Effect of lyophilization on liposomal encapsulation of salmon calcitonin

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Abstract

Purpose: The intent of this work was to assess the impact of lyophilization on the encapsulation of salmon calcitonin (sCT) into liposomes.
Methods: Four different liposomal formulations were investigated, i.e. 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 the cryoprotectant, trehalose dihydrate. The liposomes were lyophilized, reconstituted and extruded to obtain small unilamellar vesicles. Non-encapsulated sCT was separated by gel filtration. Non-lyophilized formulations and liposomes lyophilized without the cryoprotectant were used as controls. Liposomes were analyzed for particle size, polydispersity index, zeta-potential and encapsulation efficiency. ^{31}P-NMR (phosphorous nuclear magnetic resonance spectroscopy) was performed on selected formulations.
Results: Post-lyophilization, no significant change in particle sizes and zeta-potentials were noted, regardless of the presence or absence of the cryoprotectant. Encapsulation efficiencies, however, increased following lyophilization, in both PEGylated (lyophilization 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: Lyophilization resulted in an increase in encapsulation efficiency of sCT in PEGylated liposomes, even in the absence of a cryoprotectant, due to presence of micellar vesicles.

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 Paget's disease and hypercalcaemia and is currently marketed as injectable solutions (Calcimar® and Miacalcin®) (Antosova et al., 2009; Chestnut et al., 2008; Stevenson, 2009). Until recently, nasal sprays (Fortical® and Miacalcin®) were also available, but have been withdrawn from the market. Various research groups have been investigating the suitability of liposomal carriers for delivery of sCT via parenteral (Fukunaga et al., 1984), intranasal (Chen et al., 2009; Law & Shih, 2001; Law et al., 2001) and oral (Ebato et al., 2003; Song et al., 2002, 2005; Yamabe et al., 2003) routes. The main issue regarding the stability of liposomal sCT formulation arises from the fact that the peptide is amphiphilic and thus, liquid formulations cannot be expected to provide a long shelf-life (Bradshaw, 1997; Diociaiuti et al., 2006).

Extensive research has been carried out assessing the impact of lyophilization on various aspects of liposomal formulations, including the improvement in encapsulation efficiency, which is dependent on the type of lyoprotectant and its concentration (Chen et al., 2010). In addition, increased permeability of the payload across the bilayer upon rehydration, following lyophilization in the presence of a lyoprotectant, has been reported, resulting in increased encapsulation efficiency (Zhang et al., 1997). The lyophilization process is very versatile allowing the use of different concentrations of cryo/lyoprotectants, temperatures and duration of lyophilization and drying cycles, making it suitable for the most sensitive payloads (Stark et al., 2010; van Winden et al., 1997). During lyophilization, a change in gel to liquid crystalline phase transition occurs. This is when the payload is lost, and to overcome this issue, lyoprotectants are added in an attempt to avoid the phase transition. Carbohydrate lyoprotectants act by protecting membrane integrity and preventing payload leakage due to their high T_g (Chen et al., 2010). Previously, lyophilization technique has been successfully employed for insulin and VIP-containing liposomes, but freeze-drying of salmon calcitonin (sCT) liposomes has not been reported to date (Bi et al., 2008; Ohmori et al., 2006).

Therefore, this work aimed to determine the impact of lyophilization on the encapsulation efficiency of sCT in PEGylated and non-PEGylated liposomes. Also, the impact various amounts of cholesterol on lyophilization was

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analyzed. Trehalose dihydrate was used as cryoprotectant, due to its reported benefits (Chen et al., 2010; Heikal et al., 2009; Quaak et al., 2010; Schwarz & Mehnert, 1997; Stark et al., 2010). Trehalose dihydrate forms a layer around the payload, thus preventing it from the harsh effects of quick freezing and sublimation maneuvres. It also aids in the formation of a fluffy cake and easily dissolves during reconstitution. Different concentrations of trehalose dihydrate were studied either in the external medium alone or in internal and external compartment. The observed increase in encapsulation efficiency in the case of PEGylated liposomes was investigated using ^{31}P-NMR spectroscopy.

Materials and methods

Materials

Dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidylethanolamine-polyethylene glycol 2000 (DSPE-PEG_{2000}) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol (Chol), trehalose dihydrate, 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 (St. Louis, MO). Sodium chloride, potassium chloride and sodium bicarbonate were purchased from Fisher Scientific (Waltham, MA). Ultra pure water was used in all experiments and generated by a Millipore Synergy unit (Billerica, MA). A Rotavapor R-210 rotary evaporator was used for lipid film preparation (Büchi, Flawil, Switzerland). A Virtis freeze-dryer (Warminster, PA) was used to lyophilize 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 6000 Da used for gel filtration were purchased from Bio-Rad (Hercules, CA). Particle size, polydispersity index (PdI) and zeta-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) wanted to determine the impact of lyophilization on encapsulation efficiency of this formulation and one obtained by increasing cholesterol concentration with their non-PEGylated counterparts. 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 bicarbonated Krebs–Ringer buffer (KRB) of pH 3.5, 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.

Liposome preparation: lyophilization batches with and without cryoprotectant

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

In a pilot study we assessed whether it made a difference if the cryoprotectant (i.e. trehalose dihydrate) was added to the internal and external or only the external phase (Table 1). The results showed a smaller particle size and PdI as well as enhanced encapsulation efficiency for sCT in batches that had trehalose dihydrate in both compartments. Therefore, all following formulations were prepared in this way.

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Methods

Liposome preparation: non-lyophilization batches

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). From earlier studies, DPPC:Chol:DSPE-PEG$_{2000}$ (75:20:5) was the optimal formulation in terms of small particle size/poly dispersity index, high encapsulation efficiency of sCT and the ability to withstand nebulization stress (Swaminathan et al., 2014). We then wanted to determine the impact of lyophilization on encapsulation efficiency of this formulation and one obtained by increasing cholesterol concentration with their non-PEGylated counterparts. 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 bicarbonated Krebs–Ringer buffer (KRB) of pH 3.5, 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.

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Table 1. Particle size, polydispersity index (PdI) and encapsulation efficiency of DPPC:Chol (66.7:33.3) liposomes prepared with trehalose dihydrate in the external medium only and in both internal and external compartments. Data presented as means ± SD ($n = 3$).

Parameters	Particle size (nm)	PdI	Encapsulation efficiency of sCT (%)
Control	193.3 ± 5.14	0.30 ± 0.02	20.37 ± 1.10
Trehalose dihydrate – external compartment only			
1:1 lipid:trehalose	161.1 ± 0.22	0.17 ± 0.02	48.83 ± 2.07
1:5 lipid:trehalose	177.2 ± 2.19	0.26 ± 0.02	43.91 ± 9.62
1:10 lipid:trehalose	168.5 ± 1.7	0.22 ± 0.02	35.61 ± 0.15
Trehalose dihydrate – internal and external compartments			
1:1 lipid:trehalose	149.9 ± 1.48	0.08 ± 0.02	40.06 ± 3.42
1:5 lipid:trehalose	151.4 ± 0.57	0.07 ± 0.01	61.82 ± 5.46
1:10 lipid:trehalose	144.2 ± 0.27	0.02 ± 0.04	52.35 ± 3.88

Lyophilization of liposomal formulations

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

Reconstitution and extrusion of the samples

Following lyophilization, samples were reconstituted with KRB (pH 3.5) and left in the water bath maintained at 42–43 °C for 5 min. The time until complete reconstitution was noted. If the samples did not reconstituted completely following after 5-min incubation, they were vortexed for 20 s, before they were extruded and gel filtered as explained above.

Physicochemical characterization

All formulations were analyzed for their particle size, their polydispersity index (PdI) and the particle surface charge (zeta-potential). Disposable low volume cuvettes were used for particle size estimation, whereas disposable capillary cells were used for zeta-potential determination. Samples for physicochemical characterization 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 lyophilization experiments and in triplicates form 2 independent batches ($n = 6$) for release study batches.

HPLC method for the 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 two independent measurements from three liposome batches ($n = 6$) for lyophilization experiments.

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 RP-HPLC analysis. The phospholipid content was determined using Stewart's assay (Stewart, 1980). Data of the Stewart's assay were the average of three individual measurements from three independent liposome batches ($n = 9$).

Encapsulation efficiency following gel filtration was calculated using the following equation:

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

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

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. (2008). 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.

Statistical analysis

Both lyophilization and non-lyophilization batches with and without cryoprotectant were prepared in triplicates. Data are presented as mean ± SD. 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.

Results

Physicochemical characteristics of non-lyophilized and lyophilized formulations

Particle sizes, polydispersity indices and zeta-potentials of all formulations are presented in Figure 1 and Table 2, respectively. Lyophilization did not result in a significant increase in particle size in any of the studied formulations (Figure 1). In fact, lyophilized non-PEGylated DPPC:Chol (66.7:33.3) liposomes were reduced in size. The particle sizes of the non-PEGylated DPPC:Chol (80:20) formulations were higher than all other formulations tested. In general, no significant change was observed in particle size between the non-lyophilized liposomes and the lyophilized formulations. From Table 2, it can be appreciated that there was a significant ($*p \leq 0.05$) decrease in PdI in DPPC:Chol (66.7:33.3) following lyophilization, whereas all other formulations showed no significant changes. Zeta-potential values were always in the neutral range for all formulations investigated.

Encapsulation efficiency of non-lyophilized and lyophilized formulations

There was an increase in encapsulation efficiency of sCT noted in most formulations following lyophilization (Figure 2). Prior to lyophilization, DPPC:Chol:DSPE-PEG$_{2000}$ (75:20:5) vesicles showed a significantly higher encapsulation efficiency (i.e. 63.07 ± 8.51%) compared to all other systems. Lyophilization without trehalose dihydrate led to an increase in sCT entrapment in all formulations except for DPPC:Chol (66.7:33.3). However, this increase was only significant ($p \leq 0.05$) in the case of liposomes prepared from a mixture of DPPC:Chol:DSPE-PEG$_{2000}$ (65:30:5). The addition of cryoprotectant at 1:5 lipid:trehalose

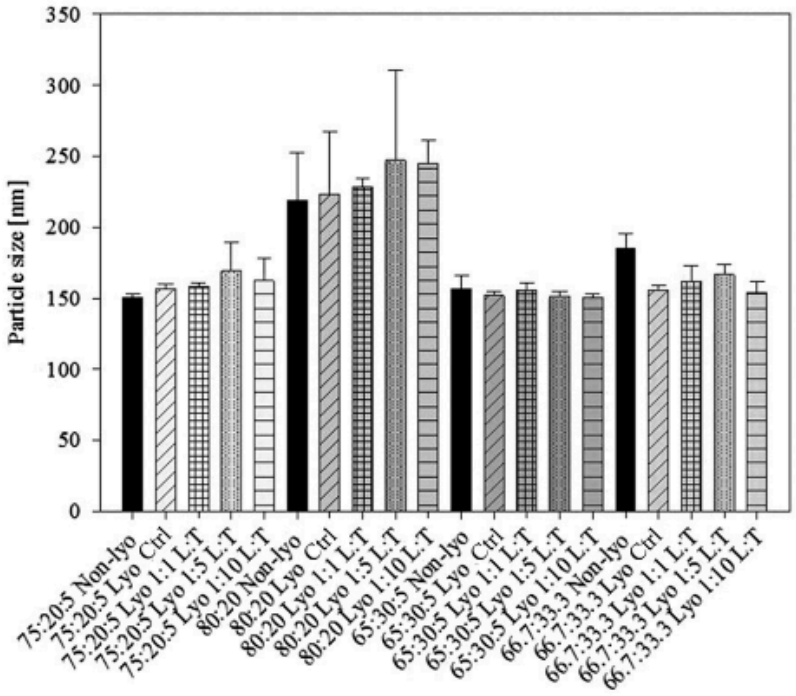

Figure 1. Particle size of non-lyophilized and lyophilized sCT encapsulated liposomal formulations. Bar graph represents particle size of DPC:Chol:DSPE-PEG_{2000} (75:20:5), DPPC:Chol (80:20), DPPC:Chol:DSPE-PEG_{2000} (65:30:5) and DPPC:Chol (66.7:33.3). Particle size of formulations that were not freeze-dried (black bars), lyophilized control formulations (bars with slanting stripes), lyophilized formulations containing lipid:trehalose dihydrate (L:T) 1:1 (chequered bars), 1:5 (dotted bars) and 1:10 (bars with horizontal stripes). Differences in particle size following lyophilization were non-significant for all formulations. Data presented as means ± SD ($n = 9$).

Table 2. Polydispersity index (PdI) and zeta-potential of non-lyophilized and lyophilized formulations following lyophilization and reconstitution.

Parameter	PdI	zeta-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

Lyo, lyophilized; Non-lyo, not lyophilized; Lip, lipid; Treh, trehalose dehydrate.

For each formulation, the lyophilized batches (i.e. control and various concentrations of trehalose dihydrate) were compared against the non-lyophilized control. No significant change in PdI occurred following lyophilization in all formulations except DPPC:Chol (66.7:33.3) where a significant reduction in PdI following lyophilization was observed. Changes in zeta-potential following lyophilization were insignificant for all batches. Data presented as means ± SD ($n = 9$).

*$p \leq 0.05$.

dehydrate ratio showed a significant ($p \leq 0.05$) increase in encapsulation efficiency in the case of DPPC:Chol (80:20) liposomes. The other ratios did not have significant effects on sCT encapsulation in any of the other formulations studied.

^{31}P-NMR spectroscopy

NMR spectra with 0.00625% w/v 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). 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 disappeared and was only barely visible at the highest concentration of Triton X-100 studied (i.e. 2% w/v) (Figure 4). Samples without Triton X-100 gave no peaks as the surfactant was required to solubilize the phosphorous content of phospholipids to yield a signal.

Discussion

The objective of this work was to assess, whether lyophilization leads to an additional increase (i.e. more than that obtained following PEGylation) in the encapsulation efficiency of salmon calcitonin in PEGylated liposomes and to determine, whether non-PEGylated liposomes show a similar increase in encapsulation efficiency of sCT following lyophilization. In addition, the impact of different concentrations of trehalose dihydrate on particle size and encapsulation

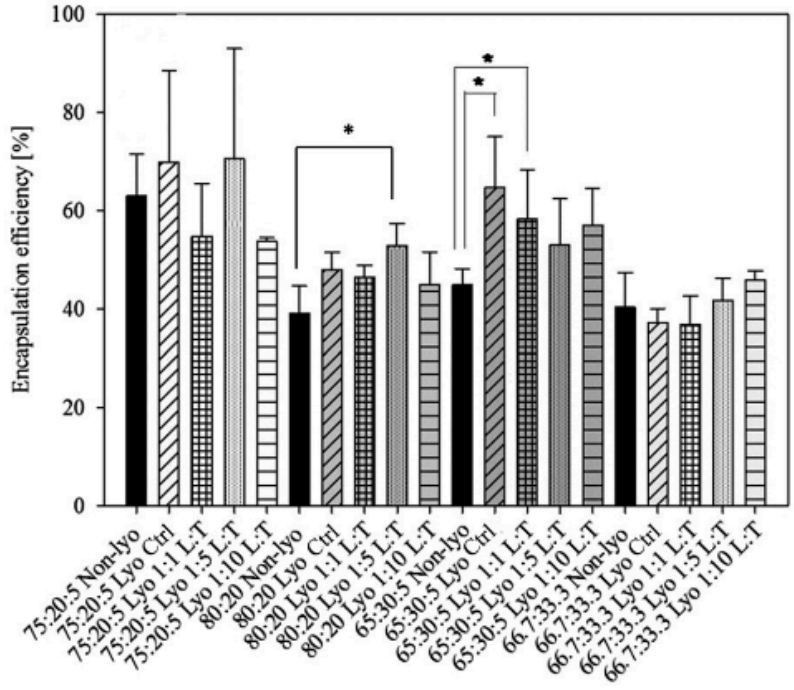

Figure 2. Encapsulation efficiency of non-lyophilized and lyophilized sCT encapsulated liposomal formulations. Bar graph represents particle size of DPC:Chol:DSPE-PEG_{2000} (75:20:5), DPPC:Chol (80:20), DPPC:Chol:DSPE-PEG_{2000} (65:30:5) and DPPC:Chol (66.7:33.3). Encapsulation efficiency of sCT in formulations that did not undergo lyophilization (black bars), lyophilized control formulations (bars with slanting stripes), lyophilized formulations containing lipid:trehalose dihydrate (L:T) 1:1 (checked bars), 1:5 (dotted bars) and 1:10 (bars with horizontal stripes). There was a significant increase in encapsulation efficiency of sCT following lyophilization in PEG formulations. Cryoprotectant (trehalose dihydrate) did not play a vital role in enhancement of encapsulation efficiency of PEGylated formulations. Data presented as means ± SD ($n = 9$); *$p \leq 0.05$.

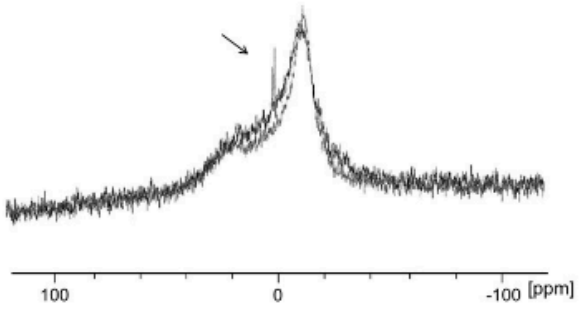

Figure 3. Representative ^{31}P-NMR spectra of PEGylated liposomes and the corresponding non-PEGylated formulations without Triton X-100. NMR spectra showing the presence of a sharp micelle peak (arrow) and a broad liposome peak in the PEGylated formulation and only a broad liposome peak for non-PEG liposomes. The formulations investigated were DPPC:Chol:DSPE-PEG_{2000} (75:20:5) and DPPC:Chol (80:20).

efficiency was studied. Lastly, the cause of the higher encapsulation efficiency observed for PEGylated liposomes in comparison to their non-PEGylated counterparts was investigated.

The observed increase in particle size in the non-PEGylated batches post-lyophilization could be attributed to the increased ability of the liposomal vesicles to fuse, which is avoided in the PEGylated formulations due to steric hindrance (Immordino et al., 2006). Enhanced encapsulation efficiency following lyophilization can be described as the result of repacking of lipid bilayers and subsequent encapsulation of the payload on reconstitution. This is in accordance with the observations by Zhang et al. (1997) who reported improved bilayer permeability of lyophilized liposomes with and without cryoprotectant upon rehydration. This might also explain why the cryoprotectant played only a minor role in enhancing the encapsulation efficiency in the case of non-PEGylated liposomes. However, the presence of cryoprotectant was important during the reconstitution of the lyophilized product. The lipid:cryoprotectant ratios 1:1 and 1:5 were reconstituted with ease (i.e. without vortexing) and faster (i.e. in <3 min) than the batches without cryoprotectant, which required vortexing following the reconstitution. The 1:10 batches did not show a significantly different reconstitution pattern from 1:5 batches. None of the batches (i.e. with/without cryoprotectant) affected the stability of sCT, as confirmed by the absence of degradation peaks in the HPLC chromatograms (data not shown). The higher encapsulation values achieved with PEGylated liposomes can be explained by data obtained from ^{31}P-NMR spectroscopy. ^{31}P-NMR measurements can give information about the size and the structure of lipid formulations. According to Burnell et al. (1980), vesicles smaller than 150 nm show symmetric line shapes. For larger liposomes, a low-field shoulder which is correlated to the presence of lipid bilayers is visible. The distance between low-field shoulder and high-field peak and the width of the spectrum can give further information

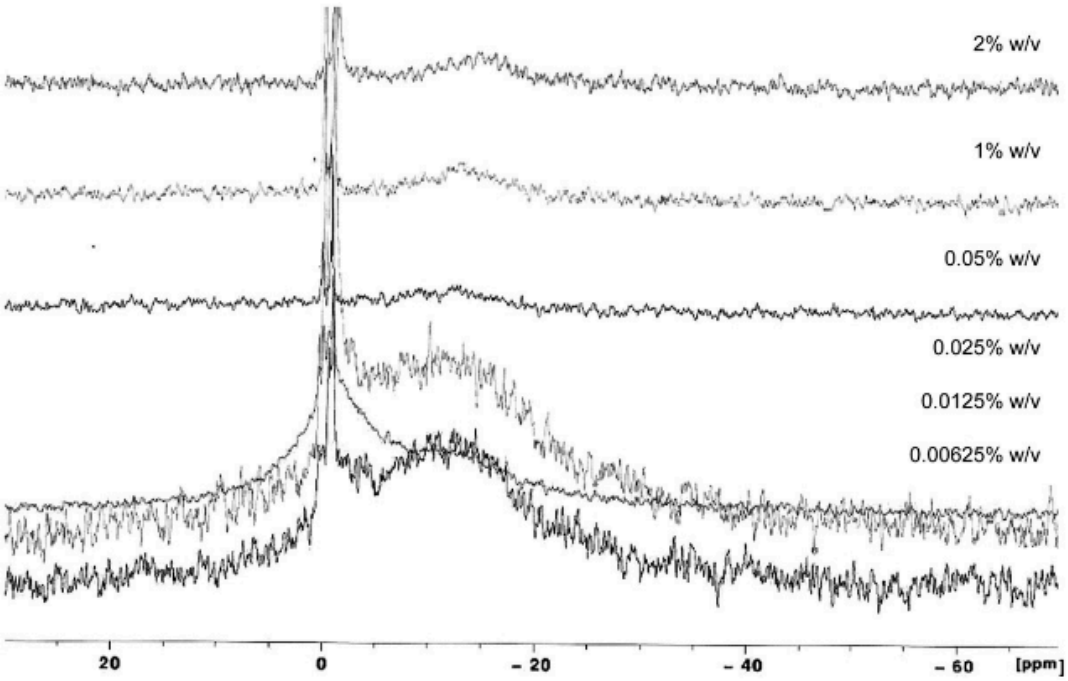

Figure 4. Representative ^{31}P-NMR spectra with varying concentrations of Triton X-100. NMR spectra of DPPC:Chol:DSPE-PEG$_{2000}$ (75:20:5) treated with varying concentrations of Triton X-100 showing two distinct populations – micelles represented by the sharp peak and liposomes represented by the broader peak. The broad liposome peak faded away with increasing concentrations of Triton X-100.

on the local order of the lipids. The ^{31}P-NMR spectrum in Figure 3 suggested the presence of two distinct vesicle populations in the 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 (Baginski et al., 2012; Leal et al., 2008). Micelle formation did not occur in non-PEGylated counterparts, which was evident from the absence of sharp peaks in the NMR spectra. The presence of micelles suggested an additional reason for the observed increased encapsulation efficiency in PEGylated formulations. This was due to the fact that micelles were not removed by the gel filtration manoeuvre that was performed to separate the non-encapsulated sCT. This micelle formation might be the result of insufficient mixing of the base lipid (DPPC) and PEG lipid (DSPE-PEG) during membrane formation, due to different chain lengths, ultimately causing ejection of PEG-lipids from the lipid bilayer and formation of micelles. These micelles are then able to encapsulate free sCT. Due to their size, the sCT micelles are not removed during gel filtration and hence, led to the alleged higher encapsulation rate observed when PEG-lipids were included in the formulation. This phenomenon was also observed by Leal et al. (2008).

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

^{31}P-NMR of samples without Triton X-100 yielded an extremely faint signal due to the absence of free phosphorus. Following the addition of a small concentration of Triton X-100 (i.e. 0.00625% w/v), the bilayer was sufficiently disrupted to obtain a signal. Hence, this value was chosen as the control.

The lyophilized 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.

Conclusions

Lyophilization of liposomes enhances the encapsulation efficiency of sensitive payloads such as peptides, in addition to augmenting their stability. Whilst the presence of trehalose dihydrate did not lead to an increase in encapsulation efficiency, it helped during the reconstitution of lyophilized vesicle. This study contributes to a better understanding of formulation and lyophilization aspects of liposomes containing peptide drugs. In addition, the formulation concepts described can be helpful for designing liposomes for other sensitive payloads.

Declaration of interest

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