liposomes) by the cryoprotectant during freezing and lyophilisation steps. Hence, all following formulations were prepared with trehalose di-hydrate in both internal compartment and external medium. (94)

The observed increase in particle size in the non-PEGylated batches could be attributed to the increased ability of the liposomal vesicles to fuse, which is overcome in the PEGylated formulations due to steric hindrance. (15) Enhanced encapsulation efficiency following lyophilisation can be described as the result of repacking of lipid bilayers and subsequent encapsulation of payload on reconstitution. This is in accordance with the observations by Zhang *et al.* who reported improved bilayer

permeability of freeze-dried liposomes with and without lyoprotectant upon rehydration. (86) This also explains why the cryoprotectant played only a minor role in increasing the encapsulation efficiency of non-PEGylated liposomes. The higher encapsulation values achieved with PEGylated liposomes can be explained by data obtained from ^{31}P-NMR spectroscopy. ^{31}P-NMR spectra suggested the presence of two distinct vesicle populations in PEGylated samples – micelles and liposomes. The big broad peak corresponded to the presence of liposomes due to their larger size and the sharp peak to micelles as they were several folds smaller in size. Micelle formation did not occur in non-PEGylated counterpart which was evident from the absence of sharp peak. The presence of micelles suggested an additional reason for increased encapsulation efficiency with PEGylated formulations apart from increased hydrophilicity observed with them. This is due to the fact that micelles were not removed by gel filtration that was performed to separate the non-encapsulated sCT. This micelle formation could be the result of insufficient mixing of the base lipid (DPPC) and PEG lipid (DSPE-PEG) due to different chain lengths causing expulsion of PEG molecules from the liposomes and formation of micelles. These micelles then encapsulated free sCT. Due to their size, the sCT micelles were not removed during gel filtration and hence lead to the allegedly higher encapsulation rate observed when PEG-lipids were included in the formulation. This phenomenon was also observed by Leal *et al.* (95)

On the addition of Triton-X 100 and with increasing concentrations, the liposomes disintegrated and more micelles were formed. This might have been the reason for the fading and disappearance of the broad liposome peak and replacement of a sharp micelle peak.

Lyophilisation enhanced the encapsulation efficiency of a sensitive payload without affecting its structure (since it was detected by RP-HPLC without any problem) which cannot be obtained by other conventional preparation methods. The lyophilised products reconstituted with ease in addition to retention of physicochemical properties of liposomes making this a preferred method over other conventional preparation methods. In case of PEGylated liposomes, formation of micelles was an added advantage leading to further increase in encapsulation efficiency.

3.6 CONCLUSIONS

Lyophilisation of liposomes caused an increase in encapsulation efficiency of salmon calcitonin in PEGylated formulations in general, and this increase was more pronounced in DPPC:Chol:DSPE-PEG_{2000} (65:30:5) liposomes in comparison to the other PEGylated and non-PEGylated counterparts. Presence of trehalose-di-hydrate did not cause an increase in encapsulation efficiency of non-PEGylated formulations. PEGylated formulations showed an increase in encapsulation efficiency even in the absence of cryoprotectant, though some cryoprotectant batches showed significantly high encapsulation efficiencies. Micelle formation was the reason behind high encapsulation efficiency observed in PEGylated liposomes. Change in encapsulation efficiency with increase in Chol concentration was insignificant. DPPC:Chol:DSPE-PEG2000 (75:20:5) was the best compromise between small particle size and high encapsulation efficiency. The next thesis chapter will provide an insight into its *in vitro* bioactivity and *in vivo* PK parameters.

4. sCT LIPOSOMES: RELEASE OF SCT FROM LIPOSOMES, *IN VITRO* AND *IN VIVO* STUDIES

Parts of this chapter will be submitted as:

J.Swaminathan, O. Gobbo, F. Tewes, C. Ehrhardt. sCT liposomes: Release of sCT from liposomes, *in vitro* and *in vivo* studies. *Eur J Pharm Biopharm*. Under preparation.

4.1 ABSTRACT

Purpose: Salmon calcitonin, a 32 amino acid polypeptide hormone has been marketed as intranasal and intravenous formulations for the treatment of osteoporosis, Paget's disease and hypercalcaemia. Liposomal formulations intended for pulmonary delivery have not been reported to date. Here we report *in vitro* release kinetics and bioactivity as well as *in vivo* pharmacokinetics (PK) following aerosol administration of liposomal formulations developed in Chapter 2 in rats.

Methods: PEGylated and non-PEGylated liposomal formulations which were stable to nebulisation were analysed for *in vitro* release kinetics in KRB (pH 7.4) at 37°C. Similar formulations were tested for bioactivity in a T47D cell-based scamp assay. Liposomal formulations used for *in vitro* analysis and bioactivity studies were hydrated with a loading buffer containing sCT at a concentration of 0.2 mg/ml. *In vivo* studies were performed on rats with sCT liposomes (hydrated using loading buffer containing sCT at a concentration of 0.5 mg/ml) were administered either by intratracheal nebulisation or intravenously at a sCT concentration of 50 μg/kg. Control rats were treated with sCT solution of the same dose and sCT was quantified by ELISA.

Results: PEGylated formulation released sCT at a slower rate compared to non-PEGylated formulation. Bioactivity studies revealed no change in bioactivity over a 24 h period from either formulation. The bioactivity was 52-55% in all cases. *In vivo*, following nebulisation of liposomes, sCT plasma concentration and pharmacokinetic parameters were not significantly different from that of sCT solution. Whilst following i.v. administration, liposomes showed significantly higher bioavailability and AUC_{inf} values when compared to sCT solution. Moreover, after 180 min, the plasma concentration of sCT following administration of liposomes was higher than

the minimum effective concentration of sCT which was not the case with sCT solution at the same time point.

Conclusions: Release of sCT was significantly retarded from the PEGylated formulation compared to its non-PEG counterpart. A 0.2 mg/ml sCT loading concentration was found to be more stable compared to 0.5 mg/ml. Both PEGylated liposome and its counterpart showed similar bioactivities. Following in vivo administration, liposomes were not optimal carriers for pulmonary administration of sCT which needs to be confirmed by extending the study to 180 minutes as with IV study.

4.2 INTRODUCTION

Targeted delivery of biopharmaceuticals and conventional drugs to the lungs using liposomes as carriers has been extensively investigated over the past decades. (25 & 28) Salmon calcitonin a 32-amino-acid peptide secreted by the C cells of the thyroid, is used in the clinic for the treatment of osteoporosis, Paget's disease and hypercalcaemia, and is currently marketed as injectable solutions (Calcimar®, Miacalcin®)as well as nasal sprays (Fortical®, Miacalcin®). (49, 69 & 5) Liposomes containing sCT for intranasal (65-68) and oral delivery (68, 59, 61-63) have been investigated, while we to the best of our knowledge present the first data on sCT liposomes for pulmonary delivery.

Release pattern of the peptide from liposomes, bioactivity of the peptide following *in vitro* release and *in vivo* pharmacokinetics are essential parameters for the success of any formulation. For oral sCT liposomes, *in vitro* studies performed by Song *et al.* (63 & 59) showed permeation enhancement of sCT across Caco-2 cell monolayers when sCT was formulated as proliposomes containing sodium taurodeoxycholate as

permeation enhancer. Measuring cyclic adenosine momophosphate (cAMP) levels in T47D cells (a breast cancer cell line that has sCT receptors) is an established protocol and has been reported by others in the context of characterising advanced delivery systems for sCT. (95-98) Retaining the bioactivity is paramount in the development of any controlled release system for peptide drugs, together with protecting the payload against degrading enzymes.

Chen *et al.* (67), Plessis *et al.* (68) and Law *et al.* (66) reported enhancement of PK of sCT following encapsulation into liposomes for intranasal administration in rats or rabbits. Yamabe *et al.* (61) and Ebato *et al.* (62) showed similar results for sCT liposomes following oral administration in rats.

Chapter 2 of this thesis discussed liposomal formulations that showed high encapsulation efficiency of sCT and that were stable to nebulisation making them suitable candidates for pulmonary delivery. The aim of this chapter is to report the release pattern of sCT *in vitro*, the bioactivity after encapsulation and release and the *in vivo* pharmacokinetics after aerosol delivery to rats.

4.3 MATERIALS AND METHODS

4.3.1 Materials

Lipids like DPPC and DSPE-PEG_{2000} were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA) while Chol, SA, glucose, HEPES, calcium chloride dihydrate, sodium dihydrogen phosphate, iron (III) chloride hexahydrate, ammonium thiocyanate, chloroform, acetonitirile, magnesium sulphate heptahydrate, RPMI 1640 with HEPES modification, l-glutamine, 3-isobutyl-1-methyl xanthine (IBMX), forskolin, non-essential amino acids and fetal calf serum were procured from Sigma-Aldrich (Dublin, Ireland). Sodium chloride, potassium chloride and sodium

bicarbonate were purchased from Fisher Scientific (Dublin, Ireland). Ultra pure water was used in all experiments and generated by a Millipore Synergy unit (Cork, Ireland). A Rotavapor R-210 rotary evaporator was used for lipid film preparation (Büchi, Flawil, Switzerland). The Lipex thermobarrel extruder and the poly carbonate filters used in the extrusion step were bought from Northern Lipids (Burnaby, British Columbia, Canada) and Whatman (Maidstone, UK), respectively. Econo-pac columns with an exclusion limit of 6,000 Da used for gel filtration were purchased from Alpha Technologies (Wicklow, Ireland). Float-a-lyzer dialysis bags and sCT ELISA kits used for release studies were obtained from Spectrum labs (Breda, The Netherlands) and Bachem (Weil am Rhein, Germany), respectively. The ELISA kits used for cAMP estimation in bioactivity assessment were procured from R&D systems (Abingdon, United Kingdom). Ketamine/Domitor mixture (Vetalar®/Domitor®) used to anaesthetise rats and atipamezole hydrochloride (Antisedan®) used to reverse sedation in the *in vivo* experiments were procured from Pfizer (Dublin, Ireland). Heparinised Solocath™ catheters were purchased from Linton Instrumentation (Palgrave Norfolk, UK).Intratracheal administration of sCT liposomes was achieved using an AeroProbe™ intracorporeal nebulising catheter controlled by a LabNeb™ unit (Trudell Medical International, London, UK). Particle size, polydispersity index (PdI) and ζ-potential were measured using a ZetasizerNanoseries ZS (Malvern Instruments, Malvern, UK). The concentration of sCT was determined using a Shimadzu HPLC (Kyoto, Japan), comprising a LC-10AT pump, auto sampler SIL-10AD, degasser DGU-14A, UV-Vis detector SPD-10A and system controller SCL-10A. Jones chromatography (Cardiff, UK) reverse phase C-18 column, 150×4.6mm, with 4-μm packing was used.

4.3.2 Methods

4.3.2.1 Liposome preparation

Liposomes were prepared by the lipid film hydration method. Briefly, lipids were dissolved in chloroform and subsequently, the solvent was removed using a rotary evaporator to obtain a dry lipid film. This film was then hydrated with bicarbonated Krebs-Ringer buffer of relevant pH value, which contained sCT at a concentration of 0.2 mg/ml for *in vitro* sCT bioactivity and 0.5 mg/ml for *in vivo* studies, to a final lipid concentration of 10 μmol/ml. The multi lamellar vesicles thus obtained were subjected to size reduction using a thermobarrel extruder. Polycarbonate filters of pore sizes ranging from 100 to 400 nm were used to generate small unilamellar vesicles. Hydration and extrusion were carried out either at room temperature or 42-43°C, depending on the T_g of the lipids used in the formulation. Non-encapsulated sCT was removed by gel filtration on pre-packed columns.

4.3.2.2 Physicochemical characterisation

All formulations were analysed for their particle size, PdI and the particle surface charge (ζ-potential). Disposable low volume cuvettes were used for particle size determination, whereas disposable capillary cells were used for ζ-potential measurements. Samples for physicochemical characterisation were prepared by diluting liposomal suspensions with KRB (pH 7.4). The measurements were based on dynamic light scattering at 173° backscatter detection. Each experiment was repeated in triplicates from 3 independent batches (n = 9).

4.3.2.3 HPLC method for determination of sCT content

The concentration of salmon calcitonin in liposome samples was determined by RP-HPLC. This method was used primarily for encapsulation efficiency determination and had a limit of detection of 3 μg/ml. An isocratic elution method was used with a flow rate of 1 ml/min. The mobile phase was prepared by mixing 660 ml ultra pure water, 340 ml acetonitrile, 1.16 g sodium chloride and 320 µltrifluoroacetic acid. UV absorption of sCT was detected from 190 to 340 nm, and the actual spectrophotometric measurement carried out at 215 nm. Each HPLC result was the average of 2 independent measurements from 3 liposome batches (n = 6).

4.3.2.4 Encapsulation efficiency

Samples were prepared by addition of Triton X-100 to liposomal suspensions such that the final concentration of the detergent in the sample was 1%. The samples were then incubated at 37°C for 30 min, before being subjected to HPLC analysis. The phospholipid content was determined using Stewart's assay. (76) Each value of Stewart's assay was the average of 3 individual measurements from 3 independent liposome batches (n = 9).

Encapsulation efficiency following gel filtration and following nebulisation was calculated using the following equations

$$\frac{(\mu\text{g sCT}/\mu\text{mol total lipid}) \text{ after gel filtration} \cdot 100}{(\mu\text{g sCT}/\mu\text{mol total lipid}) \text{ before gel filtration}} \qquad \text{(Eq. 1)}$$

Encapsulation efficiency was the average of 2 individual determinations from three independent batches (n = 6).

4.3.2.5 In vitro sCT release

Release studies were carried out to determine the impact of PEGylation on the release profile of the peptide. The three formulations investigated were thus DPPC:Chol:SA:DSPE-PEG_{2000} (55:20:20:5) at 0.5 mg/ml sCT concentration in loading buffer, DPPC:Chol:DSPE-PEG_{2000} (75:20:5) at 0.5 mg/ml and 0.2 mg/ml sCT concentrations in loading buffer and DPPC:Chol (80:20) at 0.2 mg/ml sCTconcentration in loading buffer. Release of sCT was determined using Float-a-lyzer dialysis bags containing 1 ml of liposomal suspension in the donor compartment. The receiver compartment constituted 50 ml of KRB (pH 7.4; 37°C) in a 100-ml volumetric cylinder, maintained under constant magnetic stirring at 100 rpm. Twenty-five millilitre samples were withdrawn serially from the receiver compartment after 1 and 24 h. The sample volume was each time replaced with fresh pre-warmed KRB. In addition, 450 µl samples were taken from the donor compartment at 0 h and 24 h to determine the initial concentration as well as to test for mass balance at the end of the study. Salmon calcitonin concentration was analysed by competitive ELISA according to the manufacturer's instructions. Since the sCT content in the samples was much lower than the limit of detection of RP-HPLC, ELISA was used to determine the sCT content in release study samples. Samples were diluted with KRB when appropriate. Release data were obtained using liposomes from 3 different batches (n = 6-9).

4.3.2.6 Assessment of sCT bioactivity

T47D human breast cancer cells expressing calcitonin receptors were maintained in RPMI-1640 medium modified with 25 mM HEPES and supplemented with 1% v/v penicillin–streptomycin, 10% v/v foetal bovine serum (FBS), 0.2 IU/ml insulin, 1%

v/v of non-essential amino acids and 2% v/v of l-glutamine (200 mM). (85) Cells were seeded on 24 well plates at an initial density of 1.0×10^6 cells/well and incubated in air with 5% of CO_2 at 37°C for 2 days. After washing with serum free RPMI-1640 on the second day, cells were pre-incubated with the same medium supplemented with the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (IBMX, 200 μM) at 37°C for 30 min on the third day. The cells were then incubated for 15 min with 0.4 ml of sCT liposome release study samples, sCT solution, positive and negative controls dissolved in serum free RPMI-1640 plus IBMX. The sCT concentration was 10^{-9} M, which was close to the EC_{50} values of sCT. Positive control constituted 10^{-5} M forskolin in RPMI-1640 medium containing IBMX and negative control was RPMI-1640 medium containing IBMX. The cells were detached with 0.4 ml of trypsin-EDTA and 0.5 ml of cold phosphate buffered saline (PBS) was added to stop the reaction. The cells were then centrifuged at 10,000 rpm for 7 min at 4°C. Supernatant was removed and the pellet was washed thrice with 0.3 ml cold PBS. After removing the supernatant, the intracellular cAMP was extracted from the cells by incubating them in cell lysis buffer and measured using a cAMP ELISA kit according to the manufacturer's protocol. Liposomal formulations studied were DPPC:Chol:DSPE-PEG_{2000} (75:20:5) and DPPC:Chol (80:20). This study was performed with three independent liposomal formulations of each type analysing three samples from each formulation each in duplicate (n = 18).

4.3.2.7 Pharmacokinetics of sCT liposomes

The experimental protocols employed in this study were approved by the Animal Research Ethics Committee, Trinity College Dublin, and the Department of Health and Children (Ireland), in accordance with the European Community Directive

(86/609). The experiments were carried out using male Wistar rats supplied by BioResources, TCD, Ireland, with a body weight of 350±50 g. The animals were housed in a thermo regulated environment with a 12-h light/dark cycle. Food and water were available *ad libitum*. Rats were anaesthetized by an intraperitoneal (*i.p.*) injection of Ketamine-Domitor mixture before a heparinised catheter was installed into the left femoral artery for blood sampling. The left femoral vein was cannulated for sCT solution administration. Liposomal formulations and sCT solution were administered intravenously or intratracheally. Intravenously a dose of sCT corresponding to 50μg/kg was injected as bolus dose. Nebulisation was performed using an AeroProbe™ intracorporeal nebulising catheter for intratracheal administration of liposomal formulations. The dose of sCT analysed was 50 μg/kg. After drug administrations, the sedation was reversed by an injection of atipamezole hydrochloride and blood sampling was carried out in freely moving animals. Arterial blood samples (200 μl) were collected in heparinised tubes at the following times: 0; 3; 10; 20; 30; 60; 90; 120; 180 min post sCT administration. Blood samples were centrifuged at 10000 rcf for 5 min. Plasma was withdrawn and kept at -20°C. Samples were assayed using a high-sensitive sCT ELISA kit according to manufacturer's specifications. Concentration was calculated by interpolation from a standard curve and assay performance was controlled with quality control samples.

Liposomal formulations investigated were DPPC:Chol:DSPE-PEG_{2000} (75:20:5) by parenteral route and DPPC:Chol:SA:DSPE-PEG_{2000} by nebulisation (55:20:20:5). This study was performed using two (parenteral) or three (nebulisation) independent liposomal formulations that were tested on six rats altogether in each case. (n = 6)

Pharmacokinetic parameters were determined for each individual rat using a two-compartment model. sCT plasma concentration (C_p) versus time profiles were fitted with Eq. 2 using the least-squares method (Excel, Microsoft, USA).

$$C_p = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-Kat} \quad \text{(Eq. 2)}$$

with $\alpha > \beta$, and $C = 0$ for profile fitting after iv administration, and $C = -(A + B)$ for profile fitting after pulmonary administration. The distribution and elimination phases of C_p versus time profiles were characterized by the half-life times $t_{1/2_\alpha} = \ln 2/\alpha$ and $t_{1/2_\beta} = \ln 2/\beta$, respectively. Area under C_p versus time profiles (AUC) were calculated using the linear trapezoidal rule. The area remaining after the last measured concentration ($C_{t\rightarrow 180}$) was extrapolated using Eq 3.

$$AUC_{t\rightarrow\infty} = \frac{C_{t\rightarrow\infty}}{\beta} \quad \text{(Eq. 3)}$$

Bioavailability (F) was calculated by comparison of the sCT AUC extrapolated to infinity obtained after pulmonary administration to that obtained after iv administration of the same dose as shown in Eq. 4.

$$F = \frac{AUC^{pulm}{}_{t\rightarrow\infty}}{AUC^{iv}{}_{t\rightarrow\infty}} \quad \text{(Eq. 4)}$$

The total body clearance of sCT (CL) was calculated as the ratio between the dose of sCT (dose_ F) and the corresponding AUC.

4.4 RESULTS

4.4.1 Physicochemical characterisation and encapsulation efficiency of *in vitro* and *in vivo* liposomal formulations

The physicochemical parameters particle size, PdI and ζ-potential and encapsulation efficiency of *in vitro* and *in vivo* formulations are represented in Table 4.1. In both groups DPPC:Chol:DSPE-PEG_{2000} (75:20:5) liposomes showed smaller particle size

and PdI with higher encapsulation efficiency of sCT, compared to their counterparts. When two different loading concentrations of sCT (0.2 mg/ml versus 0.5 mg/ml) in DPPC:Chol:DSPE-PEG_{2000} (75:20:5) formulation were compared, both showed similar physicochemical parameters and encapsulation efficiency. The change in physicochemical parameters and encapsulation efficiency following increase in sCT concentration in loading buffer was insignificant from DPPC:Chol:DSPE-PEG_{2000} (75:20:5) batches.

Table 4.1. Physicochemical parameters and encapsulation efficiency of *in vitro* and *in vivo* batches. Data are presented as mean ± SD (n = 9 for physicochemical parameters and n = 6 for encapsulation efficiency)

Formulation investigated	Particle size (nm)	PdI	Z-potential (mV)	Encapsulation efficiency (%)
***In vitro* formulations (0.2 mg/ml sCT in loading buffer)**				
DPPC:Chol:DSPE-PEG_{2000} (75:20:5)	156.6 ± 9.6	0.10 ± 0.07	-0.17 ± 0.47	50.5 ± 1.8
DPPC:Chol (80:20)	218.4 ± 14.3	0.34 ± 0.04	4.92 ± 0.63	36.2 ± 2.1
***In vivo* formulations (0.5 mg/ml sCT in loading buffer)**				
DPPC:Chol:SA:DSPE-PEG_{2000} (55:20:20:5)	190.3 ± 38.4	0.14 ± 0.04	-1.38 ± 2.14	38.9 ± 2.5
DPPC:Chol:DSPE-PEG_{2000} (75:20:5)	135.5 ± 3.3	0.07 ± 0.01	-0.25 ± 1.32	49.9 ± 3.4

4.4.2 *In vitro* sCT release

Formulations for the bioactivity assay (0.2 mg/ml sCT)

Release profiles of sCT from DPPC:Chol (80:20) and DPPC:Chol:DSPE-PEG_{2000} (75:20:5) liposomes are shown in Figure 4.1. Albeit no statistical significance was obtained ($*P \leq 0.05$), there was a clear tendency for sCT release from non-PEGylated liposomes to be faster and more complete, when compared to PEG-liposomes.

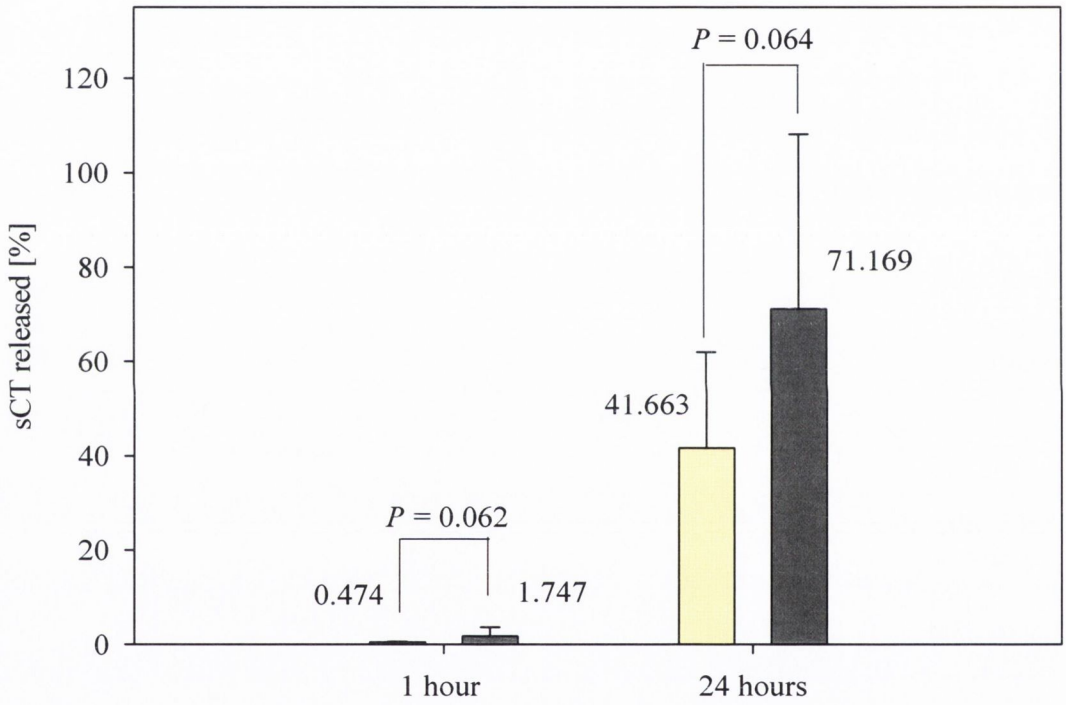


Figure 4.1. Release of sCT from liposomal formulations *in vitro*. Release of sCT from DPPC:Chol (80:20; grey bars) and DPPC:Chol:DSPE-PEG_{2000} (75:20:5; yellow bars) liposomes is shown after 1 h and 24 h. Data are presented as mean ± SD (n = 8-9).

Formulations for the pharmacokinetic study (0.5 mg/ml sCT)

Release profiles of sCT from DPPC:Chol:SA:DSPE-PEG_{2000} (55:20:20:5) and DPPC:Chol:DSPE-PEG_{2000} (75:20:5) liposomes are shown in Figure 4.2. The profiles

were quite similar with the non-SA formulation showing a slightly retarded sCT release.

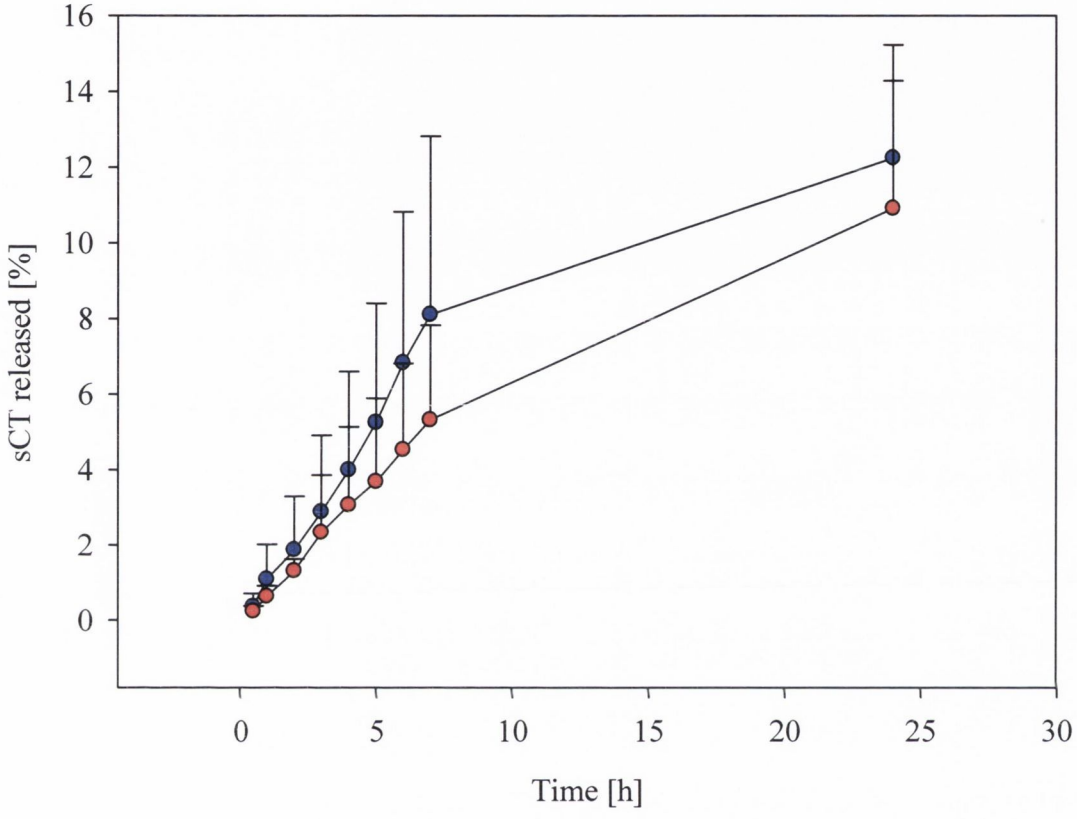


Figure 4.2. Release profile of sCT from liposomal formulations. Release of sCT from DPPC:Chol:SA:DSPE-PEG_{2000} (55:20:20:5; blue circles) and DPPC:Chol:DSPE-PEG_{2000} (75:20:5; red circles) is shown. Data are presented as mean ± SD (n = 6-9)

Comparison of release profiles of DPPC:Chol:DSPE-PEG_{2000} (75:20:5) at different sCT loading concentrations

The cumulative amount of sCT released (Figure 4.3) was similar in both loading concentrations at both time intervals considered while the percentage sCT release (Figure 4.4) was significantly ($*P \leq 0.05$) lower with the higher payload concentration.

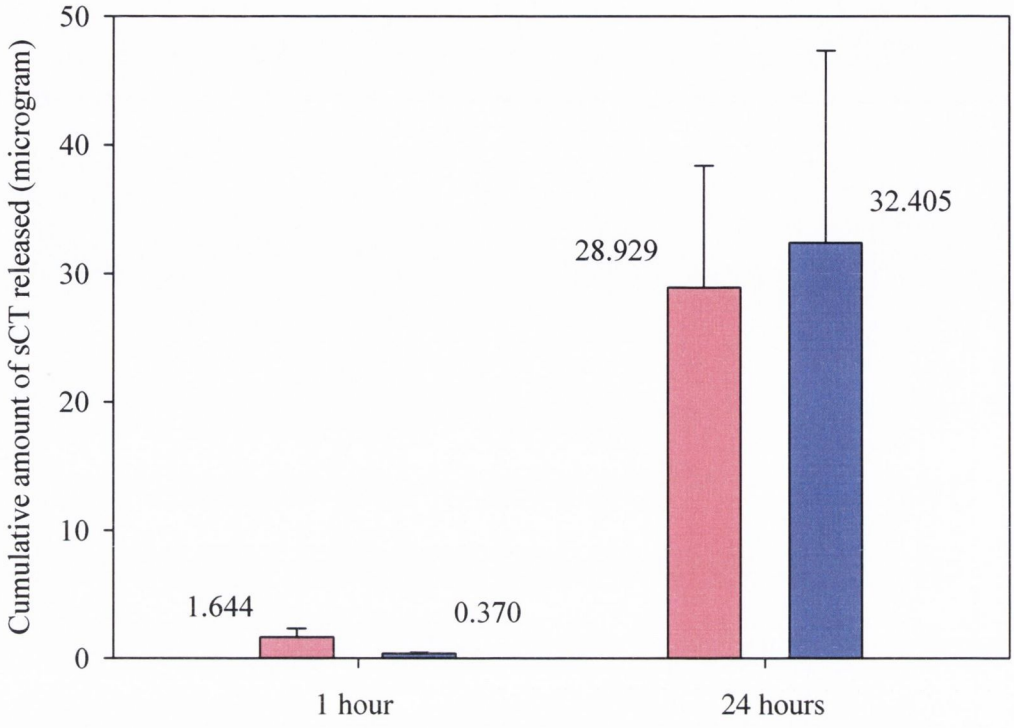


Figure 4.3. Cumulative amount of sCT released from DPPC:Chol:DSPE-PEG$_{2000}$ (75:20:5) liposomes with different initial sCT concentrations. The cumulative amount of sCT released when initial sCT concentrations were 0.5 mg/ml (pink bars) and 0.2 mg/ml (blue bars) at 1 h and 24 h. Data presented as mean ± SD (n = 6-9).

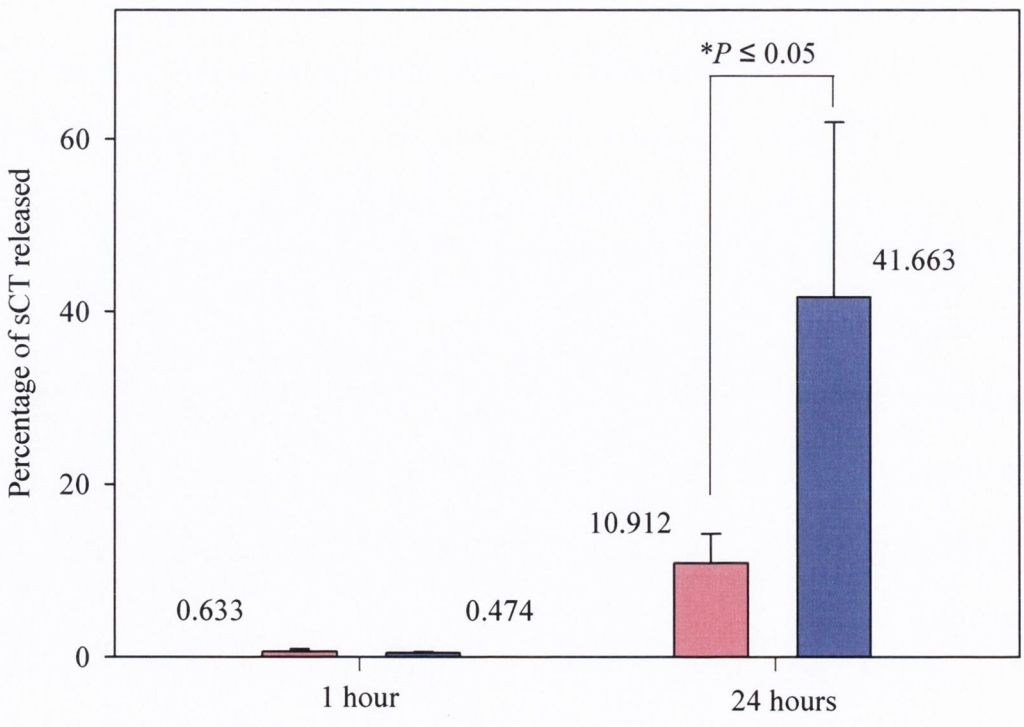


Figure 4.4. Percentage of sCT released from DPPC:Chol:DSPE-PEG$_{2000}$ (75:20:5) liposomes with different initial sCT concentrations. Percentage of sCT released when the initial sCT concentration was 0.5 mg/ml (pink bars) and 0.2 mg/ml (blue bars) at 1 h and 24 h. Data presented as mean ± SD (n = 6-9).

4.4.3 sCT bioactivity *in vitro*

The bioactivity of DPPC:Chol:DSPE-PEG$_{2000}$ (75:20:5) and its non-PEG counterpart, DPPC:Chol (80:20) at 1 h and 24 h are shown in Figure 4.5. No significant ($^*P \leq 0.05$) difference in bioactivity was noticed with either the formulations or time points.

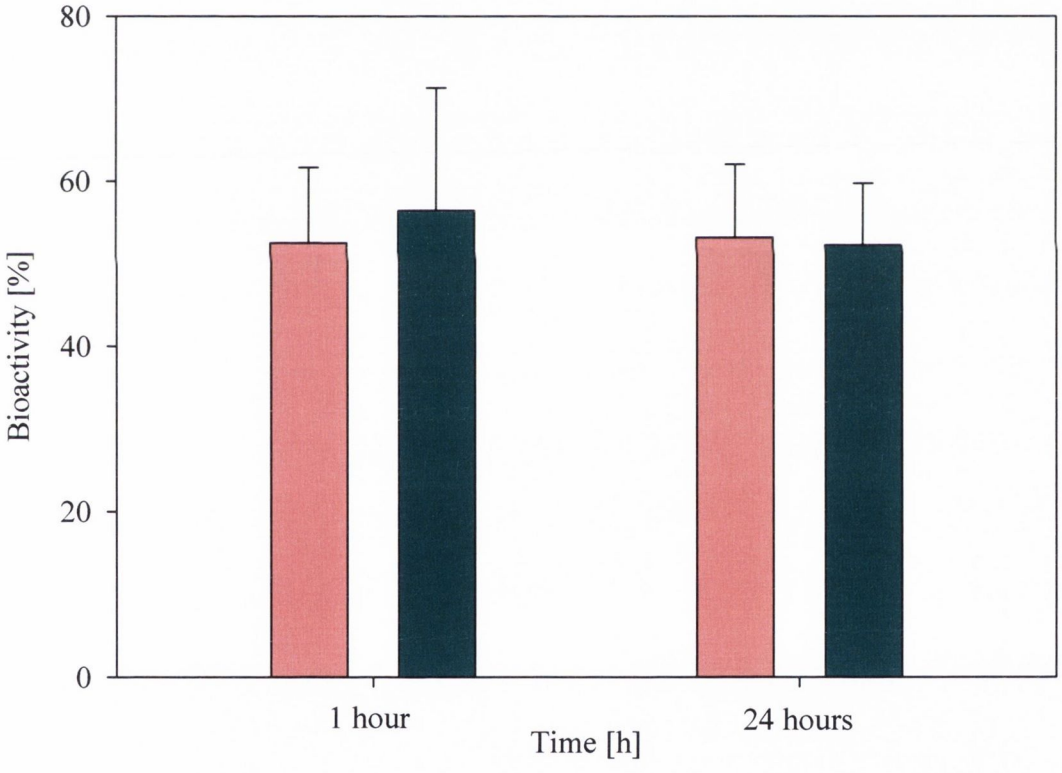


Figure 4.5. Bioactivity of liposomal formulations in T47D cells. The bioactivity of samples obtained following the *in vitro* release from liposomal formulations adjusted to 10^{-9} M sCT was investigated in T47D cells. Coral bars represent DPPC:Chol:DSPE-PEG$_{2000}$ (75:20:5) and cyan bars represent DPPC:Chol (80:20). Data are presented as mean ± SD (n = 12).

4.4.4 *In vivo* study

The sCT plasma concentration-time profile following nebulisation of DPPC:Chol:SA:DSPE-PEG_{2000} (55:20:20:5) liposomes and sCT solution is represented in Figure 4.6 and in Appendix figures A.16 and A.17. Corresponding PK parameters are shown in Table 4.2. The PK profiles (Figure 4.6) showed an initial short absorption phase with a T_{max} of 10.50±5.43 min and 15.5±9.67 min for sCT liposome and sCT solution, respectively. K_a values of both formulations were also comparable, as were distribution and elimination phases. A detectable plasma concentration was prevalent only until 60 min after administration regardless of formulation administered.

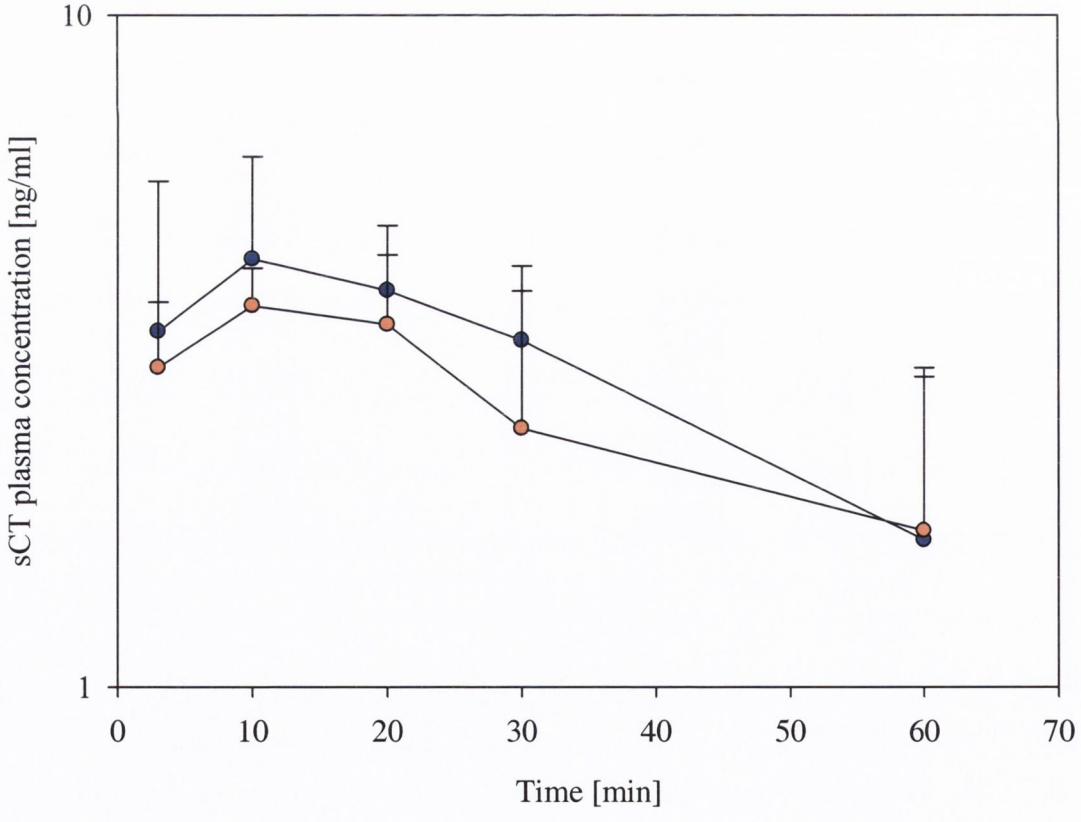


Figure 4.6. Comparison of sCT plasma profiles following nebulisation of the peptide either encapsulated into liposomes or as solution. sCT concentration in plasma following administration of liposomal formulation (orange circles) and the sCT solution (blue circles). Data represented as mean ± SD (n = 6)

Table 4.2. Pharmacokinetic parameters of *in vivo* experiments. PK parameters (AUC_{inf}– area under the curve to infinity time point, K_{el} – elimination rate constant, Cl_{inf} – clearance to infinity time point, C_{max} – maximum plasma concentration, T_{max} – time taken to reach maximum plasma concentration, $t_{1/2}$ – half life and K_a – absorption rate constant) of DPPC:Chol:SA:DSPE-PEG_{2000} (55:20:20:5) liposomes containing sCT and sCT solution following nebulisation.

Parameter	Nebulised sCT liposomes	Nebulised sCT solution
Absolute bioavailability (%)	4.63 ± 1.47	5.36 ± 3.21
Relative bioavailability (%)	83.6 ± 35.1	NA
AUC_{inf} (min.ng/ml/μg.kg)	5.50 ± 1.76	6.06 ± 3.70
K_{el} (min^{-1})	0.03 ± 0.01	0.04 ± 0.01
Cl_{inf} (ml/min/kg)	14.73 ± 3.14	11.76 ± 0.93
C_{max} (ng/ml)	3.70 ± 0.55	4.83 ± 1.57
T_{max} (min)	10.50 ± 5.43	15.5 ± 9.67
$t_{1/2}$ (min)	40.87	40.39
K_a (min^{-1})	0.35 ± 0.12	0.31 ± 0.14

Comparative PK profiles following intravenous administration of sCT encapsulated DPPC:Chol:DSPE-PEG_{2000} (75:20:5) liposome and sCT solution in rats are represented in Figure 4.7 and their corresponding single PK profiles are shown in Appendix figures A.18 and A.19 with PK parameters shown in Table 4.3.

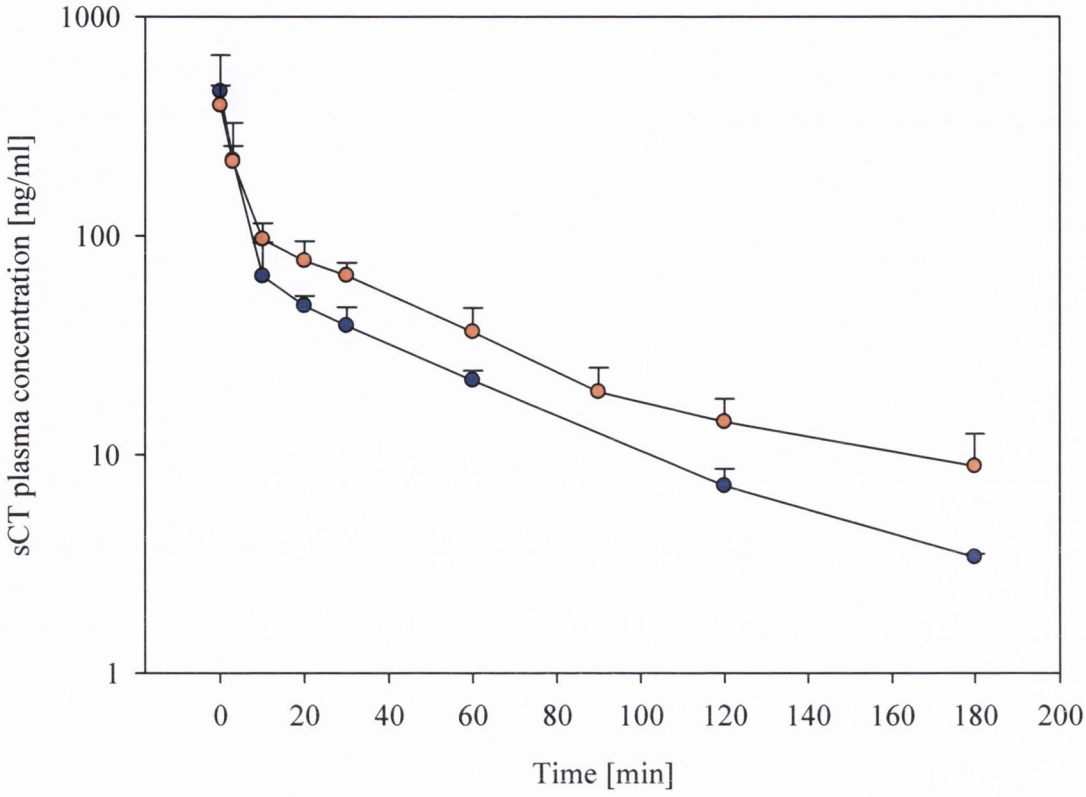


Figure 4.7. Comparison of sCT plasma profiles following intravenous injection of the peptide either encapsulated into liposomes or as solution. Rats were administered liposomal formulation (orange circles) or sCT solution (blue circles). Data represented as mean ± SD (n = 4-6).

The profiles for both sCT solution and liposomes looked similar and were marked by the presence of two phases – distribution and elimination. The distribution phase extended until 10 min post administration, succeeded by the elimination phase. From 10 to 90 min, both profiles showed identical slopes. After 90 min, the profiles looked different and had different slopes with the encapsulated sCT being eliminated slower. In general, liposomes showed higher plasma sCT concentration at all experimental time points (except T_{max}) compared to the solution. Following parenteral administration, sCT liposomes showed significantly higher bioavailability and AUC_{inf} values compared to sCT solution (Table 4.3).

Table 4.3. Pharmacokinetic parameters of *in vivo* experiments. PK parameters (AUC_{inf}– area under the curve to infinity time point, K_{el} – elimination rate constant, Cl_{inf} – clearance to infinity time point, C_{max} – maximum plasma concentration, T_{max} – time taken to reach maximum plasma concentration, $t_{1/2}$ α and $t_{1/2}$ β – half lives) of DPPC:Chol:DSPE-PEG_{2000} (75:20:5) liposomes and sCT solution following intravenous administration.

Parameter	Parenteral sCT liposomes	Parenteral sCT solution
Absolute bioavailability (%)	125.81 ± 29.55	100
AUC_{inf} (min.ng/ml/μg.kg)	139.66 ± 23.76	115.25 ± 27.84
K_{el} (min^{-1})	0.06 ± 0.01	0.09 ± 0.01
Cl_{inf} (ml/min/kg)	7.91 ± 1.30	9.40 ± 1.96
C_{max} (ng/ml)	393.15 ± 91.91	454.97 ± 211.78
T_{max} (min)	0	0
$t_{1/2}$ (min)	45.77 ± 8.53	43.01 ± 1.42

4.5 DISCUSSION

The intent of this work was to compare the sCT release profiles, *in vitro* bioactivity and *in vivo* performance of the two most promising formulations from the previous study. The formulations were DPPC:Chol:SA:DSPE-PEG_{2000} (55:20:20:5) and DPPC:Chol:DSPE-PEG_{2000} (75:20:5). Albeit both PEGylated formulations show similar physiochemical parameters, encapsulation efficiency was significantly ($P \leq 0.05$) higher for the formulation without SA. This can be explained by the higher DPPC concentration leading to higher membrane rigidity and thus better retention of an amphiphilic payload like sCT. (78 & 79) Since DPPC:Chol:DSPE-PEG_{2000} (75:20:5) liposomes showed better encapsulation efficiency, *in vitro* bioactivity of that formulation in was studied in T47D cells and its performance was compared to its non-PEG counterpart, DPPC:Chol (80:20). Finally, the pharmacokinetics of both PEGylated liposomal formulations were studied *in vivo*. In this context, two different

routes of administration were studied (i.e. i.t. and i.v.) and sCT solution was used as control in both cases. As this study was performed in parallel with lyophilisation study explained in Chapter 3, the PK of the first best formulation [DPPC:Chol:SA:DSPE-PEG_{2000} (55:20:20:5)] was checked following nebulisation. When this work was in progress, another formulation [DPPC:Chol:DSPE-PEG_{2000} (75:20:5)] showed higher encapsulation efficiency compared to the former whose PK was studied following intravenous administration in rats.

Considering the *in vitro* sCT release from the formulations, PEGylation resulted in more pronounced sCT retention compared to the plain liposomes. This is not surprising as PEG molecules are protruding from the internal and external surfaces of the liposome and their massive size leads to steric hindrance making them responsible for reduced release and thus increased retention of sCT. (99 & 100) Nearly 14-28% of the starting material could not be accounted for in the case of PEGylated formulation and 23-37% in the case of non-PEGylated formulation at the end of the 24 h *in vitro* release experiment. This was likely caused by the inherent instability of sCT in aqueous buffers at 37°C. (101) In addition, the sodium content of the release buffer and stirring of the medium will have further enhanced degradation of the peptide. (102) Higher sCT loss associated with non-PEGylated liposomes could be due to higher sCT release in them (may be an amount of sCT higher than what is reported here was released) and the part of sCT released initially aggregated due to the factors mentioned earlier which caused it to be undetectable by any of the analytical techniques. Increased sCT release after the first hour approaching statistically significant limits could be indicative of this.

Formulations intended for the *in vivo* study had a higher loading (i.e., 0.5 mg/ml) and showed a significantly ($*P \leq 0.05$) lower rate of release compared to the formulations

studied *in vitro* which contained 0.2 mg/ml of sCT. The slightly higher release from the SA-formulation was due to lower membrane rigidity due to lesser percentage of DPPC in the same. Since sCT is amphiphilic payload (78, 79) that interacted with lipid membranes to form channels (103), formulation with higher percentage of DPPC retained it better. The significant ($*P \leq 0.05$) difference in percentage sCT release between the different loading concentrations in the DPPC:Chol:DSPE-PEG_{2000} (75:20:5) formulation could be due to instability of sCT at higher concentrations which might cause aggregates that no longer can be released from liposomes in addition to not being detected by the analytical techniques employed.

Reduction in bioactivity at both time points and in both formulations could be attributed to sCT degradation that occurred during the release studies. Since the release study samples were used for bioactivity determination, a loss in bioactivity could be contemplated with presence of aggregates in the release study sample that might not have been bioactive when added to cells. Additional reason could be due to dilution of sCT (to 10^{-9} M) stored under specified storage conditions as comparator/control. If sCT solution of similar concentration as that of the liposome had been subject to release studies at the same conditions over the same duration and samples thus obtained had been analysed, different results could have been obtained. However this method was not feasible due to absence of due to absence of previous reports on the use of this method. The protocol followed in my thesis was a previously established protocol. (95-98)

Liposomes did not show a prolonged release of the payload *in vivo* following nebulisation and intravenous administration. Earlier studies by Anabousi *et al.* have confirmed the integrity of PEGylated DPPC-based liposomes in artificial lung surfactant for a period of 48 h. (45) This suggests that the liposomes stay intact for the

experimental period and release the drug very slowly. The decrease in sCT plasma concentration following nebulisation of sCT liposomes (compared to nebulised sCT solution) could be the result of peptide degradation by catabolising enzymes in the pulmonary epithelial lining fluid. There could also be a possibility of extended absorption happening post initial absorption phase suggesting slower elimination of sCT from liposome. On comparison of K_{el} values of sCT solution post nebulisation and intravenous administration, the K_{el} post nebulisation (0.04 ± 0.01 min^{-1}) is lesser than that following intravenous administration (0.09 ± 0.01 min^{-1}). This further suggests slower elimination and the possibility of absorption of sCT happening following nebulisation. However data from more time points need to be considered to further confirm this and since the sCT plasma concentration was lesser than the limit of detection of ELISA, data from samples collected at 90, 120 and 180 minute time points could not be analysed. Another issue with ELISA results was the inability to differentiate between the released sCT and encapsulated sCT in the plasma samples obtained. The wash buffer used in ELISA contains a surfactant that could disrupt the liposomes leading to estimation of toal sCT at each time point rather than the sCT released from the liposome.

Following intravenous administration, the increase in sCT plasma concentration from liposomes (compared to parenteral sCT solution) could be due to better ability of PEGylated liposome to protect and release sCT over time in comparison to administration of sCT solution. In the initial elimination phase (10 – 90 min), the elimination of sCT was faster since the sCT that was released post preparation from the liposomes was readily available for distribution and elimination. Over time (90 – 180 min), elimination of sCT from liposomes reached a steadier rate as sCT was released from the liposome slowly for being distributed and eliminated. The K_{el} value

of liposome (0.06 ± 0.01 min^{-1}) was lesser than that obtained with sCT solution (0.09 ± 0.01 min^{-1}) following i.v. administration confirming slower elimination of sCT following administration of sCT liposomes. This phenomenon was not observed with the sCT solution which showed a single continuous elimination phase. The minimum effective concentration of sCT is 3.4 ng/ml. (104) Liposomes were above the minimum effective concentration level at the end of 180 min (8.859±3.599 ng/ml), while the corresponding plasma concentration following i.v. administration of solution was 3.396±0.127 ng/ml, which was lower than the MEC of sCT suggesting liposomes to be better carriers for sCT following i.v. administration compared to sCT solution.

A significant increase in bioavailability was noticed following parenteral administration of liposomes, suggesting improved PK of sCT liposomes following this administration route compared to nebulisation. DPPC-based PEGylated liposomes proved as good carriers for sCT administration after parenteral administration. Research findings of other groups that worked on sCT liposomes are as follows. Research by Chen *et al.* is contradictory to our work that showed a 22.3±2.4% reduction in serum calcium levels following nasal administration of non-PEGylated SPC based sCT liposomes in rats. (67) Plessis *et al.* worked on Pheroid vesicles that are liposome-like bilayer vesicles and reported a 3.6 and 1.5 fold increases in C_{max} following nasal and oral administration of sCT Pheroid vesicles compared to respective controls (sCT solutions) in rats. (68) Law *et al.* reported a 2.8 fold increase in bioavailability following administration of DOPC based positively charged liposomes at a sCT concentration of 2.5 μg/kg sCT in rabbits. (66) The above mentioned research works suggested nasal mucosa to be more permeable for sCT administration. Studies on oral sCT double liposomes by Yamabe *et al.* and Ebato *et*

al. suggested the cationic charge of the inner liposome to be favourable in sCT absorption. (61 & 62) The above studies could suggest that liposomes might not be the best carriers for sCT for the administration routes investigated due to small improvements in C_{max} or bioavailability following encapsulation. This could be due to the fact that sCT is amphiphilic and penetrates to the external medium via the lipid vesicle. Even liposomes with very rigid membrane (prepared with disteraoylphosphatidylcholine or with DPPC and high concentration of cholesterol) are not able to retain sCT effectively.

4.6 CONCLUSIONS

PEGylation of liposomes increased encapsulation efficiency and prolonged *in vitro* release of sCT. The concentration of sCT that is stable for formulation of liposomes is 0.2 mg/ml. *In vitro* studies showed a decrease in bioactivity which is a consequence of degradation of sCT when release studies were performed. *In vivo*, sCT liposome showed better PK following intravenous administration compared to nebulisation however this could be confirmed by extending the nebulisation study till 180 minutes. Protocol of a method for analysing the nebulisation study plasma samples post 60 minutes needs to be set for understanding the PK following nebulisation of sCT liposomes in rats.

5. CONCLUSIONS

Though much remains unexplored with regards to the pulmonary delivery of protein and peptide drugs, the presented work provided novel insights on the potential of liposomes as a carrier for salmon calcitonin, in terms of the stability of the pay load, its release pattern, stability following nebulisation, sustainment of bioactivity and pharmacokinetic performance following aerosolisation into rat lungs *in vivo*.

One major drawback of pulmonary delivery of proteins and peptides is the actual amount of pay load that reaches deep lung following inhalation. Subsequent to this, the amount of drug that can actually pass the pulmonary epithelial barrier to reach the systemic circulation in bioactive form is unanswered yet.

One drawback of pulmonary delivery of proteins and peptides is the poor understanding of the fate of the drug following inhalation (71). Research carried out by some groups suggests that the primary source of elimination of macromolecules post inhalation and before absorption into systemic circulation is clearance by alveolar macrophages (106 & 107). Other work from our group demonstrated that the enzymatic activity in the lungs might further reduce sCT concentration before absorption. (108 & 109). These and other parameters are critical when trying to understand the PK/PD of pulmonary administered peptides, however this area has been little explored to date.

In the present work, if liposomes as carriers for pulmonary delivery of salmon calcitonin could potentially improve the performance of this peptide drug was investigated. Liposomes were chosen as drug carriers based on the fact that they can

be formulated to be biocompatible in the lung and offer sustained release properties for a large number of drugs, including peptides (27).

Initially, a number of preliminary experiments were performed to determine the best method of separation of non-encapsulated drug from liposome formulations. Among the different methods employed, gel filtration using pre-packed columns with an exclusion limit of 6,000 Da was considered the best in terms of reproducibility of results especially with an amphiphilic pay load like sCT. Further to this, the absence of dimers in laboratory working conditions was ascertained, since sCT is known to form dimers at neutral pH, presence of high concentration of the peptide and exposure to high temperature (80, 81 & 82). Following this, a formulation development study was performed during which liposomes with a rigid membrane and positive surface charge showed better encapsulation efficiency of sCT compared to their counterparts that possessed a fluid membrane, neutral or negative surface charge. Though encapsulation efficiency was higher with higher pH values of loading buffers, pH value of 3.5 was chosen to facilitate sCT stability (75). On PEGylation the formulations zeta-potential became neutral due to steric hindrance of PEG groups in addition to a significantly ($*P \leq 0.05$) higher encapsulation of sCT.

Nebulisation stability of PEGylated formulations and their non-PEG counterparts was assessed which revealed that PEGylated formulations were less stable to nebulisation. This was believed to be related to the large size of PEG molecule making the liposomes more prone to damage and release of pay load during nebulisation.

Another study performed simultaneously was the lyophilisation of liposomes to analyse, if this had a positive impact on the encapsulation efficiency. Following lyophilisation, PEGylated liposomes showed an increase in encapsulation efficiency in the absence of a cryoprotectant (trehalose-di-hydrate) and in the presence of the same at 1:5 lipid:cryoprotectant ratio. Non-PEG vesicles did not show an increase. This was explained following analysis of the samples by ^{31}P-NMR spectroscopy, which revealed micelle formation in PEGylated liposomes that did not occur in non-PEGylated liposomes. This led to increase in surface area and subsequent encapsulation of sCT.

Further to nebulisation stability and lyophilisation study, the release pattern of sCT from liposomes *in vitro* in KRB of pH value 7.4 was studied. The non-PEGylated liposomes showed significantly higher release at the end of 24 hours compared to PEGylated liposomes due to steric hindrance caused by PEG molecules that hindered sCT release. Apart from that, no mass balance of sCT was observed at the end of the study with higher quantity of sCT not being able to be accounted for from non-PEGylated liposomes. Higher sCT loss associated with non-PEGylated liposomes could be due to higher sCT release and subsequent aggregation due unsuitable pH value of the buffer (pH 7.4), high temperature (37°C) and shaking conditions (100 rpm) (101).

Since two different concentrations of sCT in loading buffer were worked with (0.2 mg/ml for all *in vitro* studies and 0.5 mg/ml in the *in vivo* studies), there arose a necessity for comparison of the amount of sCT and percentage of sCT released from the optimal liposome formulation (in terms of small particle size, high encapsulation

efficiency, good nebulisation stability, controlled release of sCT and significantly higher AUC following *in vivo* study) thus far [i.e., DPPC:Chol:DSPE-PEG_{2000} (75:20:5)] with the two loading concentrations. Intriguingly, the amount of sCT released was the same in both cases, while the percentage released was significantly lower with the higher concentration, suggesting that sCT could have aggregated or denatured and was not detected by any of the analytical methods (RP-HPLC and ELISA).

Bioactivity studies revealed only 50% bioactivity regardless of the type of formulation (PEGylated/non-PEGylated) in question. This could have been the outcome of using release study samples which already showed aggregation and degradation of sCT. The aggregated sCT was not bioactive on cells. *In vivo* studies demonstrated that sCT liposomes showed higher AUC and bioavailability compared to solution, following intravenous administration compared to pulmonary administration which was translated in the form of higher plasma concentration of sCT from liposome compared to sCT solution. The decreased sCT plasma concentration from liposomes following pulmonary administration in comparison to sCT solution might be due to sCT degradation by lung catabolising enzymes or lung macrophages. *In vivo*, sCT liposome showed better PK following intravenous administration compared to nebulisation however this could be confirmed by extending the nebulisation study till 180 minutes. Protocol of a method for analysing the nebulisation study plasma samples post 60 minutes needs to be set for understanding the PK following nebulisation of sCT liposomes in rats.

Though liposomal formulations that were stable to nebulisation that showed prolonged sCT release kinetics *in vitro* and excellent encapsulation efficiency of sCT following lyophilisation were prepared, their PK parameters following nebulisation to the lung could not be completely assessed due to incompetency of analysing method.

From the work carried out in the context of this thesis and other data obtained by our group over the past years, it may be concluded that liposomes are not the optimal carriers for the pulmonary delivery of salmon calcitonin. A comparatively high loss of biological activity and poor encapsulation efficiency compared to other delivery systems (i.e., polymeric microparticles) are one reason; the slow release from the vesicles paired with a surprisingly high enzymatic activity in the target organ another. Conclusively, no further efforts will be made to follow this line of investigations.

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APPENDIX

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2. ABBREVIATIONS 153

A1. FIGURES

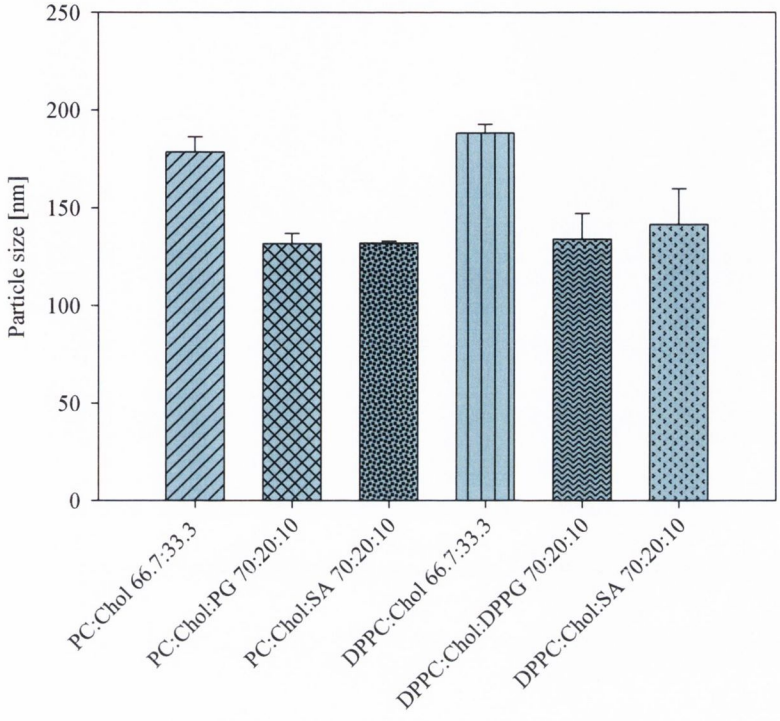


Figure A.1: Particle size of fluid/rigid membrane and neutral/negative/positive surface charged liposomes at pH value of loading buffer being 3.5

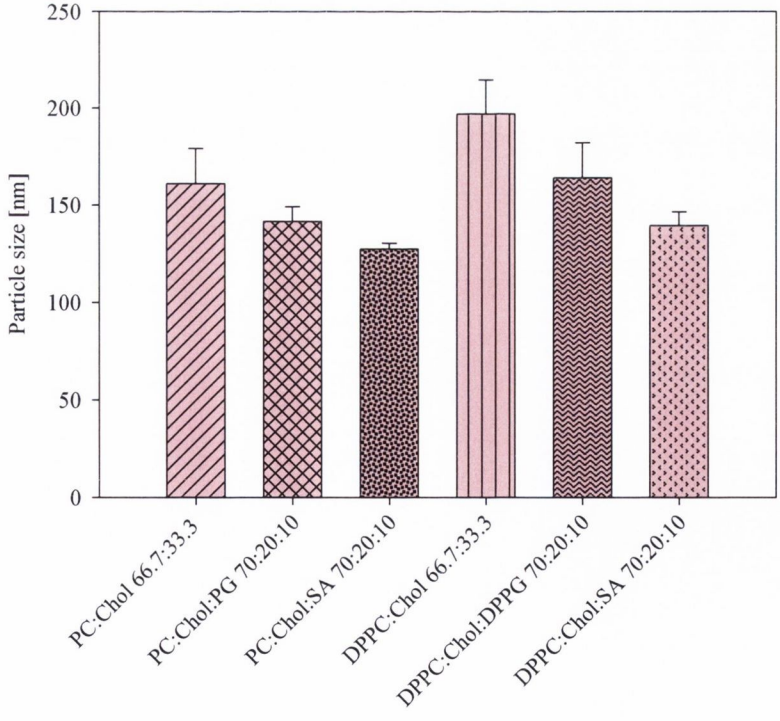


Figure A.2: Particle size of fluid/rigid membrane and neutral/negative/positive surface charged liposomes at pH value of loading buffer being 5.0

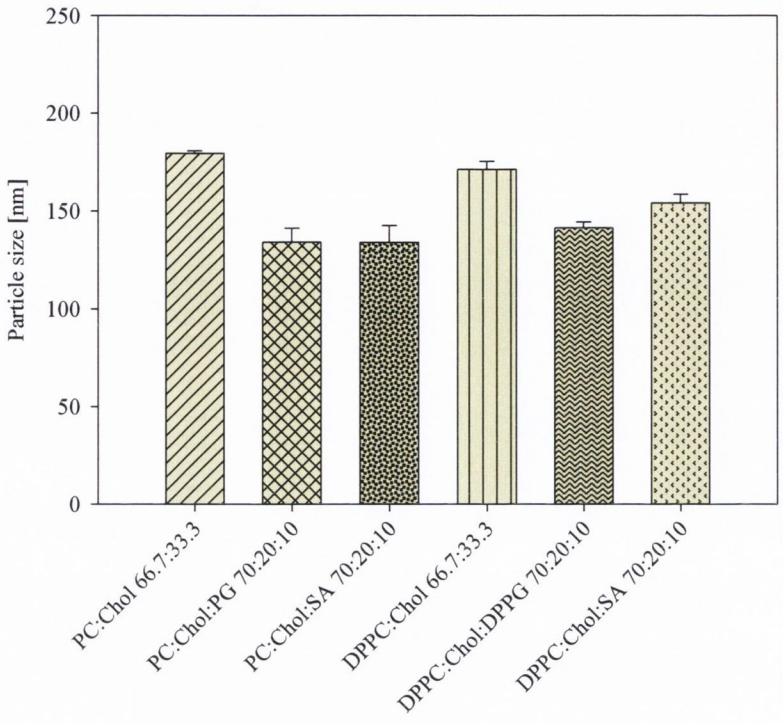


Figure A.3: Particle size of fluid/rigid membrane and neutral/negative/positive surface charged liposomes at pH value of loading buffer being 7.0

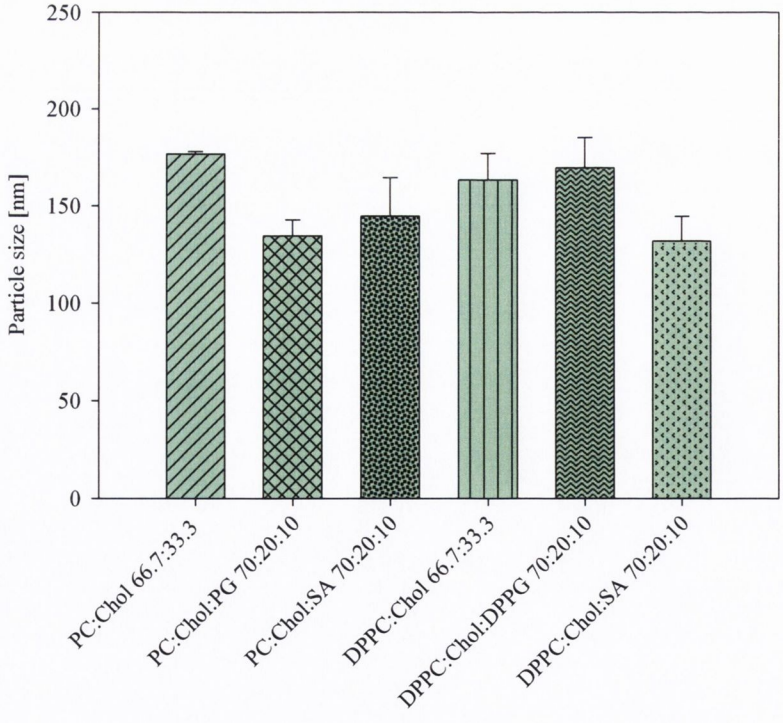


Figure A.4: Particle size of fluid/rigid membrane and neutral/negative/positive surface charged liposomes at pH value of loading buffer being 8.5

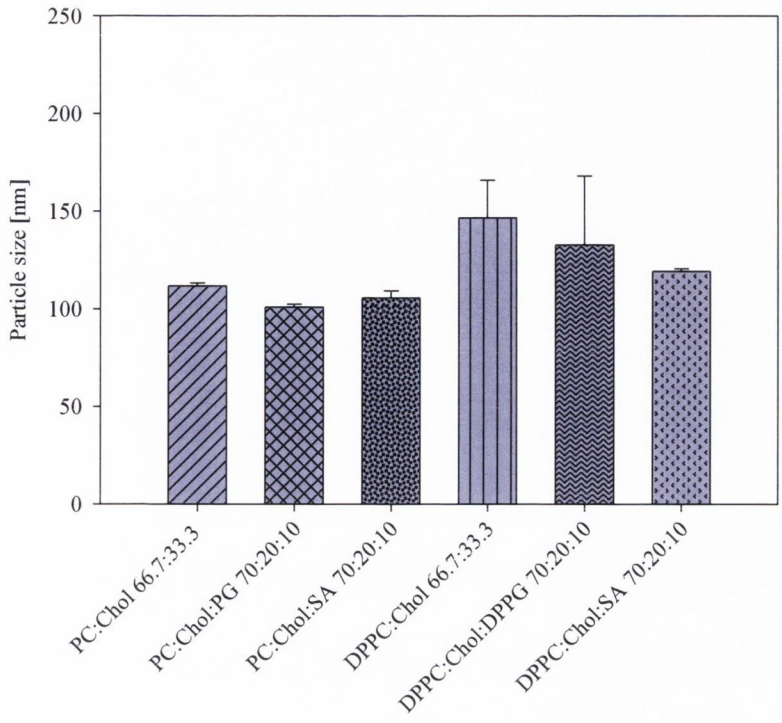


Figure A.5: Particle size of fluid/rigid membrane and neutral/negative/positive surface charged liposomes at pH value of loading buffer being 10.0

Comparison of particle size of formulations: grouping based on pH of loading buffer: In the above graphs, liposomes with neutral surface charge (irrespective of membrane fluidity) show greater particle size compared to their counterparts with negative and positive surface charge. There is no significant difference in particle size between the fluid and rigid membrane counterparts bearing all three surface charges.

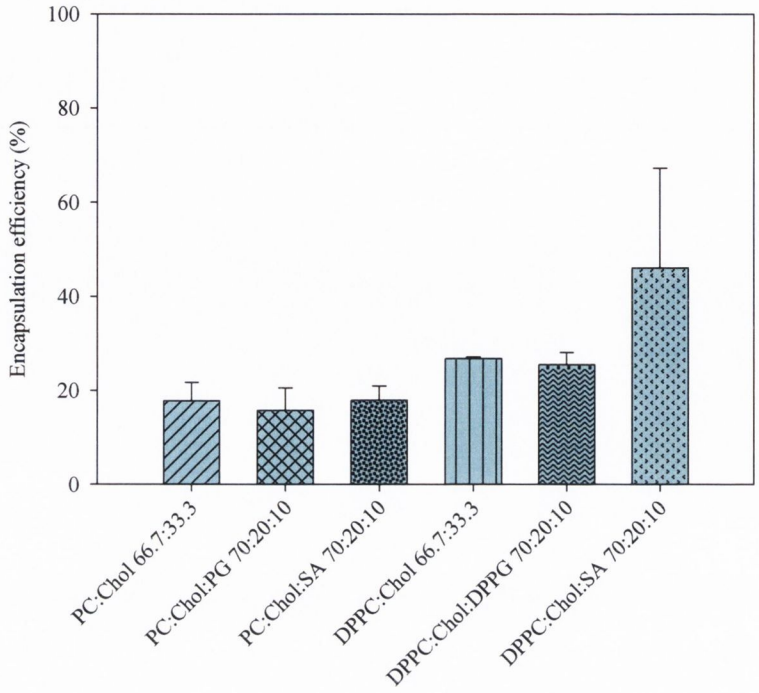


Figure A.6: Encapsulation efficiency of fluid/rigid membrane and neutral/ negative/positive surface charged liposomes at pH value of loading buffer being 3.5

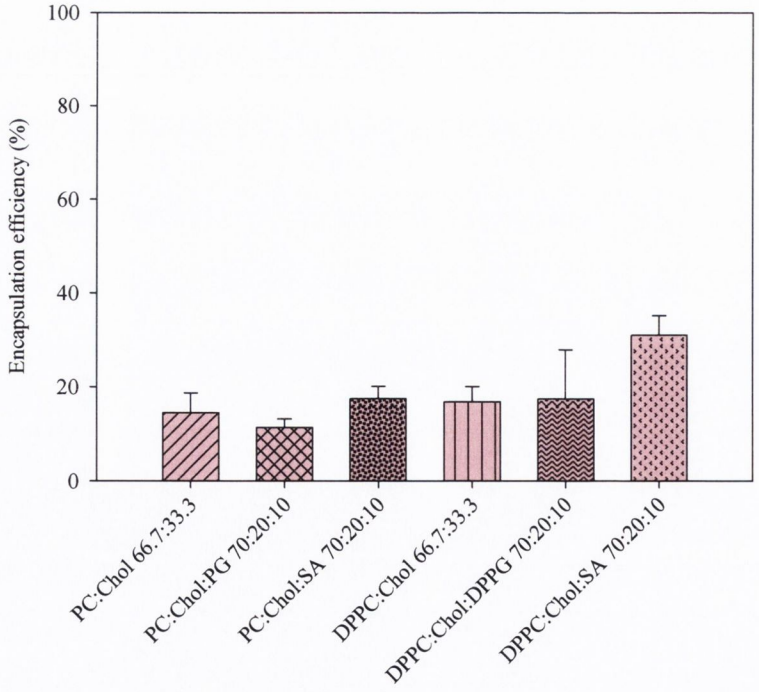


Figure A.7: Encapsulation efficiency of fluid/rigid membrane and neutral/negative/ positive surface charged liposomes at pH value of loading buffer being 5.0

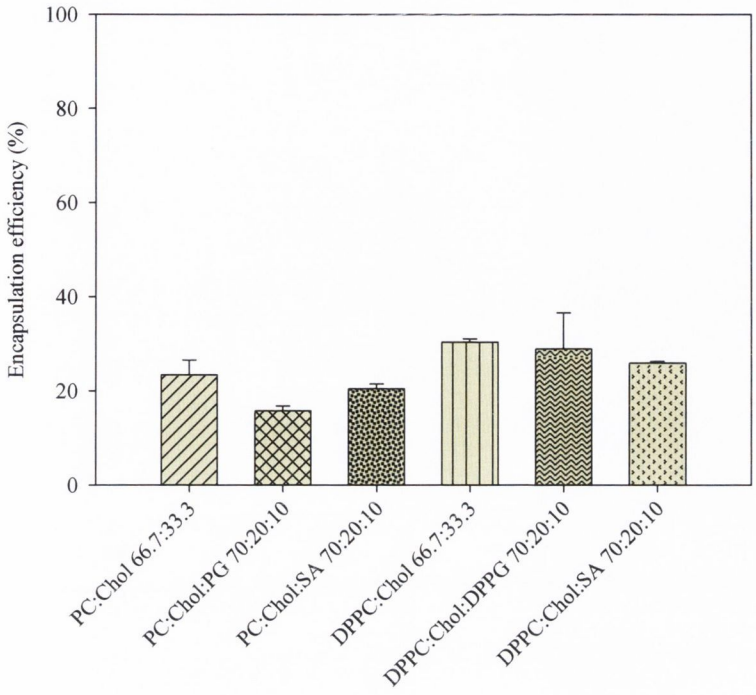


Figure A.8: Encapsulation efficiency of fluid/rigid membrane and neutral/ negative/ positive surface charged liposomes at pH value of loading buffer being 7.0

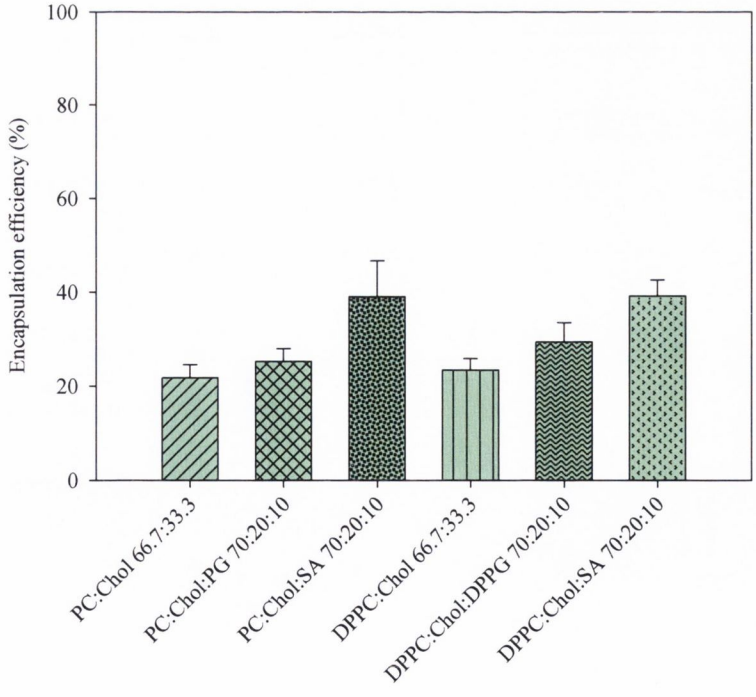


Figure A.9: Encapsulation efficiency of fluid/rigid membrane and neutral/negative/ positive surface charged liposomes at pH value of loading buffer being 8.5

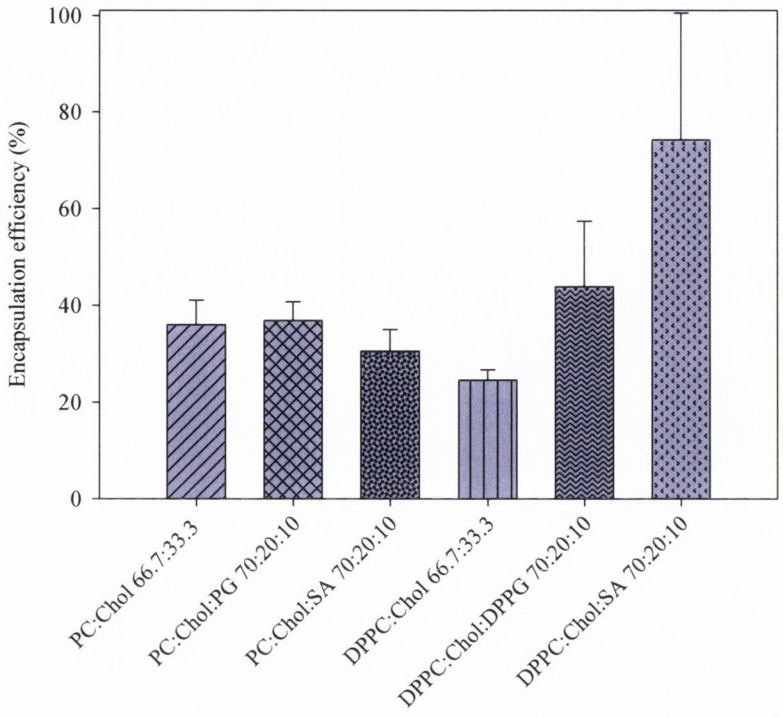


Figure A.10: Encapsulation efficiency of fluid/rigid membrane and neutral /negative/positive surface charged liposomes at pH value of loading buffer being 10.0

Comparison of encapsulation efficiency of formulations: grouping based on pH of loading buffer: In the above graphs, liposomes with positive surface charge showed higher encapsulation of sCT compared to neutral and negative counterparts regardless of the nature of membrane rigidity. Among the liposomes with positive surface charge at each pH, the rigid formulations showed better encapsulation efficiency compared to their fluid counterparts. Formulation with positive surface charge and rigid membrane [DPPC:Chol:SA (70:20:10)] showed highest encapsulation efficiency in all pH's of loading buffer.

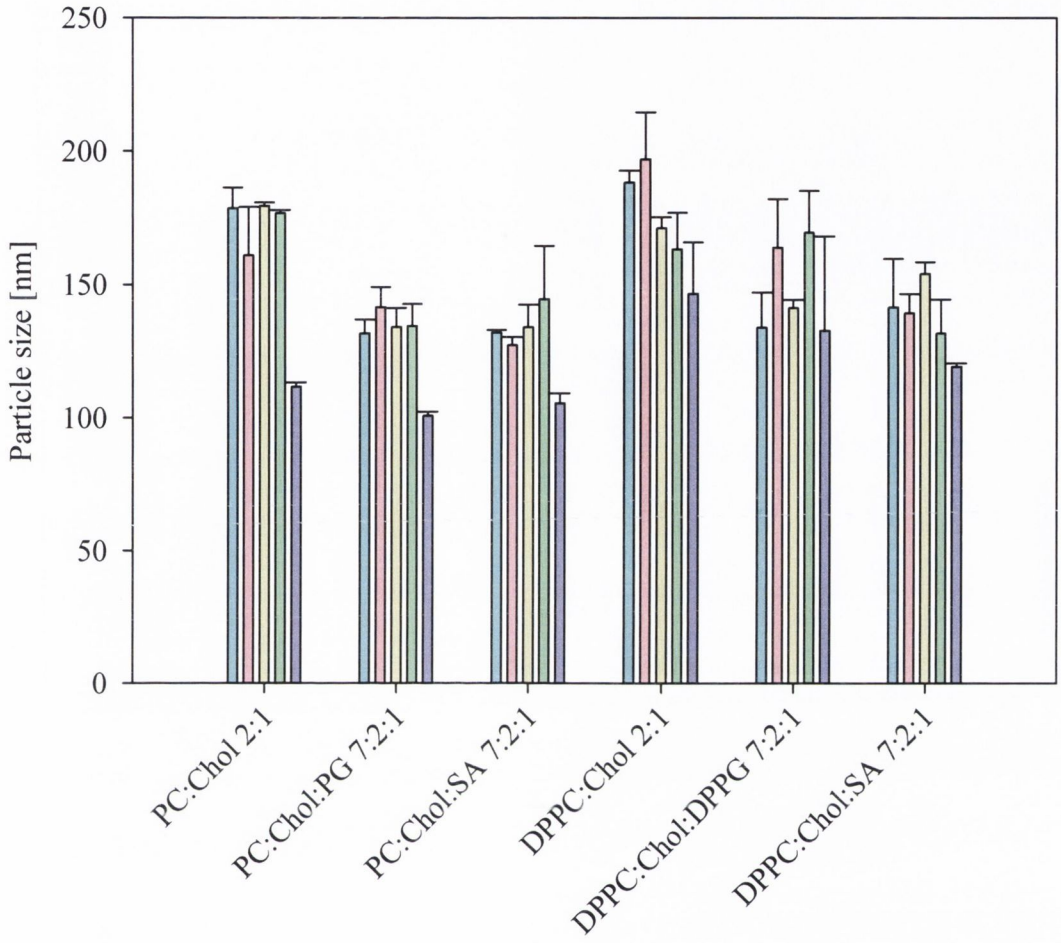


Figure A.11: Particle size of rigid/fluid formulations with neutral/negative/positive surface charges at all pH's: grouping based on formulations: Aqua bars represent pH value of 3.0, baby pink bars represent pH value of 5.0, light yellow bars represent pH value of 7.0, pale green bars represent pH value of 8.5 and lavender bars represent pH value of 10.0. No significant difference in particle size of the same formulation at different pH's.

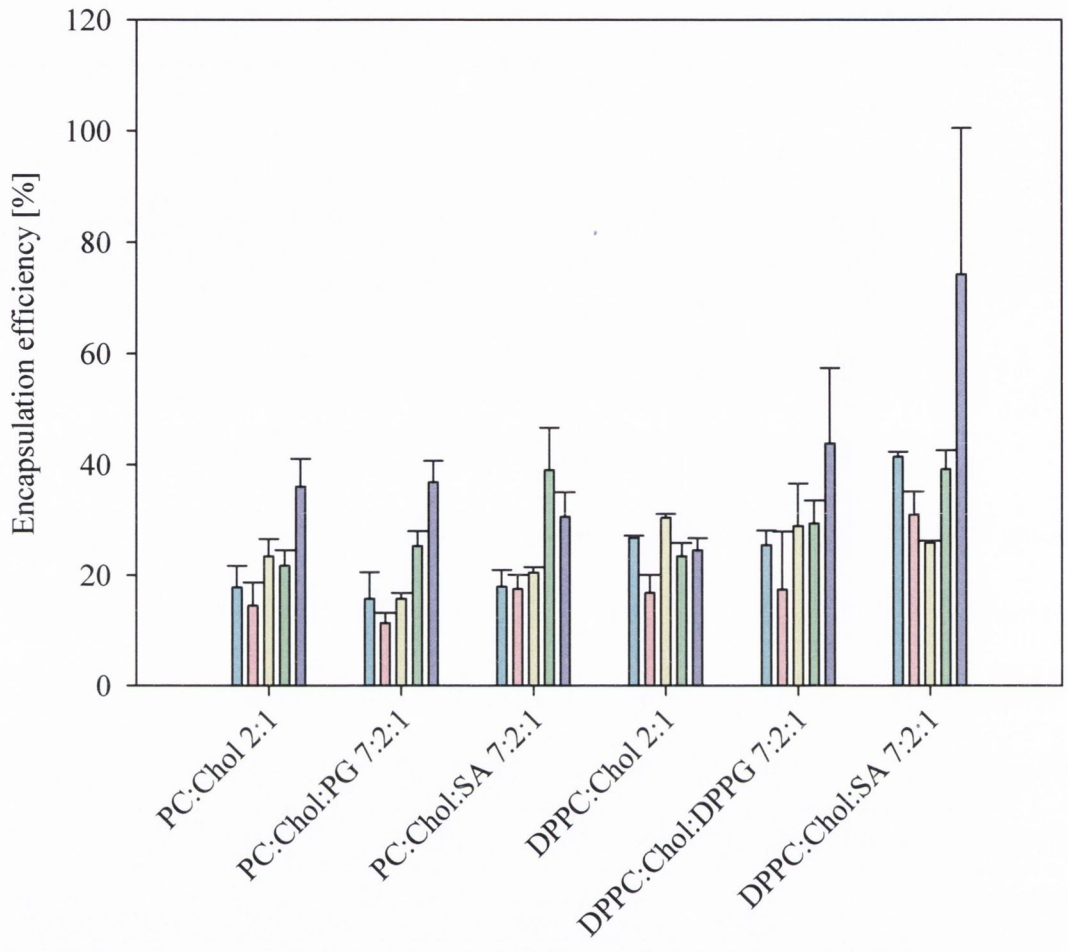


Figure A.12: Encapsulation efficiency of rigid/fluid formulations with neutral/negative/positive surface charges at all pH's: grouping based on formulations: Aqua bars represent pH value of 3.0, baby pink bars represent pH value of 5.0, light yellow bars represent pH value of 7.0, pale green bars represent pH value of 8.5 and lavender bars represent pH value of 10.0. All formulations (with an exception of PC:Chol:SA and DPPC:Chol) showed highest encapsulation efficiency when the pH value of the loading buffer was 10.0.

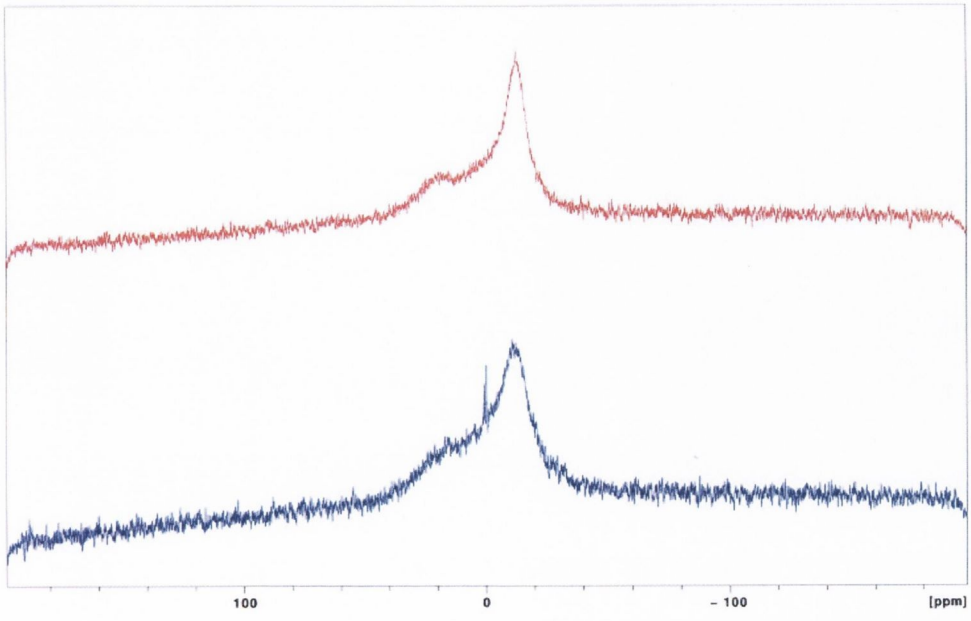


Figure A.13: Individual ^{31}P - NMR spectra of PEGylated and non-PEGylated liposomes.

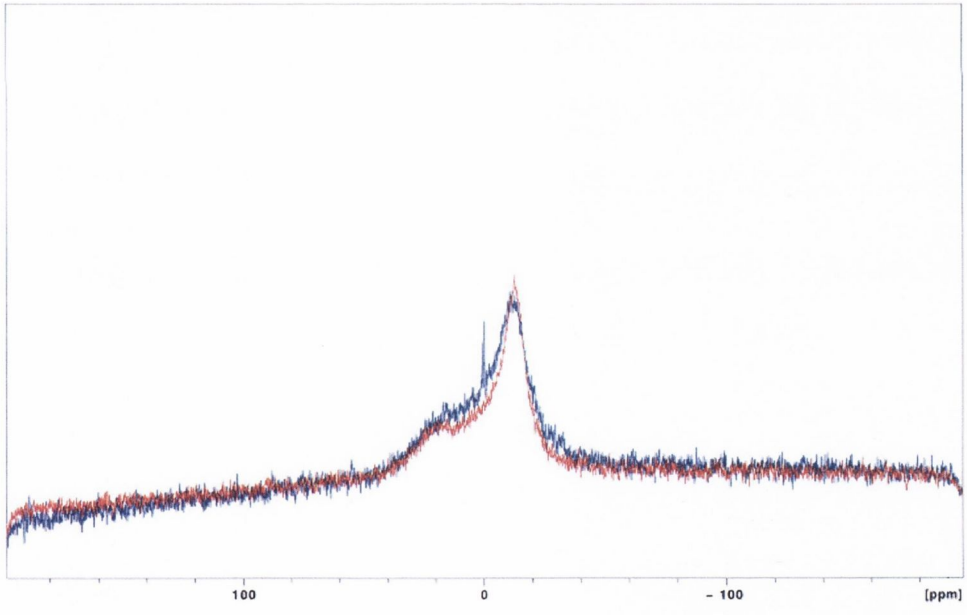


Figure A.14: Overlapping ^{31}P – NMR spectra of PEGylated and non-PEGylated liposomes.

The above graphs represent ^{31}P - NMR spectra of DPPC:Chol:DSPE-PEG_{2000} (75:20:5; blue) and DPPC:Chol (80:20; red) liposomes showed the presence of a broad peak at -10 ppm indicating the presence of liposomes in both formulations while the sharp peak at 5 ppm in PEGylated formulation indicated the presence of micelles.

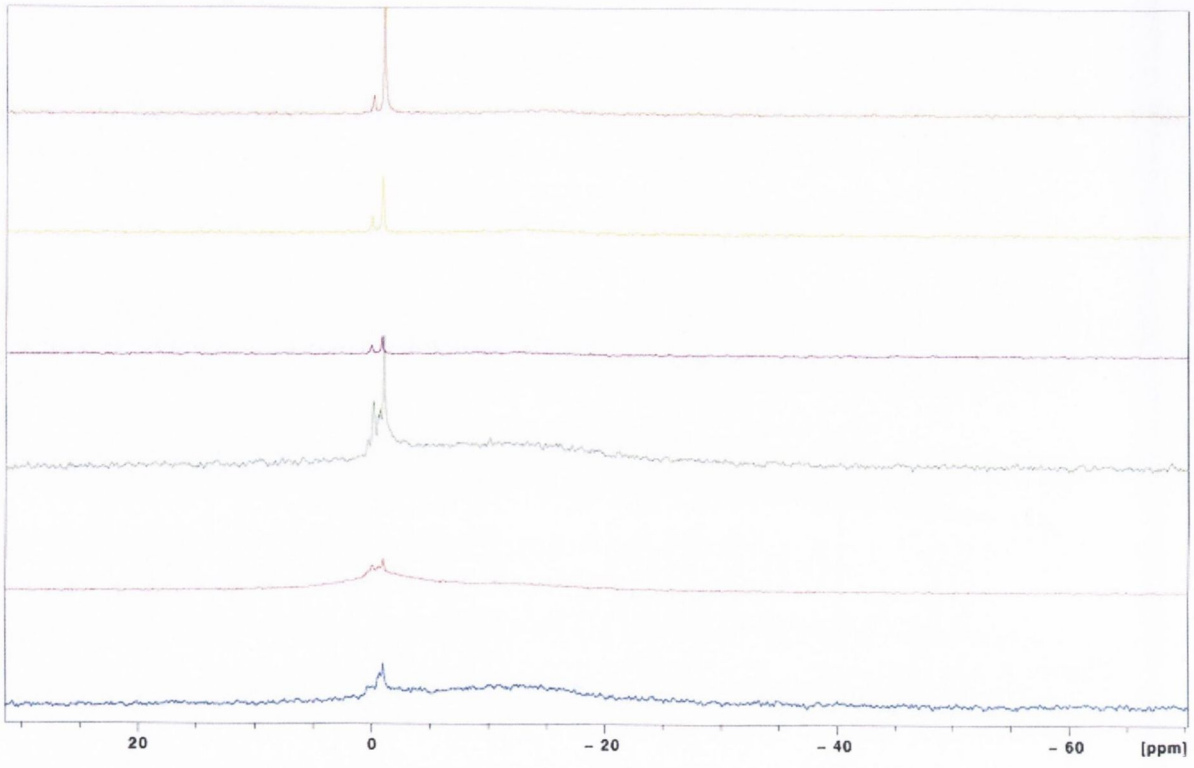


Figure A.15: Complete spectra following treatment of DPPC:Chol:DSPE-PEG_{2000} (75:20:5) liposomes with increasing concentrations of Triton-X 100. With increasing concentrations of Triton-X 100 from 0.00625 w/v (blue) to 0.0125 w/v (red), 0.025 w/v (green), 0.05 w/v (pink), 0.1 w/v (yellow) and 0.2 w/v (orange), the broad liposome peak faded away replacing it with a sharp micelle peak that suggested breakdown of liposomes to form micelles, Triton-X facilitating the process.

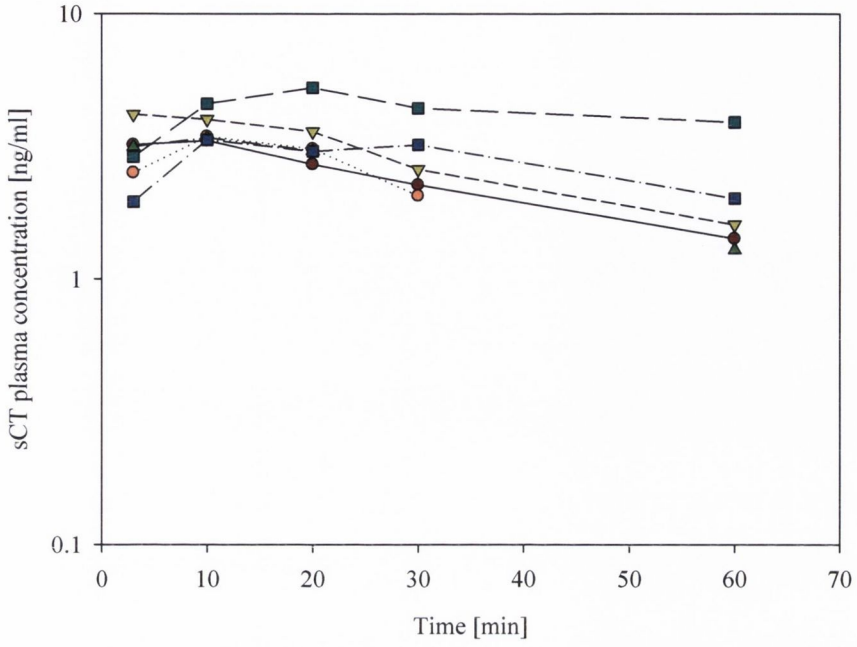


Figure A.16: Single PK profiles following nebulisation of sCT encapsulated liposome to rats. Salmon calcitonin encapsulated DPPC:Chol:SA:DSPE-PEG_{2000} (55:20:20:5) liposomes were administered to rats using Aeroneb Pro vibrating mesh nebuliser at a concentration of 50 µg/kg sCT. Plasma sCT concentration was measurable till 60 min after administration in all rats.

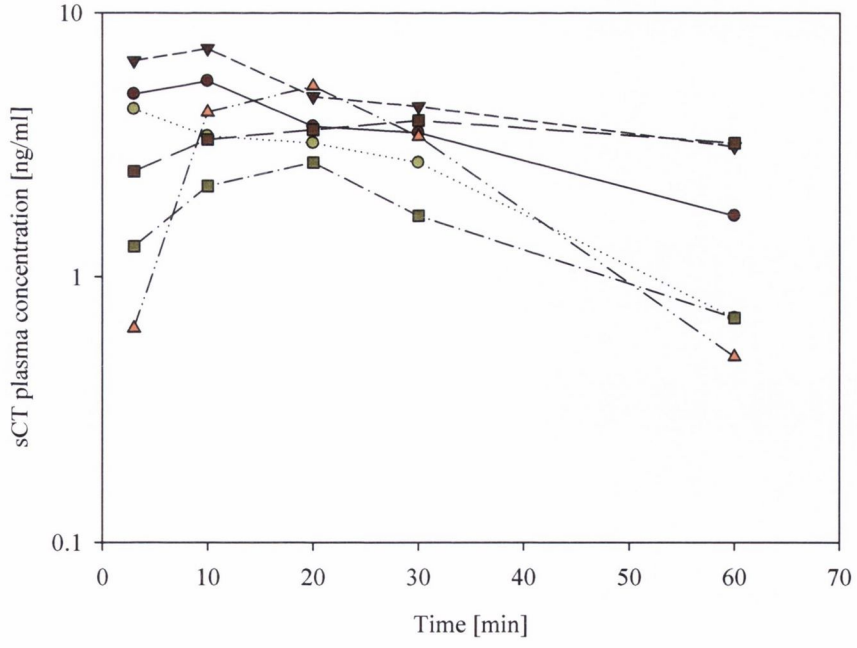


Figure A.17: Single PK profiles following nebulisation of sCT solution to rats. Salmon calcitonin solution was administered to rats using Aeroneb Pro vibrating mesh

nebuliser at a concentration of 50 µg/kg sCT. Plasma sCT concentration was measurable till 60 min after administration in all rats.

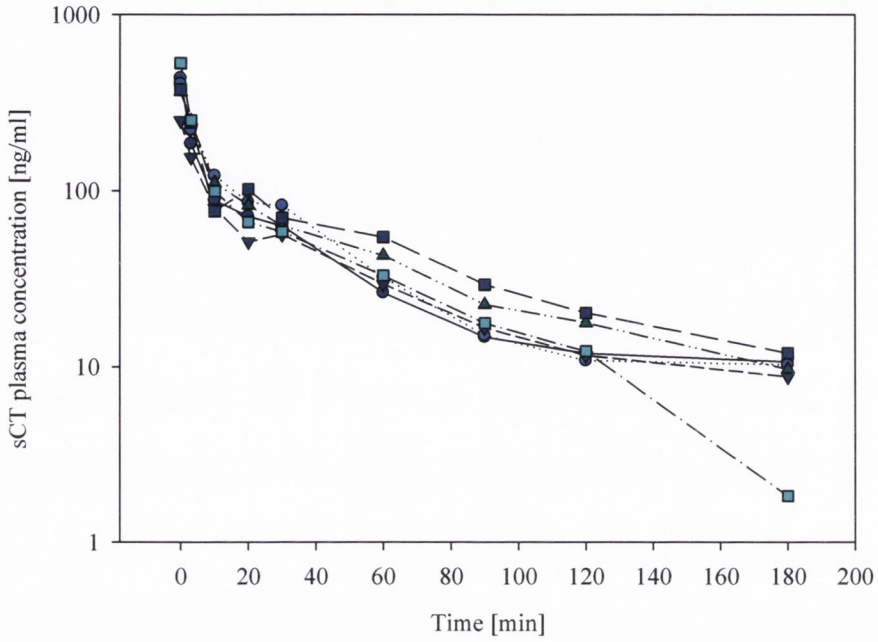


Figure A.18: Single PK profiles following intravenous administration of sCT encapsulated liposome to rats. Salmon calcitonin encapsulated DPPC:Chol:DSPE-PEG_{2000} (75:20:5) liposomes were administered to rats parenterally at a concentration of 50 µg/kg sCT. Plasma sCT concentration was measurable till 180 min after administration in all rats.

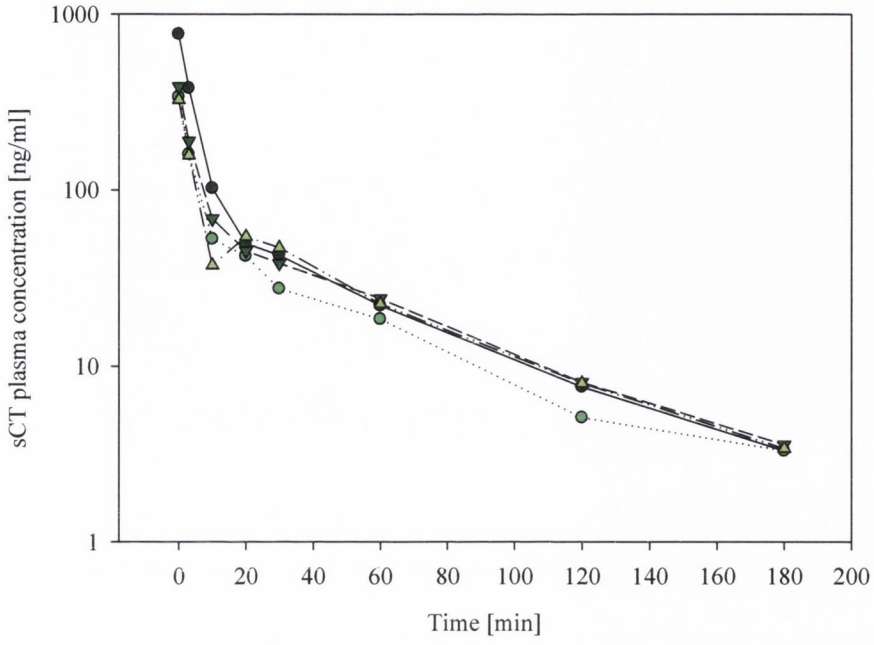


Figure A.19: Single PK profiles following intravenous administration of sCT solution to rats. Salmon calcitonin solution was administered to rats parenterally at a concentration of 50 µg/kg sCT. Plasma sCT concentration was lesser than that obtained with sCT liposomes administered parenterally and was measurable till 180 min after administration in all rats.

A2.ABBREVIATIONS

°	Degree
μ	Micro
%	Percent
±	Plus-minus
ζ	Zeta
Alveofect	Artificial lung surfactant
ANOVA	Analysis Of Variance
AUC	Area Under the Curve
AUC_{inf}	AUC at infinity time point
BA	Bioavailability
BAL fluid	Broncho Alveolar Lavage fluid
BCA assay	Bicinchoninic Acid assay
°C	degree Celsius
C-18	18 Carbon derivative
cAMP	cyclic Adenosine Mono Phosphate
Chol	Cholesterol
Cl_{inf}	Clearance at infinite time point
C_{max}	maximum plasma Concentration
CO_2	Carbon di-oxide
D_2O	Deuterated water
Da	Dalton
DC	Dodecylated Chitosan
DMPC	1,2-Dimyristyl-*sn*-glycero-3-Phosphocholine
DOPC	1,2-Dioleyl-*sn*-glycero-3-Phosphocholine
DPI	Dry Powder Inhaler

DPPC	1,2-Dipalmitoyl-*sn*-glycero-3-Phosphocholine
DPPG	1,2-Dipalmitoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (Sodium salt)
DSPC	1,2-Distearoyl-*sn*-glycero-3-Phosphocholine
DSPE-PEG2000	1,2-Distearoyl-sn-glycero-3-Phosphoethanolamine-N -[methyl (Polyethylene Glycol) – 2000] (Ammonium salt)
EC_{50}	Half maximal Effective Concentration
ed, eds	editor, editors
EE	Encapsulation Efficiency
eg	exempli gratia, for example
ELISA	Enzyme Linked Immunosorbent Assay
EPC or Egg PC	L-α-Phosphatidylcholine
Eq	Equation
et al	et alli, and others
FBS	Foetal Bovine Serum
FDA	Food and Drug Administration
g	gram
GM1	Monosialoganglioside
h	hour
hCT	human Calcitonin
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
IBMX	3-Isobutyl-1-Methyl Xanthine
i.m.	intramuscular
i.p.	intraperitoneal
i.t.	intratracheal

i.v.	intravenous
k	thousand
K_{el}	Elimination rate constant
kg	kilogram
kHz	kilo hertz
KRB	Krebs Ringer Buffer
μg	microgram
μl	microlitre
μm	micrometer
μmol	micromole
μg/μmol	microgram per micromole
μg/kg	microgram per kilogram
min	minutes
mg	milligram
ml	millilitre
mM	millimolar
mV	millivolt
mg/ml	milligram per millilitre
ml/min	millilitre per minute
mol %	mole percent
M	Molar
MHz	Millihertz
MLV	Multi Lamellar Vesicles
MPS	Mononuclear Phagocytic System
nm	nanometre

NMR	Nuclear Magnetic Resonance
Non-PEG	Non-PEGylated
P-5	Gel filtration column with an exclusion limit of 5,000 Da
P-6	Gel filtration column with an exclusion limit of 6,000 Da
P-10	Gel filtration column with an exclusion limit of 10,000 Da
PBS	Phosphate Buffered Saline
PdI	Poly dispersity Index
PEG	Polyethylene Glycol
PG	Phosphatidylglycerol
pH	negative logartithm of hydrogen ion concentration
PI	Phosphatidylinositol
PK	Pharmacokinetics
pMDI	pressurised Metered Dose Inhaler
^{31}P NMR	^{31}Phosphorous Nuclear Magnetic Resonance spectroscopy
PTH	Parathyroid Hormone
rH	Relative humidity
rpm	rotations per minute
RP-HPLC	Reverse Phase – High Performance Liquid Chromatography
SA	Stearyl Amine
sCT	Salmon Calcitonin
SD	Standard Deviation
SLN	Solid Lipid Nanoparticle
SP	Surfactant Protein
SPC	Soybean Phosphatidylcholine
SUV	Small Unilamellar Vesicles

$t_{1/2}$	half life
T_g	Glass transition temperature
TCD	Trinity College Dublin
TEER	Transepithelial Electrical Resistance
T_{max}	Time corresponding to maximum plasma concentration
UFL	Ultra Flexible Liposome
UV	Ultraviolet
vs	versus
w/v	weight per volume
ζ potential	zeta potential