Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
International Journal of Pharmaceutics 311 (2006) 237–244
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

The choice of a suitable oligosaccharide to prevent aggregation of PEGylated nanoparticles during freeze thawing and freeze drying

W.L.J. Hinrichs [a,*], F.A. Manceñido [b], N.N. Sanders [c], K. Braeckmans [c], S.C. De Smedt [c], J. Demeester [c], H.W. Frijlink [a]

[a] *Department of Pharmaceutical Technology and Biopharmacy, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands*
[b] *Department of Pharmacy and Pharmaceutical Technology, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain*
[c] *Laboratory of General Biochemistry and Physical Pharmacy, Ghent University, Harelbekestraat 72, 9000 Ghent, Belgium*

Received 1 November 2005; received in revised form 16 December 2005; accepted 19 December 2005
Available online 25 January 2006

Abstract

In a previous study we have shown that the oligosaccharide inulin can prevent aggregation of poly(ethylene glycol) (PEG) coated plasmid DNA/cationic liposome complexes ("PEGylated lipoplexes") during freeze thawing and freeze drying [Hinrichs et al., 2005. J. Control. Release 103, 465]. By contrast, dextran clearly failed as stabilizer. These results were ascribed to the fact that inulin and PEG are compatible while dextran and PEG are not. In this study the stabilizing capacities of inulin and dextran (of various molecular weights) during freeze thawing and freeze drying of four different types of nanoparticles, each type with different amounts of PEG at their surface, were investigated. Freeze drying and freeze thawing of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)/dioleoyl-phosphatidyl-ethanolamine (DOPE) liposomes and egg phosphatidyl choline (EPC)/cholesterol (CHOL) liposomes showed that inulins are excellent stabilizers even for highly PEGylated liposomes while (especially higher molecular weight) dextrans dramatically lost their stabilizing capacity when increasing the degree of PEGylation of the liposomes. The same results were obtained for plasmid DNA/DOTAP/DOPE complexes. Finally, both inulin and dextran could prevent full aggregation of plasmid DNA/polyethylenimine (PEI) complexes independent whether PEI was PEGylated or not. It is concluded that inulins are preferred as stabilizers over dextrans for various types of PEGylated nanoparticles due to their compatibility with PEG.

Keywords: Freeze drying; Inulin; Nanoparticles; PEGylation; Particle size

1. Introduction

Formulation of drug substances into nanoparticles is one of the upcoming technologies in modern pharmaceutics (Bhadra et al., 2002; Heurtault et al., 2003; Mehnert and Mader, 2001; Monfardini and Veronese, 1998; Otsuka et al., 2003; Papahadjopoulos, 1996). A common disadvantage of many nanoparticles is that after intravenous administration they are rapidly cleared from the bloodstream which limits the duration of the therapeutic effect. One way to solve this problem is to link poly(ethylene glycol) (PEG) to the surface of the particles (PEGylation) (Bhadra et al., 2002; Otsuka et al., 2003). PEGylation reduces recognition by the immune system through which PEGylated nanoparticles have strongly increased circulation times (Bhadra et al., 2002; Kichler, 2004; Moghimi, 2002; Monfardini and Veronese, 1998; Ogris et al., 1999; Otsuka et al., 2003; Papahadjopoulos, 1996; Photos et al., 2003; Srinath and Diwan, 1994).

Nanoparticles are usually prepared in an aqueous environment. A major drawback of these dispersions is their thermodynamic driven tendency to lower their interfacial surface area with the environment and thus to aggregate. Due to a steric stabilization mechanism, PEGylated nanoparticles have an increased in vitro stability as compared with their non-PEGylated counterparts (Kichler, 2004; Nicolazzi et al., 2003; Riley et al., 1999; Sanders et al., 2002). Despite of that, the shelf life of PEGylated nanoparticles is still often limited. Therefore, it would be advantageous to store the PEGylated nanoparticles in a dry and stable state. However, when a dispersion is dried without a protectant, the nanoparticles are concentrated and aggregation is

* Corresponding author. Tel.: +31 50 363 2397; fax: +31 50 363 2500.
E-mail address: W.L.J.Hinrichs@rug.nl (W.L.J. Hinrichs).

0378-5173/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2005.12.032

facilitated. Despite of their increased stability, also PEGylated nanoparticles require stabilizers to prevent aggregation during freeze drying (Armstrong et al., 2002; Lenter et al., 2004; Ogris et al., 2003). In some studies it has even been found that PEGylation actually can induce aggregation during freeze drying (De Jaeghere et al., 1999, 2000; Zambaux et al., 1999).

It is well-known that sugars can be applied to prevent aggregation of nanoparticles during drying and storage (Schwarz and Mehnert, 1997; Talsma et al., 1997; van Winden, 2003). When properly dried the particles are incorporated in a sugar glass. The stabilization of materials in sugar glasses has been explained by the particle isolation theory (Allison et al., 2000) and vitrification theory (Molina et al., 2004). The particle isolation hypothesis refers to the formation of a sugar matrix which acts as a physical barrier between the particles. The vitrification theory refers to the formation of a glassy sugar matrix in which diffusion on a relevant time scale is inhibited. Both the physical barrier and lack of translational movement prevent aggregation.

A literature review reveals that in most studies disaccharides like sucrose and trehalose are used as stabilizers for various types of nanoparticles (Armstrong and Anchordoguy, 2004; Brus et al., 2004; Kuo and Hwang, 2004; Liao et al., 2002; Strauss et al., 1986; Sun et al., 1996). In a previous study we have evaluated the stabilization of lipoplexes by sugar glasses during subzero temperature storage and freeze drying followed by storage (Hinrichs et al., 2005). It was shown that oligosaccharides like inulin and dextran can also act as excellent stabilizers. It can be assumed that oligosaccharides stabilize the lipoplexes by the same mechanisms as disaccharides, i.e. by particle isolation and vitrification as described above. Due to their high glass transitions temperatures and high glass transitions temperature of the maximally freeze concentrated fractions, oligosaccharides were even preferred over disaccharides in certain cases. Both inulin and dextran acted as excellent stabilizers for non-PEGylated lipoplexes. However, when PEGylated lipoplexes were considered, the stabilization by inulins was better than by dextrans in most cases. The poor stabilizing capacity of dextrans was ascribed to the well-known incompatibility between dextrans and PEG (Albertsson, 1986; Hinrichs et al., 2005). When a dispersion of PEGylated lipoplexes in a solution of dextran is frozen, the concentration of both lipoplex and dextran is strongly increased by ice formation. As a result, the dextran molecules should penetrate the PEG shell present around the particles. However, because of the incompatibility of dextran and PEG, dextran will diffuse away from the PEG shell. Due to this freeze induced phase separation between the lipoplexes and dextran, the sugar molecules can no longer act as a stabilizer resulting in aggregation of the particles. Similar stability problems occurring during freeze drying of solutions of PEGylated proteins and excipients that are incompatible with PEG were envisaged by Heller et al. (1997, 1999). This hypothesis was further supported by the observation that inulin which is compatible with PEG (Hinrichs et al., 2005) prevents PEGylated lipoplexes from aggregation during freeze thawing or freeze drying (Hinrichs et al., 2005).

The aim of this study was to further investigate cryo- and lyoprotection of PEGylated nanoparticles by oligosaccharides that are either compatible or incompatible with PEG. Therefore, the aggregation behavior of nanoparticles with various degrees of PEGylation dispersed in inulin or dextran solutions with various molecular weights, during freeze thawing and freeze drying was studied. Four different types of nanoparticles were evaluated: (1) positively charged liposomes, (2) slightly negatively charged liposomes, (3) lipoplexes and (4) polyplexes.

2. Materials and methods

2.1. Materials

The lipids 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), dioleoyl-phosphatidyl-ethanolamine (DOPE), distearoylphosphatidylethanolamine–poly(ethylene glycol) (DSPE–PEG; with a molecular weight of PEG being 2 kDa), egg phosphatidyl choline (EPC) and cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Dextran 1.5 kDa was obtained from Fluka (Zwijndrecht, The Netherlands). Dextran 5 and 40 kDa were purchased from Dextran Products Limited (Scarborough, Ont., Canada). Branched polyethylenimine 25 kDa (PEI) was purchased from Sigma (St. Louis, MO, USA). Branched polyethylenimine (25 kDa), with each PEI molecule grafted with 11 PEG chains (8 kDa; PEGylated PEI) was a generous gift of Dr. S. Vinogradov of the Nebraska Medical Center, Omaha, NE, USA. Inulin 1.8 and 4 kDa (inulin types HD001111 and TEX!803, respectively) were a gift of Sensus, Roosendaal, The Netherlands. Plasmid DNA (pDNA) of 5803 base pairs encoding the reporter gene secretory alkaline phosphatase (SEAP) was amplified in *Escherichia coli*, purified and dissolved in HEPES buffer (20 mM HEPES pH 7.4) as previously described (Sanders et al., 2001). All other chemicals were of reagent or analytical grade and purchased from commercial suppliers.

2.2. Preparation of liposomes

Two types of unilamellar liposomes were prepared, each type with different amounts of PEG at their surface. The first type of liposomes were composed of DOTAP, DOPE and DSPE–PEG. The molar ratios of DOTAP, DOPE and DSPE–PEG in these liposomes were as follows: 50/50/0 (DOTAP/DOPE liposomes), 48.1/48.1/3.8 (3.8% PEGylated DOTAP/DOPE liposomes) and 45.5/45.5/9 (9% PEGylated DOTAP/DOPE liposomes). The second type of liposomes were composed of EPC, Chol and DSPE–PEG. The molar ratios of EPC, Chol and DSPE–PEG in these liposomes were as follows: 67/33/0 (EPC/Chol liposomes) and 57/33/10 (10% PEGylated EPC/Chol liposomes). The liposomes were prepared by the thin film-extrusion method as described previously (Sanders et al., 2001). Briefly, appropriate amounts of lipids were dissolved in chloroform and pipetted in a round bottom flask. The solvent was removed by rotary evaporation at 40 °C followed by purging the flask with dry nitrogen for 30 min at room temperature. The lipids were hydrated by adding 20 mM HEPES pH 7.4. Glass beads were added and swirled around to facilitate detachment of the lipid layer from the wall of the flask. Subsequently, the formed

vesicle dispersion was stored for 1 day at 4 °C after which it was extruded 11 times through two stacked 100 nm polycarbonate membrane filters (Whatman, Brentfort, UK) at room temperature.

For freeze thawing and freeze drying, the liposome dispersions were diluted with 20 mM HEPES pH 7.4 to a lipid concentration of about 2.5 mg/ml. Subsequently, 50 μl of the diluted liposome dispersions were mixed with either 50 μl of an oligosaccharide solution (12.6 wt% in water) or 50 μl distilled water. Eppendorf cups (1.5 ml) were used for freeze thawing and glass vials (4 ml) were used for freeze drying.

2.3. Preparation of lipoplexes

DOTAP/DOPE and PEGylated DOTAP/DOPE liposomes were used to prepare lipoplexes at a +/− charge ratio of 4 which corresponds to a weight ratio DOTAP/pDNA of 8.5. A pDNA solution (in 20 mM HEPES pH 7.4) was added to the liposomes dispersion (in 20 mM HEPES pH 7.4) and then vortexed for a few seconds as described previously (Sanders et al., 2001). The final plasmid concentration was 126 μg/ml and the final lipid concentrations were 2.21 mg/ml when DOTAP/DOPE liposomes were used, 2.46 mg/ml when 3.8% PEGylated DOTAP/DOPE liposomes were used and 2.82 mg/ml when 9% PEGylated DOTAP/DOPE liposomes were used.

For freeze thawing and freeze drying, 50 μl of the lipoplex dispersions were mixed with either 50 μl of an oligosaccharide solutions (12.6 wt% in water; thus final weight ratio oligosaccharides/plasmid was 1000) or 50 μl distilled water. Eppendorf cups (1.5 ml) were used for freeze thawing and glass vials (4 ml) were used for freeze drying.

2.4. Preparation of polyplexes

PEI and PEGylated PEI were used to prepare polyplexes at a nitrogen/phosphate ratio of 10 which corresponds to a weight ratio PEI/pDNA of 1.3 and PEGylated PEI/pDNA of 6.2. A solution of pDNA in 20 mM HEPES pH 7.4 was added to a solution of PEI or PEGylated PEI and then vortexed. The final pDNA concentration was 50 μg/ml while the final polymer concentrations were 65 μg/ml (PEI) or 308 μg/ml (PEGylated PEI).

For freeze thawing and freeze drying, 250 μl of the polyplex dispersions were mixed with either 250 μl 5.0 wt% solutions of oligosaccharides in water (thus the final weight ratio oligosaccharides/pDNA was 1000) or 250 μl distilled water. Eppendorf cups (1.5 ml) were used for freeze thawing and glass vials (4 ml) were used for freeze drying.

2.5. Freeze thawing

The samples were frozen in a refrigerator of −20 °C (sample temperature was decreased to below −15 °C within 20 min) and stored for 6 days. The liposomes and lipoplexes were rapidly thawed by adding 0.9 ml HEPES (20 mM pH 7.4) at room temperature and then analyzed. The polyplexes were thawed by placing the vials in a water bath of 37 °C.

2.6. Freeze drying

The samples were frozen by placing the glass vials in a refrigerator of −85 °C (sample temperature was decreased to below −50 °C within 10 min). After 16–24 h, the glass vials were placed in the freeze dryer (Amsco-Finn Aqua GT4 freeze dryer) and subsequently lyophilized for 2 h at shelf temperature of −35 °C and a pressure of 0.9 mBar. Then, the shelf temperature was raised to −15 °C while keeping the pressure the same. After 13 h the pressure was decreased to 0.15 mBar while the shelf temperature was gradually raised to 10 °C during 9 h. The condensor temperature was −60 °C during the whole freeze drying procedure. After freeze drying in the presence of oligosaccharides the samples appeared as porous cakes indicating a successful freeze drying procedure. Freeze dried liposomes and lipoplexes were rehydrated with 1.0 ml HEPES (20 mM, pH 7.4). Freeze dried polyplexes were rehydrated with 0.5 ml distilled water.

2.7. Particle size measurements

The *z*-average particle size was determined by dynamic laser scattering (DLS) using a Malvern 4700 system equipped with a helium–neon laser (Malvern Ltd., Worcestershire, UK) at a temperature of 25 °C. The data were analyzed using the viscosity and refractive index of water. Control experiments showed that the presence of oligosaccharides at concentrations used in this study did not significantly change the viscosity and refractive index of the dispersing medium. The performance of the instrument was checked with polystyrene standard spheres with a particle size of 220 ± 6 nm (Duke Scientific Corporation, Palo Alto, CA, USA). As a measure of particle size distribution, the DLS instrument reports a polydispersity index (pd). This index ranges from 0 for a monodisperse sample up to 1 for an entirely polydisperse sample. All formulations were measured at least in duplicate and each measurement consisted of five runs. The results in this study were obtained using different batches for each type of nanoparticle. The particle size varied slightly from batch to batch. Therefore, the particle size after a particular treatment was expressed as a percentage of the size of the particles of the same batch before treatment. Samples were considered as "fully aggregated" when one of the three following observations was made: (1) large aggregates visible by eye, (2) a pd of 1 in more than two out of the five runs, (3) an increase in the *z*-average particle size by at least a factor three in combination with an average pd higher than 0.9.

2.8. Zeta potential measurements

The zeta potential of the particles was measured by determining their electrophoretic mobility using a Malvern zeta-sizer 2000 (Malvern Ltd.) at a temperature of 25 °C. The zeta potential was calculated by the Smoluchowski equation: $\zeta = 12.8 \times \mu_e$ in which ζ is the zeta potential and μ_e is the electrophoretic mobility. The performance of the instrument was verified using a dispersion of carboxyl modified polystyrene nanospheres with a zeta potential of −50 ± 5 mV (DTS5050, Malvern Ltd.). In

control experiments it was found that the presence of sugars at concentrations used in this study did not significantly change viscosity and refractive index of the dispersing medium. In this study the zeta potential of the nanoparticles after a particular treatment was expressed as the percentage of the zeta potential of same batch before treatment. All formulations were measured at least in duplicate and each measurement consisted of five runs. The zeta potential of samples that were considered as fully aggregated (see above) was not measured.

3. Results and discussion

3.1. Size and zeta potential of the nanoparticles

Size and zeta potential of the nanoparticles used in this study are listed in Table 1. Liposomes of varying compositions were prepared by the thin film-extrusion method yielding unilamellar particles with a diameter of 120–155 nm. The zeta potential of the DOTAP/DOPE liposomes was about 44 mV. Since DOPE, being a zwitter ion at pH 7.4, does not bear a net charge, the positive zeta potential can be ascribed to the positive charged amine group of DOTAP. The decrease in the zeta potential upon PEGylation can be ascribed to two different mechanisms. Firstly, DSPE–PEG contains a negatively charged phosphate group decreasing the zeta potential. Secondly, and most importantly, the PEG shell shifts the plane of shear around the nanoparticles further away from their surface through which the positive charge is increasingly neutralized by ions present in the dispersion medium (Riley et al., 1999; van Steenis et al., 2003). The zeta potential of the EPC/Chol liposomes was close to zero which was expected because both constituents do not bear a charge. The zeta potential of PEGylated EPC/Chol liposomes was slightly negative (about −11 mV) which can be ascribed to the negatively charged phosphate group of DSPE–PEG.

Table 1
Particle size and zeta potential of nanoparticles

	Particle size (nm)	pd	Zeta potential (mV)
DOTAP/DOPE liposomes	154 ± 3	0.13 ± 0.03	44 ± 13
3.8% PEGylated DOTAP/DOPE liposomes	126 ± 6	0.10 ± 0.01	22 ± 10
9% PEGylated DOTAP/DOPE liposomes	127 ± 1	0.08 ± 0.03	16 ± 1
EPC/Chol liposomes	142 ± 1	0.15 ± 0.04	−3 ± 1
10% PEGylated EPC/Chol liposomes	121 ± 14	0.06 ± 0.00	−11 ± 0
DOTAP/DOPE lipoplexes	231 ± 20	0.15 ± 0.01	47 ± 8
3.8% PEGylated DOTAP/DOPE lipoplexes	216 ± 36	0.19 ± 0.11	16 ± 5
9% PEGylated DOTAP/DOPE lipoplexes	189 ± 52	0.15 ± 0.10	13 ± 0
PEI polyplexes	85 ± 11	0.26 ± 0.08	nd
PEGylated PEI polyplexes	135 ± 4	0.47 ± 0.02	nd

Complexation of DOTAP/DOPE, 3.8% PEGylated DOTAP/DOPE and 9% PEGylated DOTAP/DOPE liposomes with pDNA at a +/− charge ratio of four yielded lipoplexes with a diameter of 190–230 nm. The much larger size of the lipoplexes compared to the size of the liposomes from which they were prepared, indicates that the lipoplex contains more than one of the original liposome. The zeta potential of the (PEGylated) lipoplexes did not differ significantly from the zeta potential of the (PEGylated) liposomes. This indicates, as also observed by others, that most of the pDNA is incorporated in the core and not exposed at the surface of the complex (Sanders et al., 2001; Zelphati and Szoka, 1996).

Complexing PEI with pDNA at a nitrogen/phosphate ratio of 10 yielded polyplexes that were much smaller (about 85 nm) than the lipoplexes. Many other researchers have also found that polyplexes are often smaller than lipoplexes (Cherng et al., 1999; De Smedt et al., 2000; Kuo and Hwang, 2004; van de Wetering et al., 1999). PEGylated PEI polyplexes prepared at the same charge ratio were substantially larger (about 135 nm). The zeta potential of the polyplexes could not be measured properly because of irreproducible results most likely due to an insufficient intensity of the scattered light (as also experienced by Riley et al., 1999).

3.2. Behavior of DOTAP/DOPE liposomes upon freeze thawing and drying

Without stabilizer not only the DOTAP/DOPE liposomes but also the PEGylated DOTAP/DOPE liposomes fully aggregated both upon storage of the frozen dispersions for 6 days at −20 °C as well as upon freeze drying (see Fig. 1a and b). Aggregation of the DOTAP/DOPE liposomes is not surprising since it has been shown in many studies that non-PEGylated liposomes require a protectant during freeze thawing and freeze drying (Crowe et al., 1985; Madden et al., 1985). In contrast, to our knowledge no such studies have been published on PEGylated liposomes. Due to steric stabilization by the PEG shell, increased stability may have been envisaged. However, as reported for other types of PEGylated particles (Armstrong et al., 2002; De Jaeghere et al., 1999, 2000; Lenter et al., 2004; Ogris et al., 2003; Zambaux et al., 1999), cry- or lyoprotection seem to be required during, respectively, freeze thawing and freeze drying of PEGylated DOTAP/DOPE liposomes.

As shown in Fig. 1a and b, both inulins and dextrans were able to prevent full aggregation of DOTAP/DOPE liposomes upon storage of the frozen dispersions at −20 °C or freeze drying as the particle size was 100–200% of their original value. The particles sizes were substantially smaller after freeze drying than after storage at −20 °C. These differences may be explained as follows. Preceding storage at −20 °C for 6 days, the dispersions were frozen by placing them at −20 °C. Consequently, the cooling rate was relatively low (see Section 2). The dispersions to be freeze dried, however, were rapidly cooled by placing them at −85 °C. Possibly, during slow cooling to −20 °C, there is sufficient time available for moderate diffusion and conse-

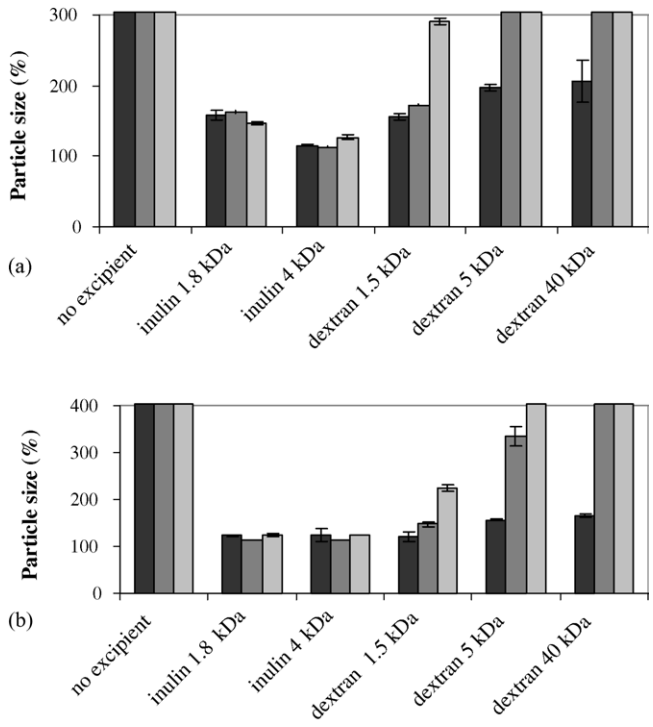

Fig. 1. (a) Particle size, as percentage of the value before storage, after storage at −20 °C of the frozen dispersions for 6 days of DOTAP/DOPE liposomes (■), 3.8% PEGylated DOTAP/DOPE liposomes (▩) and 9% PEGylated DOTAP/DOPE liposomes (□). Fully aggregated liposomes are indicated by bars that pass the upper axis. (b) Particle size, as percentage of the value before storage, after freeze drying of DOTAP/DOPE liposomes (■), 3.8% PEGylated DOTAP/DOPE liposomes (▩) and 9% PEGylated DOTAP/DOPE liposomes (□). Fully aggregated liposomes are indicated by bars that pass the upper axis.

quently limited aggregation during freeze concentration, which is apparently not the case when rapidly cooled to −85 °C. Since the particles are not only exposed to freezing stress but also to drying stress during freeze drying, the results indicate that inulin and dextran are not only excellent cryoprotectants but also excellent lyoprotectants for the DOTAP/DOPE liposomes.

PEGylation of the DOTAP/DOPE liposomes did not interfere with the stabilizing capacities of inulins for these type of particles (see Fig. 1a and b). When dispersed in dextran 1.5 kDa, 3.8% PEGylated DOTAP/DOPE liposomes also showed no full aggregation after storage for 6 days at −20 °C or freeze drying. However, 9% PEGylated DOTAP/DOPE liposomes dispersed in dextran 1.5 kDa substantially increased in size during these treatments. Both dextrans of higher molecular weight could not prevent full aggregation of the two types of PEGylated DOTAP/DOPE liposomes.

In conclusion, the extent of aggregation of the DOTAP/DOPE liposomes increased with both increasing molecular weight of dextran and increasing degree of PEGylation of the DOTAP/DOPE liposomes. Dextran 1.5 kDa shows some stabilizing capacity for PEGylated DOTAP/DOPE liposomes while dextran 5 and 40 kDa do not. This can be explained by the fact that, although dextran 1.5 kDa and PEG are incompatible (Hinrichs et al., 2005), the degree of incompatibility of dextran and PEG increases with increasing molecular weight of dextran (Stenekes et al., 1998). Moreover, with increasing degree of PEGylation the effects of incompatibility is also more pronounced. Knowing that inulin and PEG are compatible while dextran and PEG are not, these results clearly support the proposed mechanism that oligosaccharides can only prevent aggregation of PEGylated nanoparticles when the oligosaccharide and PEG are compatible.

3.3. Behavior of EPC/Chol liposomes upon freeze thawing and drying

Similar to DOTAP/DOPE liposomes, EPC/Chol liposome dispersions without stabilizer fully aggregated upon storage of the frozen dispersions for 6 days at −20 °C (see Fig. 2a). In the presence of inulins or dextrans, only moderate aggregation of these liposomes was observed upon this treatment as the particle size increased to 150–180% of their original value. No significant differences between the inulins and dextrans were found indicating that the stabilizing capacities of both types of oligosaccharides are the same for EPC/Chol liposomes during freeze thawing.

PEGylated EPC/Chol liposomes are clearly more stabile as they did not grow when stored without stabilizer at −20 °C

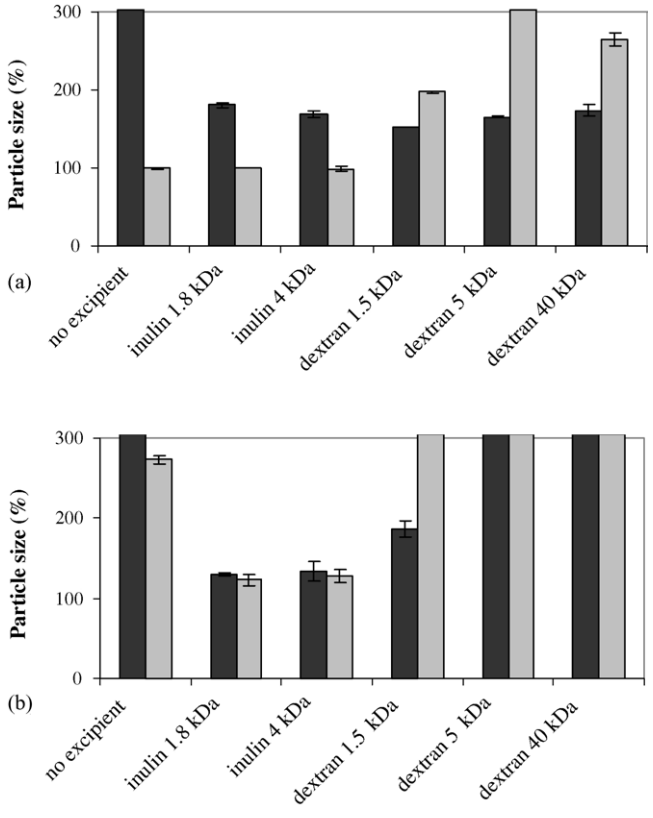

Fig. 2. (a) Particle size, as percentage of the value before storage, after storage at −20 °C of the frozen dispersions for 6 days of EPC/Chol liposomes (■) and 10% PEGylated EPC/Chol liposomes (□). Fully aggregated liposomes are indicated by bars that pass the upper axis. (b) Particle size, as percentage of the value before storage, after freeze drying of EPC/Chol liposomes (■) and 10% PEGylated EPC/Chol liposomes (□). Fully aggregated liposomes are indicated by bars that pass the upper axis.

for 6 days (see Fig. 2a). Two mechanisms may account for the increased stability of the PEGylated EPC/Chol liposome compared to its non-PEGylated counterpart. First, PEGylation results in an increased (negative) zeta potential (see Table 1). Consequently, electrostatic repulsion will increase the stability of the liposomes. Secondly, PEGylation results in steric stabilization. Although it has been found that steric stabilization does not guarantee maintenance of particle size during freeze thawing per se (e.g. results with PEGylated DOTAP/DOPE liposomes, see above), it may contribute to an increased stability in this particular case. Also dispersed in the inulins, PEGylated EPC/Chol liposomes did not show a significant increase in particle size upon storage of the frozen dispersions for 6 days at −20 °C (see Fig. 2a). However, when dispersed in dextrans, PEGylated EPC/Chol liposomes showed moderate (dextran 1.5 kDa) to full (dextran 5 and 40 kDa) aggregation. As PEGylated EPC/Chol liposomes dispersed in distilled water without stabilizer did not aggregate it can be concluded that dextrans are not only poor stabilizers for PEGylated EPC/Chol liposomes during freeze thawing, they even have an adverse effect as they actually induce aggregation.

Freeze drying gave somewhat different results (see Fig. 2b). It was found that both EPC/Chol liposomes and PEGylated EPC/Chol liposomes require a lyoprotectant as without stabilizer they fully aggregated during freeze drying. Apparently, freeze drying is a more stressful process for PEGylated EPC/Chol liposomes than storage for 6 days at −20 °C. Probably these nanoparticles did not aggregate during the freezing step (the freezing rate during the freeze drying procedure was much higher than in the freeze thawing process) but aggregation may have occurred during the drying phase. Apparently, the PEGylated EPC/Chol liposomes are in particular during dehydration susceptible to aggregation. Aggregation of both EPC/Chol liposomes and PEGylated EPC/Chol liposomes during freeze drying could be prevented by the inulins as the particle size increased to only 120–140% of the original values. In contrast, both EPC/Chol liposomes and PEGylated EPC/Chol liposomes dispersed in dextrans fully aggregated during freeze drying. Only EPC/Chol liposomes dispersed in a dextran 1.5 kDa solution showed moderate aggregation although also in this case the particle size was substantially larger than in case the liposomes were dispersed in the inulins (see Fig. 2b).

It can be concluded that dextrans are only good stabilizers when freeze thawing of EPC/Chol liposomes is concerned. In all other cases dextrans failed as a protectant against aggregation (except for dextran 1.5 kDa during freeze thawing of PEGylated EPC/Chol liposomes and freeze drying of EPC/Chol liposomes). By contrast, inulins are excellent stabilizers for both EPC/Chol liposomes and PEGylated EPC/Chol liposomes during freeze thawing and freeze drying. Again, these results are in agreement with the proposed requirement that oligosaccharides should be compatible with PEG when they are used for the stabilization of PEGylated nanoparticles. However, dextrans also showed poor stabilizing capacities for EPC/Chol liposomes during freeze drying. Most likely, the EPC/Chol liposomes and PEGylated EPC/Chol liposomes are highly susceptible to aggregation during dehydration. These results indicate that also for non-PEGylated nanoparticles inulins are preferred as stabilizers over dextrans.

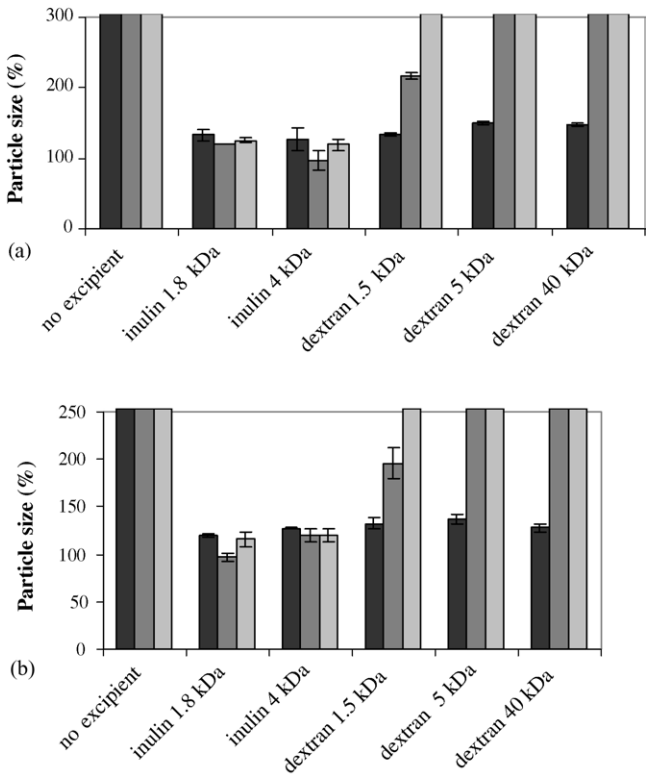

Fig. 3. (a) Particle size, as percentage of the value before storage, after storage at −20 °C of the frozen dispersions for 6 days of DOTAP/DOPE lipoplexes (■), 3.8% PEGylated DOTAP/DOPE lipoplexes (■) and 9% PEGylated DOTAP/DOPE lipoplexes (□). Fully aggregated lipoplexes are indicated by bars that pass the upper axis. Part of these results have been published before (Hinrichs et al., 2005). (b) Particle size, as percentage of the value before storage, after freeze drying of DOTAP/DOPE lipoplexes (■), 3.8% PEGylated DOTAP/DOPE lipoplexes (■) and 9% PEGylated DOTAP/DOPE lipoplexes (□). Fully aggregated lipoplexes are indicated by bars that pass the upper axis. Part of these results have been published before (Hinrichs et al., 2005).

3.4. Behavior of DOTAP/DOPE lipoplexes upon freeze thawing and drying

The aggregation behavior of DOTAP/DOPE lipoplexes during freeze thawing and freeze drying (see Fig. 3a and b) was similar to the behavior of DOTAP/DOPE liposomes (see Fig. 1a and b). Without stabilizer DOTAP/DOPE lipoplexes and the two types of PEGylated DOTAP/DOPE lipoplexes fully aggregated upon storage of the frozen dispersions for 6 days at −20 °C or freeze drying. In contrast, inulins were able to prevent complete aggregation of all three types of DOTAP/DOPE lipoplexes during these treatments as the particle size increased to only 100–130% of their original values. DOTAP/DOPE lipoplexes could also be stabilized by all three dextrans as only a slight increase in particle size was measured after storage of the frozen dispersions for 6 days at −20 °C or freeze drying. However, dextran 1.5 kDa was not able to preserve the particle size of 3.8% PEGylated DOTAP/DOPE lipoplexes as substantial aggregation was observed after these treatments. With increasing degree of

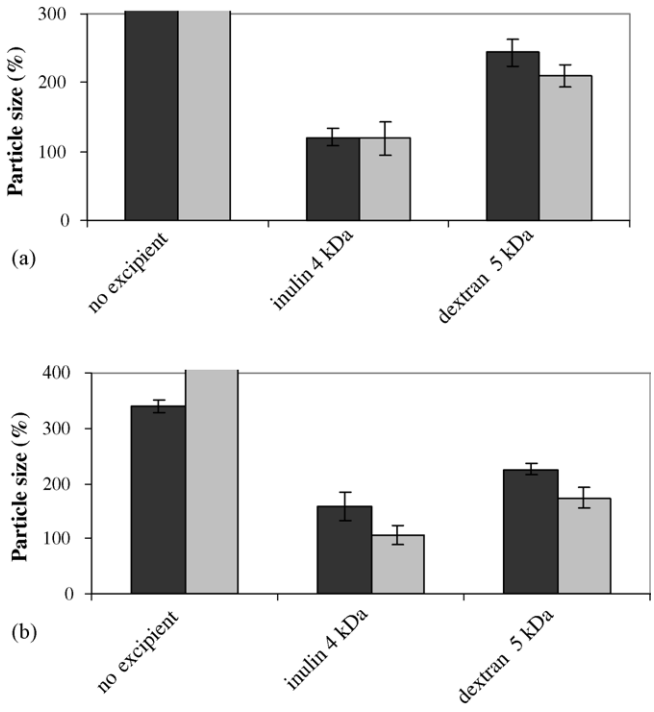

Fig. 4. (a) Particle size, as percentage of the value before storage, after storage at −20 °C of the frozen dispersions for 6 days of PEI polyplexes (■) and 10% PEGylated PEI polyplexes (□). Fully aggregated polyplexes are indicated by bars that pass the upper axis. (b) Particle size, as percentage of the value before storage, after freeze drying of PEI polyplexes (■) and 10% PEGylated PEI polyplexes (□). Fully aggregated polyplexes are indicated by bars that pass the upper axis.

PEGylation of the lipoplexes (9% PEGylated DOTAP/DOPE lipoplexes) aggregation could not be prevented by dextran 1.5 kDa. Furthermore, both dextran 5 and 40 kDa could not prevent full aggregation of both PEGylated lipoplexes. In conclusion, similar to what has been observed with DOTAP/DOPE liposomes, the effects of incompatibility between PEG and dextran are more pronounced when either or both the molecular weight of dextran and the degree of PEGylation increases.

3.5. Behavior of PEI polyplexes upon freeze thawing and drying

The effect of PEGylation of PEI on the aggregation behavior of PEI polyplexes in the presence of dextran or inulin during storage for 6 days at −20 °C or freeze drying was evaluated. Due to a limited availability of PEGylated PEI, only the negative control (no stabilizer), inulin 4 kDa and dextran 5 kDa were tested. As found by others (Lenter et al., 2004; Ogris et al., 2003), without stabilizer, both PEI and PEGylated PEI polyplexes fully aggregated during storage for 6 days at −20 °C or freeze drying indicating the necessity of using a protectant (see Fig. 4a and b). Complete aggregation of both PEI polyplexes and PEGylated PEI polyplexes during storage for 6 days at −20 °C or freeze drying could be prevented by both inulin 4 kDa and dextran 5 kDa. However, stabilization by inulin 4 kDa was somewhat better than by dextran 5 kDa as after treatment the polyplex size was 120–160 and 170–240% of their original values, respectively. Therefore, it can be concluded that inulin 4 kDa is a somewhat better protectant than dextran 5 kDa for PEI polyplexes. Furthermore, it can be concluded that PEGylation does not affect the aggregation behavior of PEI polyplexes, even if an oligosaccharide that is incompatible with PEG, i.e. dextran 5 kDa, is used as a protectant. These results are clearly anomalous to those obtained with the lipid based nanoparticles as described above. Possibly, the structural arrangement of the PEG chains at the surface of the polyplexes strongly deviate with that of the lipid based nanoparticles, which makes these PEGylated particles less vulnerable to the effects of incompatibility with dextran.

3.6. Effects of freeze thawing and drying on zeta potential of nanoparticles

The zeta potential of all batches, which were not fully aggregated during freeze thawing or freeze drying, were somewhat scattered and were 50–200% of the original values (data not shown). However, because there was no clear trend and given the accuracy of the apparatus (±5 mV), changes of the zeta potential induced by freeze thawing or freeze drying were not considered as being significant.

4. Conclusions

This study clearly illustrates that various PEGylated nanoparticles can be stabilized by oligosaccharides provided that the oligosaccharide is compatible with PEG. Inulins, which are compatible with PEG, were capable to prevent full aggregation of DOTAP/DOPE liposomes, EPC/Chol liposomes, DOTAP/DOPE lipoplexes and PEI polyplexes during freeze thawing or freeze drying, irrespective of the degree of PEGylation of these nanoparticles. By contrast, dextran is incompatible with PEG and its incompatibility increases with increasing molecular weight (Stenekes et al., 1998). As a consequence, the dextrans were not able to prevent full aggregation of the nanoparticles when they were PEGylated (except for PEI based polyplexes). Furthermore, stabilization by dextran became worse with increasing molecular weight of dextran and with increasing degree of PEGylation of the nanoparticles emphasizing the effects of compatibility on stability.

References

Albertsson, P.A., 1986. Partition of Cell Particles and Macromolecules. Wiley-Interscience, New York.

Allison, S.D., Molina, M.D.C., Anchordoquy, T.J., 2000. Stabilization of lipid/DNA complexes during the freezing step of the lyophilization process: the particle isolation hypothesis. Biochim. Biophys. Acta 1468, 127.

Armstrong, T.K., Anchordoguy, T.J., 2004. Immobilization of nonviral vectors during the freezing step of lyophilization. J. Pharm. Sci. 93, 2698.

Armstrong, T.K., Girouard, L.G., Anchordoquy, T.J., 2002. Effects of PEGylation on the preservation of cationic lipid/DNA complexes during freeze-thawing and lyophilization. J. Pharm. Sci. 91, 2549.

Bhadra, D., Bhadra, S., Jain, P., Jain, N.K., 2002. Pegnology: a review of PEG-ylated systems. Pharmazie 57, 5.

Brus, C., Kleemann, E., Aigner, A., Czubayko, F., Kissel, T., 2004. Stabilization of oligonucleotide-polyethylenimine complexes by freeze-drying:

physicochemical and biological characterization. J. Control. Release 95, 119.
Cherng, J.Y., Talsma, H., Verrijk, R., Crommelin, D.J., Hennink, W.E., 1999. The effect of formulation parameters on the size of poly-((2-dimethylamino)ethyl methacrylate)-plasmid complexes. Eur. J. Pharm. Biopharm. 47, 215.
Crowe, L.M., Crowe, J.H., Rudolph, A., Womersley, C., Appel, L., 1985. Preservation of freeze-dried liposomes by trehalose. Arch. Biochem. Biophys. 242, 240.
De Jaeghere, F., Allemann, E., Feijen, J., Kissel, T., Doelker, E., Gurny, R., 2000. Freeze-drying and lyopreservation of diblock and triblock poly(lactic acid)-poly(ethylene oxide) (PLA-PEO) copolymer nanoparticles. Pharm. Dev. Technol. 5, 473.
De Jaeghere, F., Allemann, E., Leroux, J.C., Stevels, W., Feijen, J., Doelker, E., Gurny, R., 1999. Formulation and lyoprotection of poly(lactic acid-co-ethylene oxide) nanoparticles: influence on physical stability and in vitro cell uptake. Pharm. Res. 16, 859.
De Smedt, S.C., Demeester, J., Hennink, W.E., 2000. Cationic polymer based gene delivery systems. Pharm. Res. 17, 113.
Heller, M.C., Carpenter, J.F., Randolph, T.W., 1997. Manipulation of lyophilization-induced phase separation: implications for pharmaceutical proteins. Biotechnol. Prog. 13, 590.
Heller, M.C., Carpenter, J.F., Randoph, T.W., 1999. Conformational stability of lyophilized PEGylated proteins in a phase-separated system. J. Pharm. Sci. 88, 58.
Heurtault, B., Saulnier, P., Pech, B., Proust, J.E., Benoit, J.P., 2003. Physicochemical stability of colloidal lipid particles. Biomaterials 24, 4283.
Hinrichs, W.L.J., Sanders, N.N., De Smedt, S.C., Demeester, J., Frijlink, H.W., 2005. Inulin is a promising cryo- and lyoprotectant for PEGylated lipoplexes. J. Control. Release 103, 465.
Kichler, A., 2004. Gene transfer with modified polyethylenimines. J. Gene Med. 6 (Suppl. 1), S3.
Kuo, J.H., Hwang, R., 2004. Preparation of DNA dry powder for non-viral gene delivery by spray-freeze drying: effect of protective agents (polyethyleneimine and sugars) on the stability of DNA. J. Pharm. Pharmacol. 56, 27.
Lenter, M.C., Garidel, P., Pelisek, J., Wagner, E., Ogris, M., 2004. Stabilized nonviral formulations for the delivery of MCP-1 gene into cells of the vasculoendothelial system. Pharm. Res. 21, 683.
Liao, Y.H., Brown, M.B., Nazir, T., Quader, A., Martin, G.P., 2002. Effects of sucrose and trehalose on the preservation of the native structure of spray-dried lysozyme. Pharm. Res. 19, 1847.
Madden, T.D., Bally, M.B., Hope, M.J., Cullis, P.R., Schieren, H.P., Janoff, A.S., 1985. Protection of large unilamellar vesicles by trehalose during dehydration: retention of vesicle contents. Biochim. Biophys. Acta 817, 67.
Mehnert, W., Mader, K., 2001. Solid lipid nanoparticles: production, characterization and applications. Adv. Drug Deliv. Rev. 47, 165.
Moghimi, S.M., 2002. Chemical camouflage of nanospheres with a poorly reactive surface: towards development of stealth and target-specific nanocarriers. Biochim. Biophys. Acta 1590, 131.
Molina, M.C., Armstrong, T.K., Zhang, Y., Patel, M.M., Lentz, Y.K., Anchordoguy, T.J., 2004. The stability of lyophilized lipid/DNA complexes during prolonged storage. J. Pharm. Sci. 93, 2259.
Monfardini, C., Veronese, F.M., 1998. Stabilization of substances in circulation. Bioconjug. Chem. 9, 418.
Nicolazzi, C., Mignet, N., de la Figuera, N., Cadet, M., Ibad, R.T., Seguin, J., Scherman, D., Bessodes, M., 2003. Anionic polyethyleneglycol lipids added to cationic lipoplexes increase their plasmatic circulation time. J. Control. Release 88, 429.
Ogris, M., Brunner, S., Schuller, S., Kircheis, R., Wagner, E., 1999. PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. Gene Ther. 6, 595.
Ogris, M., Walker, G., Blessing, T., Kircheis, R., Wolschek, M., Wagner, E., 2003. Tumor-targeted gene therapy: strategies for the preparation of ligand-polyethylene glycol-polyethylenimine/DNA complexes. J. Control. Release 91, 173.
Otsuka, H., Nagasaki, Y., Kataoka, K., 2003. PEGylated nanoparticles for biological and pharmaceutical applications. Adv. Drug Deliv. Rev. 55, 403.
Papahadjopoulos, D., 1996. Fate of liposomes in vivo: a brief introductory review. J. Liposome Res. 6, 3.
Photos, P.J., Bacakova, L., Discher, B., Bates, F.S., Discher, D.E., 2003. Polymer vesicles in vivo: correlations with PEG molecular weight. J. Control. Release 90, 323.
Riley, T., Govender, T., Stolnik, S., Xiong, C.D., Garnett, M.C., Illum, L., Davis, S.S., 1999. Colloidal stability and drug incorporation aspects of micellar-like PLA-PEG nanoparticles. Colloids Surf. B: Biointerf. 16, 147.
Sanders, N.N., De Smedt, S.C., Cheng, C.H., Demeester, J., 2002. Pegylated GL67 lipoplexes retain their gene transfection activity after exposure to components of CF mucus. Gene Ther. 9, 363.
Sanders, N.N., Van Rompaey, E., De Smedt, S.C., Demeester, J., 2001. Structural alterations of gene complexes by cystic fibrosis sputum. Am. J. Respir. Crit. Care Med. 164, 486.
Schwarz, C., Mehnert, W., 1997. Freeze-drying of drug-free and drug-loaded solid lipid nanoparticles (SLN). Int. J. Pharm. 157, 171.
Srinath, P., Diwan, P.V., 1994. Stealth liposomes—an overview. Indian J. Pharmacol. 26, 179.
Stenekes, R.J., Franssen, O., van Bommel, E.M., Crommelin, D.J., Hennink, W.E., 1998. The preparation of dextran microspheres in an all-aqueous system: effect of the formulation parameters on particle characteristics. Pharm. Res. 15, 557.
Strauss, G., Schurtenberger, P., Hauser, H., 1986. The interaction of saccharides with lipid bilayer vesicles: stabilization during freeze-thawing and freeze-drying. Biochim. Biophys. Acta 858, 169.
Sun, W.Q., Leopold, A.C., Crowe, L.M., Crowe, J.H., 1996. Stability of dry liposomes in sugar glasses. Biophys. J. 70, 1769.
Talsma, H., Cherng, J.-Y., Lehrmann, H., Kursa, M., Ogris, M., Hennink, W.E., Cotten, M., Wagner, E., 1997. Stabilization of gene delivery systems by freeze-drying. Int. J. Pharm. 157, 233.
van de Wetering, P., Moret, E.E., Schuurmans-Nieuwenbroek, N.M., van Steenbergen, M.J., Hennink, W.E., 1999. Structure-activity relationships of water-soluble cationic methacrylate/methacrylamide polymers for non-viral gene delivery. Bioconjug. Chem. 10, 589.
van Steenis, J.H., van Maarseveen, E.M., Verbaan, F.J., Verrijk, R., Crommelin, D.J.A., Storm, G., Hennink, W.E., 2003. Preparation and characterization of folate-targeted pEG-coated pDMAEMA-based polyplexes. J. Control. Release 87, 167.
van Winden, E.C., 2003. Freeze-drying of liposomes: theory and practice. Methods Enzymol. 367, 99.
Zambaux, M.F., Bonneaux, F., Gref, R., Dellacherie, E., Vigneron, C., 1999. MPEO-PLA nanoparticles: effect of MPEO content on some of their surface properties. J. Biomed. Mater. Res. 44, 109.
Zelphati, O., Szoka Jr., F.C., 1996. Mechanism of oligonucleotide release from cationic liposomes. Proc. Natl. Acad. Sci. U.S.A. 93, 11493.