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Cyclodextrin as membrane protectant in spray-drying and freeze-drying of PEGylated liposomes

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ABSTRACT

In this study it was investigated whether hydroxypropyl-β-cyclodextrin (HPβCD) is able to stabilize the liposomal membranes during drying of long circulating polyethylene glycol (PEG) coated liposomes, as compared to the disaccharides trehalose and sucrose. PEGylated liposomes loaded with prednisolone disodium phosphate (PLP) were dried by spray-drying or freeze-drying. The dried powders were tested on their residual moisture content, glass transition temperature and amorphous character. Upon reconstitution the liposomal size, size distribution and drug retention were determined and the results were compared to the characteristics of the formulation solution before drying. In contrast to the disaccharides, HPβCD stabilizes the liposomal membranes of the PEGylated liposomes during the drying process of both spray drying and freeze-drying when present in a lipid:carbohydrate ratio of 1:6 (w/w). The resulting powder can be stored at room temperature. No changes in size and size distribution were seen upon reconstitution of the HPβCD containing formulations. Drying resulted in a minimal leaking of PLP from the liposomes. Its relatively high T_g' and T_g of HPβCD, as compared to the disaccharides, make HPβCD an excellent membrane protectant for dry PEGylated liposomal formulations.

1. Introduction

Liposomes have proven to be well tolerated drug delivery vehicles that offer the possibility of targeted drug delivery for a wide range of therapeutic agents (Metselaar and Storm, 2005). Physicochemical properties of liposomes can be changed to optimize drug delivery and retention at the target site, thus enhancing their therapeutic efficacy, and to prevent toxicity to non-target tissues (De Silva et al., 1979; Fendler and Romero, 1977; Lopez-Garcia et al., 1993; Metselaar et al., 2002). Furthermore, liposomes can offer a solution in case of formulation problems of the active compound as a result of for instance low aqueous solubility (Barratt and Bretagne, 2007; Chang and Yeh, 2012; Mazerski et al., 1982). However, the phospholipids in the liposomal membrane, especially when dispersed in water, can slowly become oxidized or hydrolyzed (Chen et al., 2010; Crommelin and van Bommel, 1984; Grit and Crommelin, 1992; Zuidam et al., 1995). This could induce fusion of liposomes, leakage of the enclosed drug compound, and structural transformations of the liposomes, which might influence their performance (Ickenstein et al., 2006). Dry products generally show higher stability, and therefore various groups have tried to develop dried liposomal formulations (Chen et al., 2010; Crowe et al., 1985; Glavas-Dodov et al., 2005; Laverman et al., 2000; Ohtake et al., 2006; Skalko-Basnet et al., 2000; van Winden and Crommelin, 1997; van Winden, 2003; Wessman et al., 2010; Wieber et al., 2012). Apart from a stabilization objective, dry liposomal formulations also offer opportunities for routes of administration other than parenteral use only, e.g. as dry powder inhalation.

Commonly applied drying techniques in pharmaceutical manufacturing are spray drying and freeze-drying. Freeze-drying of conventional liposomes (e.g. liposomes without surface modifications) has been well documented in the literature (Chen et al., 2010; Crommelin and van Bommel, 1984; Crowe and Crowe, 1988; Glavas-Dodov et al., 2005; van Winden, 2003). Though less frequently, freeze-drying of long circulating liposomes containing polyethylene glycol (PEG) has also been reported (Hinrichs et al., 2006; Hinrichs et al., 2005; Laverman et al., 2000; Wessman et al., 2010). However, as compared to lyophilization, only a limited number of reports have focused on spray-drying as a method to dry

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liposomal formulations (Chougule et al., 2007, 2008; Goldbach et al., 1993; Hauser and Strauss, 1987; Wessman et al., 2010). Most groups tried to spray-dry conventional liposomes, though Wessman et al. (2010) investigated the effect of spray-drying on the structure of PEGylated liposomes. Compared to freeze-drying, spray-drying is much faster, less expensive and more suited for production of defined particles (Ingvarsson et al., 2011). On the other hand, freeze-drying is more suited for the development of sterile drug products. Spray-drying results in a powder mass that requires subsequent handling into the final product format whereas freeze-drying offers the possibility of drying defined volumes of the aqueous formulation in the final product container.

The main issue in drying of liposomal formulations is the stability of the liposomal membranes. These membranes can be easily disrupted during the drying process, for instance due to ice crystals or phase transition of the membranes under influence of temperature, or due to sublimation of water from the liposomal surface (Chen et al., 2010; Ingvarsson et al., 2011; Siow et al., 2007). Therefore, the liposomal membranes need to be protected during the drying process. Cryo- and lyoprotectants that are often used to protect delicate structures like proteins, DNA and liposomes during drying processes are disaccharides like sucrose and trehalose (Crowe et al., 1985; Glavas-Dodov et al., 2005; Hauser and Strauss, 1987; Laverman et al., 2000; Ohtake et al., 2005, 2006). Disaccharides are able to form hydrogen bonds, thereby stabilizing the ordered conformation of the delicate structures upon removal of water molecules (water replacement theory) (Chang et al., 2005; Maitani et al., 2008).

Besides disaccharides, hydroxypropyl-β-cyclodextrin (HPβCD), a cyclic oligosaccharide, has also proven to stabilize proteins during spray-drying (Branchu et al., 1999; Iwai et al., 2007). The exact mechanism is still unknown but might be improved vitrification due to a higher vitrification temperature (the glass transition temperature of maximally cryoconcentrated solutions, T_g') and/or improved water replacement due to its large number of hydrogen donors and acceptors (Abdelwahed et al., 2006; Branchu et al., 1999; Iwai et al., 2007; Serno et al., 2011; Vega et al., 2012). HPβCD has a high aqueous solubility and a safe toxicity profile for a variety of administration routes, including parenteral use (Challa et al., 2005; Loftsson and Duchêne, 2007; Pourmokhtar and Jacobson, 2005). Several products containing HPβCD have been marketed, e.g. Sporanox® and Trisporal® (containing itraconazol) and Indocollyre® (containing indometacin) (Davis and Brewster, 2004).

In this study it was investigated whether HPβCD is able to stabilize the liposomal membranes during both spray-drying and freeze-drying of long circulating PEGylated liposomes, as compared to the disaccharides trehalose and sucrose. The PEGylated liposomes were loaded with the water-soluble drug prednisolone disodium phosphate (PLP) as a model drug. Creating a dry liposomal formulation of a water-soluble drug encapsulated in the aqueous core of the liposome is a major challenge, since the drug can leak out of the liposome during drying (van Winden, 2003). Therefore, drug leakage is a good marker for instability or even rupture of the liposomal membranes during the drying process. From our own experience we know that PLP does not leak out of the PEGylated liposomes in aqueous dispersion (Nanocort; Metselaar et al., 2003). Also, PLP solutions are chemically stable for considerable time. Based on these characteristics, we selected PLP-PEGylated liposomes as a model drug formulation. During drying, the water is removed from both outside and inside the liposomes. Therefore, it might be relevant to protect the liposomal membrane on both sides (Crowe et al., 1985; Ohtake et al., 2005). To evaluate this, liposomal formulations were prepared both with and without lyoprotectant present in the liposome core. Besides drug retention, physicochemical properties and microscopic appearance of the dried liposomal formulations were investigated.

2. Materials and methods

2.1. Preparation of the liposomes

Liposomes were prepared using a film extrusion method (Amselem et al., 1993). Briefly, dipalmitoylphosphatidylcholine (DPPC), 1,2-distearoyl-phosphatidylethanolamine-methyl-polyethyleneglycol conjugate-2000 (DSPE-PEG) (both from Lipoid GmbH, Ludwigshaven, Germany) and cholesterol (BUFA, Uitgeest, The Netherlands) were dissolved in ethanol. A lipid film was created by rotary evaporation at 65 °C. The lipid film was hydrated with a solution containing prednisolone disodium phosphate (BUFA, Uitgeest, The Netherlands) in a concentration of 139 mg/mL. Furthermore, the hydrating solutions contained either 0% or 10% of sucrose (BUFA, Uitgeest, The Netherlands), trehalose (Merck, Darmstadt, Germany) or HPβCD (Roquette Pharma, Lestrem, France) in sterile water for injections (B. Braun, Melsungen, Germany). The resulting coarse dispersion was sized by multiple extrusion steps through polycarbonate filter membranes with a pore size of 100 nm, resulting in liposomes with a diameter of about 100 nm, as was confirmed by dynamic light scattering (DLS). Unencapsulated PLP was removed by dialysis against a 10% solution of sucrose, trehalose or HPβCD using Slide-A-Lyzer dialysis cassettes (Thermo Fisher Scientific, Etten-Leur, The Netherlands) with a molecular weight cut-off of 10 kDa, with repeated changing of the dialysis medium. The lipid content of the liposomal dispersions was determined using HPLC, and the liposomal dispersions were subsequently diluted with their corresponding 10% sugar solutions to a final ratio of sugar:lipid of 6:1 (w/w, dry product), as ratios of 4:1 or higher have shown to protect the liposomes during drying in previous studies (Chaudhury et al., 2012; Crowe and Crowe, 1988; Laverman et al., 2000). The diluted dispersion is used for the drying processes.

All compounds used were of pharmaceutical (Ph. Eur) or highly pure (≥99%) grade and were used without any further purification.

2.2. Spray-drying of the liposomes

The aqueous formulations were spray-dried using a B-290 Mini Spray Drier (Büchi Labortechnik GmbH, Hendrik-Ido-Ambacht, The Netherlands). The spray-drying conditions were selected based on literature (Chougule et al., 2007, 2008; Skalko-Basnet et al., 2000) and were as follows: inlet and outlet temperatures were 100 °C and 68 °C, respectively; airflow rate was 35 m^3/h and the spray gas flow was 670 L/h; with a nozzle size of 0.7/1.5 mm the feed was set at 1 mL/min. The resulting spray-dried powders were kept in closed containers at 2–8 °C prior to characterization and further analysis.

2.3. Freeze-drying of the liposomes

1 mL aliquots of the liposomal dispersions were filled into 8R colorless glass vials (hydrolytic class type 1 Fiolax clear, Aluglas, Uithoorn, The Netherlands). Vials were partly closed using gray bromobutyl rubber lyophilization closures (West Pharmaceutical Services Inc., Lionville, PA, USA) and loaded into the freeze dryer (Model Lyovac GT4, GEA Lyophil GmbH, Hürth, Germany). The lyophilization program was based on the literature (Aso and Yoshioka, 2005; van Winden et al., 1997; van Winden, 2003). Vials were frozen to −35 °C at 0.5 °C/min in two hours. The shelf temperature of −35 °C was maintained for 24 h during the primary drying phase, while a vacuum of 10 Pa was established. At the end of primary drying the temperature was linearly increased to 0 °C in 2 h while the pressure was reduced to 0.9 Pa, to start secondary drying.

These conditions were maintained for another 48 h after which the vials were stoppered pneumatically under vacuum, removed from the freeze-dryer, and stored at 2–8 °C prior to characterization and further analysis.

2.4. Characterization of the dried powders

2.4.1. Visual inspection of the powders

The appearance, bulk density and flowability of the dried powder formulations were compared by visual inspection.

2.4.2. Differential scanning calorimetry (DSC)

DSC was performed using a Q2000 DSC equipped with a refrigerated cooling accessory (RCA) for low temperature in the T4P mode (TA instruments, New Castle, DE, USA). Temperature scale and heat flow were calibrated with indium. For determination of the glass transition temperature (T_g) powder samples of approximately 5–10 mg were transferred into TZero Aluminium pans (TA Instruments) and closed hermetically. The sample was equilibrated at 0 °C, followed by an isothermal step for 5 min. Subsequently the sample was heated to 80 °C with 2 °C/min and 1 °C/60 s modulation. An empty pan was used as a reference.

For determination of the membrane transition temperature (T_m), samples of approximately 10 mg of the reconstituted powder solutions were transferred into TZero Aluminium Hermetic pans (TA instruments) and closed hermetically. The sample was equilibrated at 10 °C, followed by an isothermal step for 5 min. Subsequently the sample was heated to 80 °C with 5 °C/min. An empty pan was used as a reference.

2.4.3. Residual moisture content

Determination of the residual moisture content of the dried product was performed using the Karl Fisher titration method. Approximately 90 mg of the dried formulation was transferred into the titration unit of a Model 658 KF Titrino apparatus (Metrohm, Herisau, Switzerland).

2.4.4. X-ray diffraction

X-ray powder diffraction measurements were performed using an X'pert pro diffractometer equipped with an X-celerator (PANanalytical, Almelo, The Netherlands). A 0.5 mm deep metal sample holder was filled with sample. The particle size of granule-like structures and crystalline materials was reduced using mortar and pestle before filling of the sample holder. Subsequently the sample was placed in the diffractometer. Samples were scanned at a current of 50 mA and a tension of 40 kV. The scanning range was 10–100° 2θ, with a step size of 0.020° and a scanning speed of 0.002° per second.

2.5. Characterization of the liposomes

The dried formulations are reconstituted with sterile water for injections (B. Braun) to their original concentrations (w/v). The resulting liposomal solutions were characterized and the results were compared to the characteristics of the formulation solution before drying.

2.5.1. Visual inspection of the formulation

The liposomal solutions were compared by visual inspection. The turbidity, the degree of opalescence and the color of the solution were compared. Additionally, the reconstitution time was determined.

2.5.2. Dynamic light scattering (DLS)

The size and size-distribution (polydispersity index, PDI) of the liposomes were determined by DLS with a Malvern ALV CGS-3 system (Malvern instruments Ltd., Malvern, Worcestershire, United Kingdom) with a scattering angle of 90° at 25 °C. Samples were diluted approximately 40 times using phosphate buffered saline (PBS) (B. Braun, Melsungen, Germany) before measurement.

2.5.3. High pressure liquid chromatography (HPLC)

PLP concentrations were determined by HPLC–UV using an 1100 series HPLC system consisting of a binary pump, Model G1312A, an autosampler Model G1367A and a UV-detector Model G1314A (all from Agilent Technologies, Amstelveen, The Netherlands). A Zorbax Eclipse-XDB-C8 analytical column (750 mm × 4.6 mm ID, particle size 5 μm, Agilent Technologies, Palo Alto, CA, USA) preceded by a guard column (reversed phase 10 mm × 3 mm, Varian, Palo Alto, CA, USA) were used. Absorbance was measured at 254 nm. Injection of 10 μL of sample was followed by a linear gradient of 5–90% acetonitrile (Biosolve B.V., Amsterdam, The Netherlands) with 10 mM ammonium formate (Fluka via Sigma–Aldrich, St. Louis, MO, USA). The pH was set at 3.6 using perchloric acid (Merck, Darmstadt, Germany). The flow rate was 1.0 mL/min. Chromatograms were processed using Chromeleon software (Dionex Corporation, Sunnyvale, CA, USA).

To determine the amount of (un)encapsulated PLP, an additional dialysis step was performed against a 10% solution of sucrose, trehalose or HPβCD using Slide-A-Lyzer dialysis cassette (Thermo Fisher Scientific, Etten-Leur, The Netherlands) with a molecular weight cut-off of 10 kDa. A 2 mL sample of the formulation solution was dialyzed against 600 mL of medium for at least 8 h. Both the permeate and the retentate were analyzed on the above mentioned HPLC–UV system.

Lipid concentrations were determined by HPLC with evaporative light scattering detection (ELSD) using an 1100 series binary HPLC pump, Model G1312A (Agilent Technologies, Amstelveen, The Netherlands), AS 3000 autosampler (Thermo Separation Products, Breda, The Netherlands) and an Alltech Varex MKIII Evaporative Light Scattering Detector (ELSD) (Grace (Alltech), Deerfield, IL, USA). An X-Bridge C18 analytical column (750 mm × 4.6 mm ID, particle size 2.5 μm, Waters Corporation, Milford, MA, USA) was used. Injection of 30 μL of sample was followed by a linear gradient of 80–100% methanol (Biosolve B.V., Amsterdam, The Netherlands) with 1% triethylamine (Merck, Darmstadt, Germany). The flow rate was 0.4 mL/min. Chromatograms were processed using Chromeleon software.

Prior to HPLC analysis the samples were diluted, if necessary, to a concentration of approximately 1 μg/mL PLP or 3 mg/mL total lipid. Subsequently, an extraction using dichloromethane (Merck, Darmstadt, Germany), sterile water for injections (B. Braun) and methanol (Biosolve) was performed on the lipid-containing samples, to separate the PLP and the lipid compounds.

2.5.4. Transmission electron microscopy (TEM)

The size and shape of the liposomes were visualized using TEM. To this end, samples diluted 1000 times were applied on Agar® formvar/carbon coated copper grids (van Loenen instruments, Zaandam, The Netherlands). The samples were negatively stained by uranyl acetate and dried on air. The samples were visualized under a Tecnai12 transmission electron microscope (Philips, Eindhoven, The Netherlands) using a GATAN 626 cryoholder (Gatan GmbH, München, Germany). Samples were observed at 120 kV. Images were recorded on TemCam-0124 camera (TVIPS GmBH, Gauting, Germany) and processed with AnalySIS software. The magnification ranged from 30,000 to 265,000 times.

Table 1
Liposomal characteristics before drying (PDI = polydispersity index). Liposomal formulations were prepared with and without the protecting carbohydrate present in the liposomal core (10% internal sugar and no internal sugar).

Formulation		Size (ø, nm)	PDI	Total lipid (mg/mL)	Molar lipid ratio (DPPC:DSPE-PEG:CHOL)	PLP content (mg/mL)
No internal sugar	Sucrose	100	0.05	13.61	2.4:0.14:1.0	0.79
	Trehalose	100	0.03	15.73	2.4:0.14:1.0	1.12
	HPβCD	112	0.04	12.10	2.6:0.15:1.0	1.54
10% internal sugar	Sucrose	104	0.09	14.22	2.4:0.13:1.0	0.86
	Trehalose	98	0.10	14.64	2.4:0.14:1.0	1.32
	HPβCD	104	0.01	13.97	2.6:0.16:1.0	1.79

3. Results

3.1. Characterization of the liposomes

PLP-PEGylated liposomes were prepared in order to evaluate the stabilizing effect of the different carbohydrates on the liposomal membranes during drying. The liposomal characteristics before drying were determined using HPLC–UV, HPLC–ELSD and DLS. All different liposomal formulations had comparable characteristics before drying (Table 1).

3.2. The effect of the drying methods

Visual inspection of the dried liposome formulations showed no differences in powder characteristics between the formulations with and without internal carbohydrate. However, differences were observed between the spray-dried formulations and the freeze-dried formulations. The powder resulting from spray-drying using HPβCD as lyoprotectant consisted of finer particles with a lower bulk density as compared to the disaccharide-containing powders, that consisted of granule-like particles. Lyophilization of the liposomal solutions resulted in white cake structures with a residual water content of approximately 1%, whereas spray-drying resulted in white powders with a residual water content of approximately 4%, irrespective of the formulation (Table 2).

X-ray diffraction analysis showed characteristic crystalline diffraction peaks in the X-ray diffraction spectra of the unprocessed disaccharides (Fig. 1: 1 in panel A and B). These were absent in the unprocessed HPβCD (Fig. 1: 1 in panel C), as well as in all dried formulations, indicating that the dried PLP-PEGylated liposome formulations are amorphous (Fig. 1: 2–5 in all panels).

The T_g of the sucrose formulations is approximately 36 °C, the freeze-dried formulations having a slightly higher T_g compared to their equivalent spray-dried formulations (Table 2). This is in correspondence with the slightly higher water content of the spray-dried formulations. The T_g of all dry trehalose formulations is 50 °C (Table 2). Apparently, the slight increase in water content does not affect the T_g of these formulations, which is a unique property of trehalose that has been reported before (Crowe et al., 1998, 1996; Kilburn et al., 2006). However, both disaccharide powders appeared to be very hygroscopic and instantly turned into its rubbery state

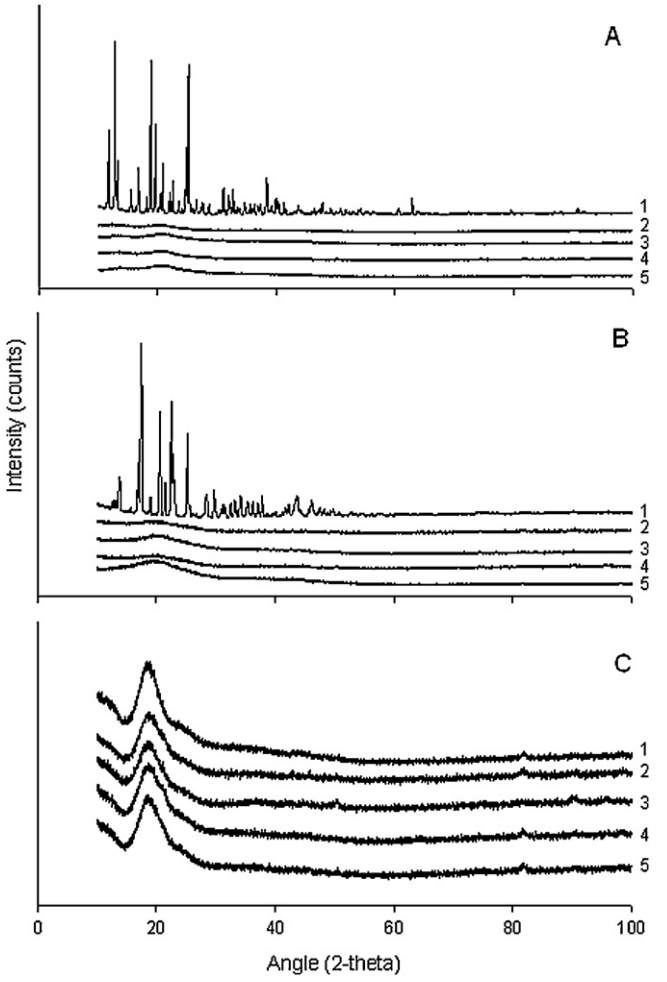

Fig. 1. X-ray diffraction patterns of the raw protecting carbohydrates and the dried formulations. (A) Sucrose formulations, (B) trehalose formulations and (C) HPβCD formulations. In each panel, 1 represents the raw carbohydrate, 2 and 3 respectively are the freeze-dried and spray-dried formulations without internal carbohydrate, 4 and 5 respectively are the freeze-dried and spray-dried formulations with 10% internal carbohydrate.

Table 2
Residual moisture content and T_g values of the dried formulation. Liposomal formulations were prepared with and without the protecting carbohydrate present in the liposomal core (10% internal sugar and no internal sugar).

Formulation		Residual moisture content (%)		T_g (°C)	
		After freeze-drying	After spray-drying	After freeze-drying	After spray-drying
No internal sugar	Sucrose	0.97	3.61	39	36
	Trehalose	1.20	3.90	48	50
	HPβCD	1.37	4.70	>100	>100
10% internal sugar	Sucrose	0.97	3.58	37	36
	Trehalose	1.06	4.04	50	50
	HPβCD	1.28	4.26	>100	>100

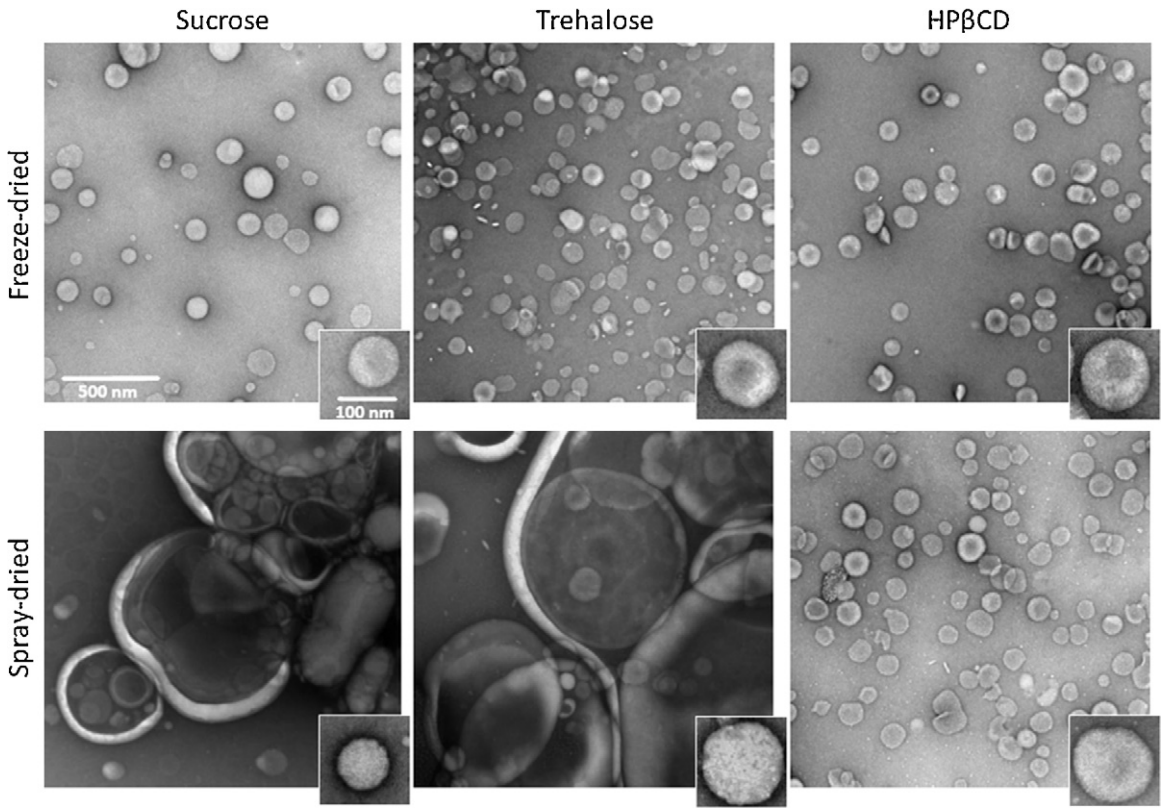

Fig. 2. TEM measurement confirms the presence of 100 nm liposomes in all formulations. No aggregates are formed in the freeze-dried formulations containing sucrose, trehalose or HPβCD, as well as in the spray-dried formulation containing HPβCD. Aggregation and rupture of liposomes occurred in the spray-dried formulations containing sucrose and trehalose, which explains their turbidity.

upon exposure to air, due to increased moisture levels and thereby reduction of the T_g to values below room temperature (Jaya and Das, 2009; Nokhodchi, 2005). This was not seen in the HPβCD formulations, since the T_g of the HPβCD formulations was found to be over 100 °C (Table 2).

3.3. Size and size distribution

Liposomal solutions containing 100 nm PLP-PEGylated liposomes are opalescent, and exhibit a characteristic red glow when inspected against visible light. This was seen in all formulation solutions before drying, and was confirmed by DLS measurement (Table 1). Reconstitution of the disaccharide-containing PLP-PEGylated liposomes took 40–75 min with manual shaking, while the reconstitution of the freeze-dried formulations and the spray-dried formulation containing HPβCD was complete within 5 min of manual shaking. Upon rehydration of all spray-dried formulations the opalescence and red glow re-appeared, an indication for the presence of 100 nm liposomes. However, with the exception of the HPβCD formulations, the formulations remained turbid upon reconstitution, indicating that also larger particles are present. Indeed this observation was confirmed by DLS analysis, which showed that the mean liposomal size as well as the PDI of all spray-dried formulations were significantly increased, with exception of the HPβCD formulations (Table 3). The PLP-PEGylated liposomal formulations containing HPβCD as lyoprotectant showed only a minor increase in size and PDI after reconstitution. This finding was confirmed by TEM analysis which showed the presence of uniform sized liposomes in the HPβCD formulations after spray- and freeze-drying, irrespective of the presence or absence of internal HPβCD (Fig. 2).

3.4. PLP leakage

PLP concentrations were measured using HPLC–UV. Before drying >90% of the PLP was encapsulated in the liposomes. Upon reconstitution of the freeze-dried formulations, the total PLP content of the formulation was comparable to the concentration before drying. However, for the sucrose and trehalose containing formulations only about 50% of the drug was still encapsulated in the liposomes, while for the HPβCD containing formulations this was still >90% (see Fig. 3).

For the spray-dried formulations, the results for drug retention were comparable to freeze-drying. For the HPβCD containing formulations approximately 100% of this drug is still encapsulated in the liposomes, while for the disaccharides up to 70% has leaked out of the liposomes.

The composition of the liposomes that are present in the dried substances was comparable to the liposomes in the aqueous formulation (comparable drug to lipid ratio, molar lipid composition DPPC:DSPE-PEG:CHOL 2.4:0.15:1.0), indicating that the leaking of the drug from the liposomes was not a result of liposome degradation. Clearly, the disaccharides were not able to stabilize the liposomal membranes during the drying process, resulting in drug leakage.

4. Discussion

PLP loaded liposomes were prepared in order to compare the stabilizing effect of HPβCD to commonly used disaccharides on the liposomal membranes during drying. The formulations were dried by spray-drying or freeze-drying. Three temperatures are of importance during drying of liposomes: the glass transition temperature (T_g) of the drying formulation, the vitrification temperature (T_g') of

Table 3
Effect of the drying method on the size (*z*-average) and size distribution of the liposomes. Liposomal formulations were prepared with and without the protecting carbohydrate present in the liposomal core (10% internal sugar and no internal sugar).

Formulation		Mean size ø (PDI)		
		Before drying	After spray-drying	After freeze-drying
No internal sugar	Sucrose	100 nm (0.05)	664 nm (0.76)	94 nm (0.11)
	Trehalose	100 nm (0.03)	1102 nm (0.64)	100 nm (0.15)
	HPβCD	112 nm (0.04)	138 nm (0.18)	107 nm (0.12)
10% internal sugar	Sucrose	104 nm (0.09)	503 nm (0.88)	95 nm (0.07)
	Trehalose	98 nm (0.10)	1159 nm (0.66)	101 nm (0.11)
	HPβCD	104 nm (0.01)	132 nm (0.14)	108 nm (0.17)

the aqueous formulation and the transition temperature (T_m) of the liposomal membrane. The T'_g and the T_g are primarily determined by the lyoprotectant used (Ingvarsson et al., 2011; Ohtake et al., 2006; van Winden et al., 1998, 1997; van Winden, 2003). The T'_g is important during freezing of the solution. Carbohydrates, like disaccharides, are used to protect the liposomal membranes during freezing, by forming a protective amorphous network around the liposomes. This vitrification occurs at temperatures below the T'_g (van Winden, 2003). For sucrose and trehalose, the T'_g is −32 °C and −30 °C, respectively (van Winden, 2003), while for HPβCD this is about −15 °C (Vega et al., 2012). The T_g is important during drying and storage of the formulation. Temperatures higher than the T_g of the (drying) powder could result in collapse of the powder. The T_g of dried sucrose and trehalose (e.g. without residual water) are about 60 °C and 100 °C respectively, and decrease with increased moisture content (Cummins et al., 2006; Hatley, 1997). The T_g of HPβCD is much higher as compared to the disaccharides (about 220 °C for dry HPβCD (Zheng and Chow, 2009)). The T_m mainly depends on the composition of the liposome and is similar for all tested formulations as the same lipid composition was used (Table 1). Due to the high content of cholesterol in the liposomal membrane, the T_m could not be determined (Ohtake et al., 2005). However, the T_m of similar liposomes with less cholesterol is typically around 41 °C in hydrated state (Hashizaki et al., 2006), and is not expected to change significantly upon dehydration (Crowe et al., 1985; Koster et al., 1994).

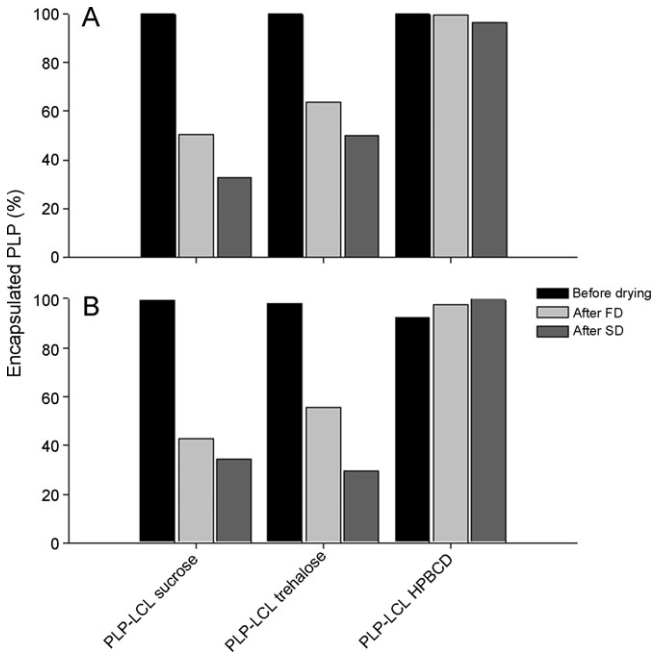

Fig. 3. Encapsulated % of PLP in formulations without internal sugar (A) and with 10% internal sugar (B).

The size and PDI of the liposomes in the spray-dried formulations containing disaccharides increased dramatically, while for the HPβCD formulations no changes in size and PDI were seen. This was also reflected by the 70% of drug leakage from the disaccharide formulations, while for the HPβCD formulations approximately 100% of the drug is still encapsulated in the liposomes. Apparently, spray-drying induces instability of the liposomal membranes that cannot be stabilized by disaccharides. This is likely due to the high drying temperatures obtained during spray-drying. The final T_g of the dried liposomal formulations was 35–50 °C, and has been below these values during the drying process. The outlet temperature of the spray drier was 68 °C, so the drying particles were temporarily heated to temperatures above their T_g. This could have caused transformation of the outer layer of the particles into a viscous liquid state, thereby enhancing its mobility, which could have resulted in partial collapse of the powder particles. The protecting vitrified structure is lost, resulting in instability of the liposomal membranes. Since the T_g of HPβCD is much higher as compared to the disaccharides, collapse of the cyclodextrin formulations is prevented. This indicates the importance of selection of proper settings of the spray-drying parameters. These settings should be based on the temperature characteristics of the selected lyoprotectant and lipid composition used (Ingvarsson et al., 2011).

Previously Hauser and Straus reported no significant structural changes after spray-drying of non-PEGylated small unilamellar vesicles. 90% of the originally entrapped materials remained entrapped in the liposomal cavity during spray-drying, when using sucrose as lyoprotectant (Hauser and Strauss, 1987). Additionally, Chougule et al. (2007, 2008) developed nanoliposomal dry powder formulations for inhalation, containing tacrolimus and dapsone. Conventional liposomes of approximately 140 nm were spray-dried with sucrose, trehalose or lactose as lyoprotecting agents at spray-drying conditions comparable to the settings used in our study. Drug retention of 97% for both formulations was reported. However, Wessman et al. (2010) showed that size and size distribution increased after spray-drying of 100 nm PEGylated liposomes with lactose as lyoprotectant. To the best of our knowledge, drug retention upon reconstitution of spray-dried PEGylated liposomes has not been reported thus far, but the occurrence of drug leakage in our own study is in line with the results obtained by Wessman et al.

Freeze-drying is performed at lower drying temperatures (up to maximal 0 °C during secondary drying) and therefore collapse due to exceeding the T_g during drying is not observed. Although no changes in size and PDI were found, approximately 50% of the drug leaked out of the liposomes during freeze-drying of the disaccharide formulations. This indicates that the liposomal membranes have been instable at some timepoint during the drying process or the rehydration with disaccharides as lyoprotectant. In contrast, in the HPβCD formulations no PLP leakage was observed. In freeze-drying, both the formation of ice crystals during freezing and the sublimation of the water from the liposomal surface could cause damage to the liposomal membranes (Siow et al., 2008; van Winden

et al., 1997; van Winden, 2003). Due to its higher T_g' the protecting vitrification networks were formed at higher temperatures during freezing of the HPβCD formulations, as compared to the formulations containing the disaccharides. This difference might have resulted in larger ice crystals during freezing of the disaccharide containing formulations, ultimately leading to membrane damage and drug leakage. The drying temperature during primary drying is well below all T_g' values, therefore no differences in membrane stability are expected during primary drying.

Structure bound (adsorbed) water is removed in the secondary drying phase of the lyophilization cycle (van Winden, 2003). In this process, the water molecules at the liposomal surface are replaced by molecules of the lyoprotecting agent, to stabilize the membrane structure in the dried state. Carbohydrates replace water by the formation of hydrogen bonds with liposomal surface structures (Crowe and Crowe, 1988; Crowe et al., 1985). Apparently, the lipid:disaccharide ratio of 1:6 (w/w) does not provide sufficient hydrogen replacement to stabilize the PEGylated liposomal membranes, resulting in drug leakage upon reconstitution. HPβCD has a unique structure with a large number of hydrogen donors and acceptors (Serno et al., 2011; Vega et al., 2012) and is therefore probably very efficient in stabilizing the liposomal membranes.

Leakage percentages of water-soluble compounds of 0% to up to 60% have been reported when using a variety of disaccharides to stabilize the liposomal membranes during freeze-drying (Crommelin and van Bommel, 1984; Crowe and Crowe, 1988; Glavas-Dodov et al., 2005; Talsma, 1991; van Winden et al., 1997). According to Crowe and Crowe, 100% drug retention in 100 nm liposomes could be obtained using a lipid:disaccharide ratio (trehalose or sucrose) of approximately 1:4 (w/w). Additionally, they have demonstrated that changes in both the lipid composition and the drying protocol can result in differences in the stability of the dried liposomes (Crowe and Crowe, 1988). To the best of our knowledge, no drug leakage results from freeze-dried PEGylated liposomes have been reported thus far.

HPβCD can form complexes by inclusion of lipophilic drugs into its cavity. The formation of a drug-HPβCD complex potentially can affect the pharmacokinetic profile of the drug, and therefore the drug release profile of the liposomal formulation should be investigated when HPβCD is added to the formulation. The effect of this possible complex formation on the pharmacokinetic behavior of the formulation is very much depending on the strength of the complexation. Although HPβCD is not able to form a complex with the liposome itself (diameter cavity HPβCD is 6–6.5 Å, whereas diameter liposome is 100 nm), complexation of drug entrapped in the liposomal core is possible when using HPβCD as internal lyoprotectant. Indeed, complexation of prednisolone (the underivatized glucocorticoid) with HPβCD has been described (Ghuzlaan et al., 2009). However, based on a complexation constant of 4300 L mol^{-1} for prednisolone, it is very unlikely that the much better water-soluble PLP forms a more stable complex with HPβCD in solution and be of any significance with respect to its pharmacokinetic profile. Nonetheless, it could be advised to stabilize the liposomal membranes only externally with HPβCD to prevent complex formation, since no differences were observed in stability of the formulations with and without internal lyoprotectant.

With respect to the storage conditions of the dried liposomal formulations, a storage temperature of 20–50 °C below the T_g is required. Therefore, the disaccharide containing formulations have to be stored at least refrigerated (+2 to 8 °C) (Chang et al., 1996; Duddu and Dal Monte, 1997; Hancock et al., 1995). Additionally, the hygroscopic behavior of the dried disaccharide formulations requires them to be stored under cold and dry conditions (Jaya and Das, 2009; Nokhodchi, 2005). Since the T_g of the HPβCD formulations is over 100 °C, these formulations can be stored at room temperature.

5. Conclusion

In conclusion, HPβCD has proven to stabilize the liposomal membranes during both spray drying and freeze-drying. Likely, its relatively high T_g' protects the membranes against damage by ice crystal formation during the freezing phase of the lyophilization cycle, while its relatively high T_g prevents the drying powder from collapse during spray-drying. Additionally, the large number of hydrogen donors and acceptors in the structure of HPβCD likely attributes to the efficiency of replacement of the water molecules at the liposomal surface during drying of the formulation, thereby protecting the liposomal membranes from damage and keeping its structure intact.

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