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International Journal of Pharmaceutics 188 (1999) 87–95

international journal of pharmaceutics

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Factors affecting the size distribution of liposomes produced by freeze–thaw extrusion

Jonathan D. Castile, Kevin M.G. Taylor *

Department of Pharmaceutics, School of Pharmacy, University of London, Brunswick Square, London WC1N 1AX, UK

Received 22 March 1999; received in revised form 25 May 1999; accepted 9 June 1999

Abstract

This paper describes the development of a protocol for the production of liposomes using a freeze–thaw extrusion methodology. Laser diffraction particle size analysis showed that the median diameter of freeze–thawed egg phosphatidylcholine multilamellar vesicles (eggPC MLVs) was increased when cholesterol was included in the bilayers. Using a freeze–thaw cycle of 3 min freezing in liquid nitrogen at −196°C followed by 3 min thawing at 50°C resulted in an anomalously large particle size for eggPC/cholesterol formulations. When liposomes were repeatedly freeze–thawed a maximum size was achieved after five freeze–thaw cycles. Dispersion of liposomes in sodium chloride solutions promoted size increases following freeze–thawing, suggesting that vesicles had aggregated or fused. Poloxamers P338 and P407 inhibited the size increases observed during freeze–thawing for eggPC MLVs dispersed in 1.0 M NaCl, probably through steric prevention of aggregation and fusion.

Keywords: Extrusion; Freeze–thaw; Liposome; Particle size; Poloxamer

1. Introduction

Much of the potential damage to liposomes during freezing and subsequent thawing is directly related to the behaviour of water in the dispersions (Talsma et al., 1991). Repeated freezing and thawing of multilamellar vesicles (MLVs) produces physical disruption of the liposomal phospholipid bilayers, probably due to ice crystals formed during the freezing process. This also serves to break apart the closely spaced lamellae of the vesicles thereby raising the trapping efficiency by increasing the ratio of aqueous solute to lipid (Hope et al., 1985; Mayer et al., 1985). Extrusion of frozen and thawed MLVs (FATM-LVs) results in production of unilamellar liposomes more readily than those made by conventional techniques (Mayer et al., 1986). Elorza et al. (1993) showed, using 5,6 carboxyfluorescein as an aqueous marker, that frozen and thawed extruded liposomes were a monodispersed population with an internal volume higher than LUVs prepared solely by extrusion of MLVs through polycarbonate filters of equivalent pore size.

* Corresponding author. Tel.: +44-171-753-5853; fax: +44-171-753-5920.

E-mail address: ktaylor@cua.ulsop.ac.uk (K.M.G. Taylor)

0378-5173/99/$ - see front matter
PII: S0378-5173(99)00207-0

Cooling rate, liposome size, phospholipid concentration and localisation of any additives, all affect the crystallization behaviour of water in liposome dispersions (Talsma et al., 1991). Fast freezing rates impose a greater force of stress on liposomes than slower rates because intra-liposomal ice formation is likely at freezing rates greater than 10°C/min, leading to expansion of vesicles' aqueous phase (Özer et al., 1988). For cooling rates faster than 20°C/min, the probability of intra-liposomal ice formation is 100%. Thawing is also an important parameter because the freeze–thawing is only effective at improving MLV swelling, and hence aqueous entrapment values, if the sample is thawed at temperatures above the temperature of the phospholipid main transition (T_c) of the liposome suspension (Hope et al., 1986; MacDonald and MacDonald, 1993).

Little has been published about the effect of varying the number of freeze–thaw cycles on the structure of liposomes. ^{31}P NMR has been used to demonstrate the influence of the number of freeze–thaw cycles on distribution of lipid within liposomes, with an equilibrium distribution between lipid and solute (Mn^{2+}) being observed after five cycles (Mayer et al., 1985). Hope et al. (1986) demonstrated that the trapped volume of egg phosphatidylcholine (eggPC) MLVs increased in relation to the number of freeze–thaw cycles. A maximum trapped volume of 9 μl water per μmol of lipid was recorded after nine cycles, after which no further increase in trapped volume was observed. No data have been published reporting the effect of varying the time length of each freeze–thaw cycle. However, Fransen et al. (1986) observed a time-dependent leakage of a drug from a liposome suspension at −30°C, but below −40°C (homogeneous nucleation temperature of water) the dispersion was shown to be stable over a long period.

Cryoprotective agents such as trehalose (and other disaccharides), dimethyl sulphoxide and glycerol are known to protect phospholipid bilayers from damage during freeze–thawing and freeze–drying. Cryoprotectants are thought to depress the phospholipid T_c, bind water, and interact with polar head groups of phospholipids. Water molecules associated with polar head groups of hydrated phospholipid bilayers may be replaced by sugar molecules which protect liposomes from aggregation and fusion during freeze–thawing or freeze–drying by holding individual vesicles in a vitrified matrix (Crowe et al., 1987, 1988). Certain cryoprotectants, such as trehalose, have also been shown to maintain phospholipids in a fluid-like state in the absence of water, avoiding passage through the gel to liquid-crystalline phase transition and subsequent bilayer disruption during freeze–drying (Crowe et al., 1987), and may prevent fusion of vesicles by decreasing the surface tension at the liposome surface (Koster et al., 1994).

The purpose of this study was to systematically develop a protocol for the production of freeze–thaw extruded MLVs. The inclusion in the liposome dispersions of sodium chloride and poloxamer surfactants was also investigated. Poloxamers are non-ionic surface active ABA block copolymers having two hydrophilic polyoxyethylene (POE) moieties and a hydrophobic polyoxypropylene (POP) moiety. They reduce phagocytosis when included in model colloidal systems (Illum et al., 1987; Rudt and Müller, 1993), and interact with the bilayers of liposomes to some extent, although the nature of the interaction is not well understood (Jamshaid et al., 1988; Moghimi et al., 1991; Kostarelos et al., 1995; Castile et al., 1999).

2. Materials and methods

2.1. Development of a freeze–thaw protocol for liposome preparation

EggPC (Lipoid, Italy) was refined by chromatographic purification (Bangham et al., 1974). MLVs were prepared as previously described (Castile et al., 1999) by weighing appropriate amounts of eggPC (up to 300 mg), with or without cholesterol (99%+ purity, Sigma, UK) at a 1:1 mol ratio, into a round-bottomed flask, and adding chloroform (HiPerSolv, BDH, UK) to dissolve the phospholipid. Chloroform was removed by rotary evaporation under vacuum,

in a water bath at 55°C for 15 min. The flask was then flushed with nitrogen for 1–2 min to remove traces of residual solvent. An appropriate volume of filtered (100-nm pore filter [Poretics, USA]), bi-distilled, deionised water (Model WP 700, Whatman, UK), further purified by passing through an Elgastat Ultra High Quality Purification System (Elga, UK) was added to the dry film in the flask to give a final phospholipid concentration of 10 mg/ml. Glass beads were added to aid dispersion, the flask flushed with nitrogen, gently rotated for 30 min in the water bath, and shaken to produce MLVs. The suspension was annealed for a further 2 h in the water bath before storage under nitrogen in a refrigerator at 4°C.

2.2. The effect of repeated heating and cooling on the thermal profile of liposomes

EggPC and eggPC/chol (1:1) MLVs were extruded 10 times through a 2-μm polycarbonate filter (Cyclopore, UK), held in a 25-mm syringe filter holder, to standardize the size of the liposome population, and sized by laser Fraunhofer diffraction using a magnetically stirred cell and a 63-mm lens (Malvern 2600C, Malvern Instruments, UK). The instrument's software expresses particle size as the volume median diameter (VMD), i.e. the equivalent sphere diameter above and below which 50% of the volume of particles lies and the size distribution is expressed as a span value [(90% undersize − 10% undersize)/50% undersize)]. Sizing data are presented as the mean ± S.D. of three independent analyses.

Aliquots from each dispersion (4 ml) were dispensed into 20-ml liquid scintillation vials (FBG-Trident, UK.) and quench frozen in liquid nitrogen at −196°C for the specified duration (1–5 min). The vials were transferred immediately to a water bath and held at 50°C for an equal duration. The freezing and thawing process was repeated for a specified number of cycles. The FATMLVs were kept at room temperature in sealed containers for 1 h before size analysis by laser diffraction. All experiments were performed in triplicate.

2.3. Standardised freeze–thaw procedure

Subsequent experiments used a standardised freeze–thaw protocol to investigate the influence of external agents on the size distribution of freeze–thawed liposomes. MLVs were extruded through polycarbonate filters, as described in Section 2.1. Each dispersion (4 ml) was frozen for 3 min at −196°C in liquid nitrogen and then thawed for 3 min at 50°C in a water bath. The cycle was repeated a further four times. In additional experiments eggPC or eggPC/chol MLVs, produced by dispersion of lipid mixtures in aqueous sodium chloride (BDH, UK) solutions at concentrations of 0.1, 0.5 and 1.0 M NaCl, were subjected to the freeze–thaw protocol.

2.4. The effect of poloxamer concentration on the size distribution of FATMLVs

EggPC MLVs were produced using 1.0 M NaCl solution as the aqueous phase, and extruded through a 2-μm pore filter as described in Section 2.1. Poloxamers P338 (ICI, France) and P407 (Blagden Chemicals, UK) were added to some formulations at concentrations of 0.05, 0.1, 0.2, 0.3, 0.5 and 1.0% w/v and incubated at 25°C for 2 h in a shaking water bath (Grant Instruments, UK) before being subjected to five freeze–thaw cycles, as described in Section 2.2.

3. Results and discussion

3.1. Parameters associated with freeze–thaw extrusion

The mean VMD of eggPC MLVs was not altered by freeze–thawing, and the duration of each freeze–thaw cycle had no clear effect on the mean VMD of eggPC FATMLVs (Fig. 1). Small but significant increases in diameter were detected following 1- and 3-min freeze–thaw cycles but not at other cycle lengths ($P < 0.05$).

The mean VMD of all eggPC/chol MLVs significantly increased following repeated freeze–thawing ($P < 0.05$) (Fig. 1), with the greatest size increase observed for samples subjected to ten

'3-min' freeze–thaw cycles. The analysis was repeated an additional five times for this data point (i.e. $n = 8$), and the observed increase in size was reproduced, suggesting that the presence of cholesterol resulted in the formation of larger liposomes or aggregates during freeze–thawing, particularly with a 3-min cycle. Cholesterol modifies the physical structure of phospholipid bilayers, restricting bilayer permeability and the movement of the hydrocarbon chains at temperatures above the T_c (Oldfield and Chapman, 1972). Therefore eggPC/chol bilayers are more rigid than eggPC bilayers. The inclusion of cholesterol reduces the water permeability and compressibility of liposome bilayers (Finklestein and Cass, 1967), decreasing their ability to withstand internal expansion due to ice formation during fast-freezing (Morris and McGrath, 1981; MacDonald and MacDonald, 1993; van Winden et al., 1997), and causing bilayers to rupture during freeze–thawing, with new liposomes forming as bilayer fragments re-assemble. In addition, cholesterol has been reported to induce aggregation and fusion on freeze–thawing of DPPC liposomes (Henry-Michellard et al., 1985). The observed increase in mean size for eggPC MLVs when cholesterol was included indicates that larger structures were formed, which may have been due to aggregation and subsequent fusion of vesicles.

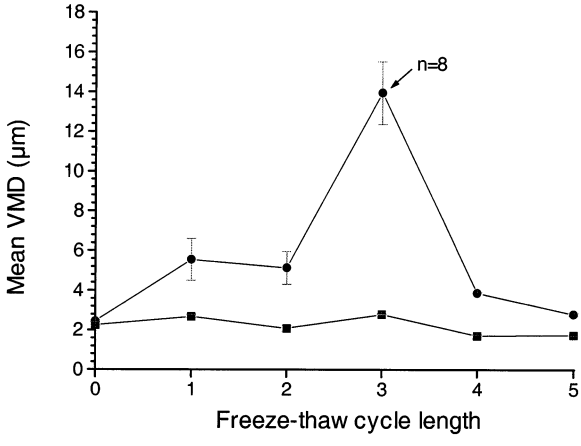

Fig. 1. Influence of freeze–thaw cycle length on the mean VMD of MLVs extruded 10 times through a 2-μm pore filter then freeze–thawed 10 times. ■, eggPC; ●, eggPC/chol (1:1). Freeze–thaw cycle length is the number of minutes preparations were held at −196°C and subsequently at 50°C ($n = 3 \pm$ S.D.).

It was observed macroscopically, that samples exposed to 1- and 2-min freeze–thaw cycles did not completely melt during the thaw stage of each cycle. Consequently, a proportion of vesicles may not have been exposed to damaging conditions during re-freezing. Preparations exposed to 3-min freeze–thaw cycles did melt completely during each thaw stage. However, the preparations may not have been warmed completely, with incomplete mixing, possibly resulting in insufficient time for disrupted bilayers to re-anneal. Therefore liposome fragments may have suffered further freeze–thaw damage before re-assembling. Vesicles exposed to 4- and 5-min freeze–thaw cycles had more time to re-assemble during each thaw stage and this may have been reflected by smaller increases in mean diameter.

Extruded liposomes had a relatively homogeneous size distribution, with small distribution span values of 0.83 ± 0.02 and 0.99 ± 0.03 for eggPC and eggPC/chol preparations respectively. Freeze–thawing increased polydispersity, with the mean distribution spans of freeze–thawed eggPC and eggPC/chol MLVs significantly higher ($P < 0.05$) than those of non-freeze–thawed preparations, with the exception of preparations subjected to the '2-min' freeze–thaw protocol. Span values for eggPC preparations ranged from 1.36 to 1.61 and for eggPC/chol MLVs from 1.36 to 1.77.

3.2. Effect of changing the number of freeze–thaw cycles

The mean VMDs of eggPC MLVs increased after one freeze–thawing cycle, but did not change significantly ($P < 0.05$) between one and 20 cycles. However, the mean VMD of eggPC/chol MLVs increased following the process up to five cycles (Fig. 2). Subsequent increases in the number of cycles produced no significant change in vesicle diameter ($P < 0.05$), indicating an equilibrium situation after which further freeze–thaw cycles had little or no effect on the mean diameter for these liposomes.

After one freeze–thawing cycle, the mean distribution spans of eggPC and eggPC/chol MLVs

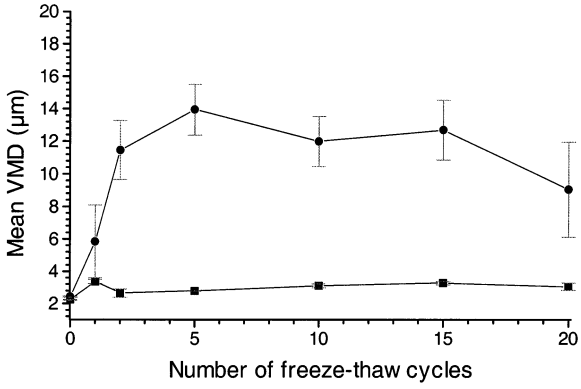

Fig. 2. Influence of the number of '3-min' freeze–thaw cycles on the mean VMD of MLVs (previously extruded 10 times through a 2-μm pore filter). ■, eggPC; ●, eggPC/chol (1:1) ($n = 3 \pm$ S.D.).

extruded through 2-μm pore filters were significantly increased ($P < 0.05$) from 0.83 ± 0.02 to 1.39 ± 0.05 for eggPC MLVs and from 0.99 ± 0.03 to 1.51 ± 0.05 for eggPC/chol MLVs. The eggPC/chol liposomes exhibited greater increases in mean distribution span between one and 20 freeze–thaw cycles than the eggPC MLVs. Span values after 20 cycles were 1.60 ± 0.04 and 1.99 ± 0.26 for eggPC and eggPC/chol preparations respectively. Thus the increased rigidity of eggPC bilayers due to the inclusion of cholesterol seemed to have a destabilizing effect during freeze–thawing, causing the liposomes to rupture and subsequently re-assemble to form a new population of liposomes with a higher mean VMD and wider and more variable size distribution.

3.3. Effect of preparing liposomes in sodium chloride solutions

The mean VMD of eggPC MLVs dispersed in NaCl solutions increased after five freeze–thawing cycles by a greater extent than liposomes dispersed in water ($P < 0.05$) (Fig. 3). Mean distribution spans of all preparations dispersed in NaCl solutions increased significantly; the increase in distribution span for MLVs dispersed in 0.5 M NaCl (for instance, an increase from 0.82 ± 0.06 before freeze–thawing to 1.55 ± 0.15 after freeze–thawing was typical). Freeze–thawing dioleoylphosphatidylcholine MLVs for 10 cycles in 0.1 M NaCl solutions has previously been reported to result in their fragmentation into a population of smaller vesicles having a mean size less than 200 nm (MacDonald et al., 1994). Such fragmentation was not observed when water was used as the aqueous phase, suggesting that an osmotic removal of water from the vesicles, in the presence of electrolyte solutions, was responsible for the dehydration and subsequent fragmentation of bilayers. Such fragmentation, though to a lesser extent, has also been described for other phospholipids (Morris and McGrath, 1981), whilst loss of the barrier properties of bilayers during freeze–thawing, without major fragmentation has also been reported (Oku and MacDonald, 1983; Mayer et al., 1985). Freeze–thawing eggPC in the presence of high concentrations (> 1 M) of some alkali metal chlorides has been reported to result in the production of 'giant' uni- and oligolamellar liposomes having diameters greater than 10 μm (Oku and MacDonald, 1983). The tendency for large liposomes to be formed depends on the alkali metal chloride being used, such vesicles being most prevalent with KCl and RbCl, and on the eutectic temperature of the frozen alkali metal chloride solutions. 'Giant' liposomes of eggPC were produced in the presence of NaCl, but at higher concentrations of

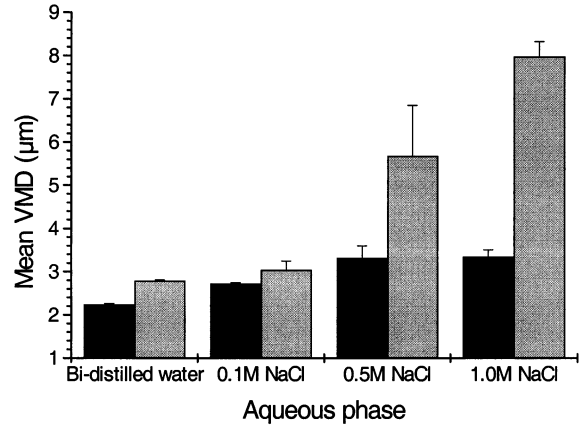

Fig. 3. Influence of NaCl concentration on the mean VMD of eggPC MLVs before and after five freeze–thaw cycles. ■, before freeze–thawing; ▨, after freeze–thawing ($n = 3 \pm$ S.D.).

both salt and phospholipid than used in the present studies (Oku and MacDonald, 1983).

EggPC MLVs reversibly aggregate in the presence of alkali metal cations, including Na^+ (Nagata et al., 1986; Mosharraf et al., 1995). Hydration forces (due to adsorption of hydroxyl groups) are the main repulsive force in dispersions of eggPC MLVs (Lis et al., 1982; Mosharraf et al., 1995). Close association is a prerequisite of vesicle fusion. These results may indicate that liposomes suspended in NaCl can locate in closer proximity to neighbouring vesicles than those dispersed in bi-distilled water due to reduction of the net negative charge by the presence of Na^+ ions. When bilayer damage occurs during freeze–thawing, damaged vesicles will aggregate and possibly fuse with vesicles with which they come into contact (Lasic, 1988). Additionally, exposure to high temperatures during thawing provides thermal energy for liposomes to become closely associated, and thus would be more likely to overcome repulsive, hydration forces and so be more likely to aggregate.

Addition of electrolyte after liposome manufacture provides an osmotic gradient and promotes an efflux of water from the aqueous compartments of vesicles. Even though liposomes were hydrated in NaCl solutions, an osmotic gradient may exist because phospholipid bilayers are semi-permeable membranes in which the outer bilayers hinder the progress of solutes to the inner aqueous compartments resulting in non equilibrium solute distributions and an osmotic gradient across the bilayer (Gruner et al., 1985). Freeze–thawing of MLVs produces equilibrium distributions of solute, and consequently enhances vesicle swelling (Mayer et al., 1985). Efflux of water due to solute imbalances prior to freeze–thawing could cause osmotic shrinkage of vesicles, producing areas where the bilayer dilates or shrinks, causing local changes to the area occupied by each phospholipid molecule with a significant effect on bilayer stability.

3.4. Effect of poloxamer concentration on the size distribution of FATMLVs

A 1.0 M NaCl solution was used as the aqueous phase in this study since it enhanced the freeze–thaw induced increase in mean diameter eggPC MLVs (Fig. 3). Following extrusion through 2-μm filters, and prior to freeze–thawing, all samples had mean VMDs of between 2.4 and 3.2 μm. The mean VMD of poloxamer-free samples was slightly higher than all poloxamer-containing samples, possibly due to aggregation before freeze–thawing. Differences, though small, were significant at all concentrations of P407 and $>0.1\%$ w/v P338 ($P<0.05$).

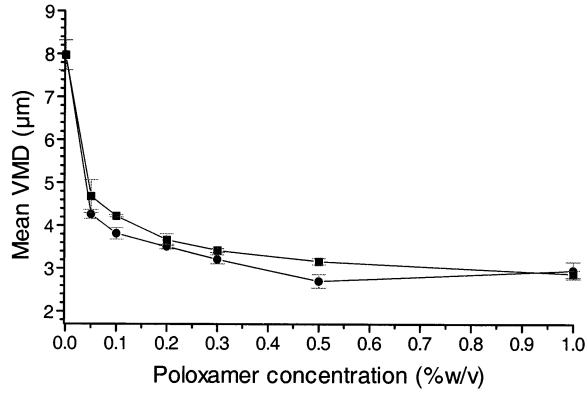

Fig. 4. Effect of poloxamer concentration on the mean VMD of eggPC MLVs following five freeze–thaw cycles. ■, P338; ●, P407 ($n=3\pm$S.D.).

Fig. 4 shows the mean VMDs of eggPC FATMLVs after five freeze–thaw cycles. The poloxamer-free sample had a mean VMD of 8.0 ± 0.4 μm following freeze–thawing. Inclusion of poloxamer P338 or P407 inhibited the size increase associated with freeze–thawing. Low poloxamer concentrations were less effective than higher concentrations at inhibiting the size increase, but the median sizes of all poloxamer-containing formulations were significantly smaller than the poloxamer-free preparations ($P<0.05$). There was no significant difference ($P<0.05$) in the mean VMD of formulations containing 0.5 and 1.0% w/v of either poloxamer.

The size distribution of FATMLVs was less polydispersed as poloxamers concentrations were increased from 0 to 0.3% w/v, but then the distribution span increased as poloxamer concentrations were increased further (Fig. 5). On closer

analysis of the data (not presented), it was apparent that this was due to a reduction of the 10% undersize value (the diameter below which 10% of the volume of particles lies). The 90% undersize value of these formulations was similar to that of samples containing lower concentrations of poloxamers, which indicates that the increase in span at higher poloxamer concentrations is caused by the formation of smaller vesicles.

The presence of poloxamer molecules within liposome bilayers may sterically inhibit contact between two vesicles and subsequently prevent aggregation and fusion. As poloxamer concentration was increased, vesicles may have been increasingly inhibited from associating with other vesicles during bilayer repair. This would account for the correlation between the increasing presence of small vesicles at higher poloxamer concentration. It is also possible that higher poloxamer concentrations may destabilize phospholipid membranes, making them more susceptible to freeze–thaw damage although still inhibited from fusing with other liposomes. Jamshaid et al. (1988) showed that bilayer permeability of eggPC SUVs was increased by the presence of poloxamers, possibly due to the formation of 'pores' or regions of enhanced membrane fluidity caused by inclusion of poloxamer within the bilayer.

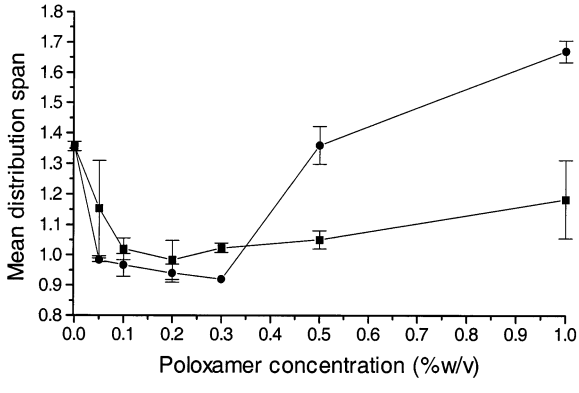

Fig. 5. Effect of poloxamer concentration on the mean distribution span of eggPC MLVs following five freeze–thaw cycles. ■, P338; ●, P407 ($n = 3 \pm$ S.D.).

4. Conclusion

The median size and size distribution of 2 μm eggPC MLVs increased following freeze–thawing, suggesting that a new population of liposomes had been formed. Inclusion of cholesterol further enhanced these increases, possibly due to aggregation and fusion of vesicles, indicating that it had a destabilizing influence on eggPC bilayers during the freeze–thaw procedure.

Liposome bilayers are damaged by internal ice formation during freezing. Bilayers may break off or partially fragment. Damaged bilayers will reassemble due to the 'hydrophobic effect' and form new liposomes possibly of a different size. Reformation may involve fusion with other liposomes (Lasic, 1988). Each freeze–thaw cycle would provide an opportunity for this process to occur, both in previously unaffected liposomes and those damaged by a previous freeze–thaw cycle. However, the proportion of vesicles that are affected and the extent of the damage to each bilayer cannot be determined from size analysis.

Preparations dispersed in NaCl solutions increased in diameter following freeze–thawing to a greater extent than those dispersed in water. It seems likely that if there was any repulsion between liposomes dispersed in bi-distilled water (from hydration or electrostatic forces), it was inhibited by NaCl, resulting in increased aggregation and fusion of vesicles as bilayers recovered from freeze–thaw damage. Significantly, NaCl may have caused an osmotic gradient across liposome bilayers resulting in areas of bilayer instability due to osmotic shrinkage of vesicles. These areas may be more susceptible to freeze–thaw damage. Poloxamers P338 and P407 inhibited the size increases observed during freeze–thawing for eggPC MLVs dispersed in 1.0 M NaCl. This suggests that the poloxamers may have sterically stabilized the liposome formulations. Poloxamers may associate with the surface of liposomes, possibly through penetration of the POP moiety into the hydrophobic region of the phospholipid bilayer or by adsorption to the liposome surface (Castile et al., 1999). Liposomes may still have been disrupted during freeze–thawing, but the presence of strongly hydrated POE groups ex-

tending from the liposome surface may have sterically inhibited contact between vesicles. Subsequently aggregation and fusion associated with freeze–thawing may then be inhibited. A greater proportion of small vesicles was detected as the poloxamer concentration was increased, suggesting that vesicles were still disrupted by freeze–thawing, but may have been increasingly inhibited from associating with other vesicles during bilayer repair.

Acknowledgements

The authors wish to thank BBSRC for financial support.

References

Bangham, A.D., Hill, M.W., Miller, N.G.A., 1974. Preparation and use of liposomes as models of biological membranes. Methods Membr. Biol. 1, 56–57.

Castile, J.D., Taylor, K.M.G., Buckton, G., 1999. A high sensitivity differential scanning calorimetry study of the interaction between poloxamers and dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine liposomes. Int. J. Pharm. 182, 101–110.

Crowe, J.H., Crowe, L.M., Carpenter, J.F., Wistrom, C.A., 1987. Stabilization of dry phospholipid bilayers and proteins by sugars. Biochem. J. 242, 1–10.

Crowe, J.H., Crowe, L.M., Carpenter, J.F., Rudolph, A.S., Wistrom, C.A., Spargo, B.J., Anchordoguy, T.J., 1988. Interactions of sugars with membranes. Biochim. Biophys. Acta 947, 367–384.

Elorza, B., Elorza, M.A., Sainz, M.C., Chantrez, J.R., 1993. Comparison of particle size and encapsulation parameters of three liposomal preparations. J. Microencapsul. 10, 237–248.

Finklestein, A., Cass, A., 1967. The effect of cholesterol on the water permeability of thin lipid membranes. Nature 216, 717–718.

Fransen, G.J., Salemink, P.T.M., Crommelin, D.J.A., 1986. Critical parameters in freezing of liposomes. Int. J. Pharm. 33, 27–35.

Gruner, S.M., Lenk, A.S., Janoff, A.S., Ostro, M.J., 1985. Novel multilayered lipid vesicles: comparison of physical characteristics of multilamellar liposomes and stable plurilamellar vesicles. Biochemistry 24, 2833–2842.

Henry-Michellard, S., Ter-Minassian-Saraga, L., Poly, P.A., Delattre, J., Pusieux, F., 1985. Lyophilization and rehydration of liposomes. Colloids Surfaces 14, 269–276.

Hope, M.J., Bally, M.B., Webb, G., Cullis, P.R., 1985. Production of large unilamellar vesicles by a rapid extrusion procedure. Characterisation of size distribution, trapped volume and ability to maintain a membrane potential. Biochim. Biophys. Acta 812, 55–65.

Hope, M.J., Bally, M.B., Mayer, L.D., Janoff, A.S., Cullis, P.R., 1986. Generation of multilamellar and unilamellar phospholipid vesicles. Chem. Phys. Lipids 40, 89–107.

Illum, L., Jacobsen, L.O., Müller, R.H., Mak, E., Davis, S.S., 1987. Surface characteristics and the interaction of colloid particles with mouse peritoneal macrophages. Biomaterials 8, 113–117.

Jamshaid, M., Farr, S.J., Kearney, P., Kellaway, I.W., 1988. Poloxamer sorption on liposomes: comparison with polystyrene latex and influence on solute efflux. Int. J. Pharm. 48, 125–131.

Kostarelos, K., Luckham, P.F., Tadros, T.F., 1995. Addition of block copolymers to liposomes prepared using soybean lecithin. Effects on formation, stability and the specific localisation of the incorporated surfactants investigated. J. Liposome Res. 5, 117–130.

Koster, K., Webb, M.S., Bryant, G., Lynch, D.V., 1994. Interactions between soluble sugars and POPC (1-palmitoyl-2-oleoylphosphatidylcholine) during dehydration: vitrification of sugars alters the phase behaviour of the phospholipid. Biochim. Biophys. Acta 1193, 143–150.

Lasic, D.D., 1988. The mechanism of vesicle formation. Biochem. J. 256, 1–11.

Lis, L.J., McAlister, M., Fuller, N., Rand, R.P., Parsegian, V.A., 1982. Interaction between neutral phospholipid bilayer membranes. Biophys. J. 37, 657–665.

MacDonald, R.C., MacDonald, R.I., 1993. Applications of freezing and thawing in liposome technology. In: Gregoriadis, G. (Ed.), Liposome Technology, vol. 1. CRC Press, Boca Raton, FL, pp. 209–228.

MacDonald, R.C., Jones, F.D., Qiu, R., 1994. Fragmentation into small vesicles of dioleoylphosphatidylcholine bilayers during freezing and thawing. Biochim. Biophys. Acta 1191, 362–370.

Mayer, L.D., Hope, M.J., Cullis, P.R., 1986. Vesicles of variable sizes produced by a rapid extrusion procedure. Biochim. Biophys. Acta 858, 161–168.

Mayer, L.D., Hope, M.J., Cullis, P.R., Janoff, A.S., 1985. Solute distributions and trapping efficiencies observed in freeze–thawed multilamellar vesicles. Biochim. Biophys. Acta 817, 193–196.

Moghimi, S.M., Porter, C.J.H., Illum, L., Davis, S.S., 1991. The effect of poloxamer-407 on liposome stability and targeting to bone marrow: comparison with polystyrene microspheres. Int. J. Pharm. 68, 121–126.

Morris, G.J., McGrath, J.J., 1981. The response of multilamellar liposomes to freezing and thawing. Cryobiology 18, 390–398.

Mosharraf, M., Taylor, K.M.G., Craig, D.Q.M., 1995. Effect of calcium ions on the surface charge and aggregation of phosphatidylcholine liposomes. J. Drug Target. 2, 541–545.

Nagata, M., Yotsuyanagi, T., Ikeda, K., 1986. NaCl induced aggregation of egg phosphatidylcholine liposomes. Chem. Pharm. Bull. 34, 1391–1394.

Oku, N., MacDonald, R.C., 1983. Differential effects of alkali metal chlorides on formation of giant liposomes by freezing and thawing and dialysis. Biochemistry 22, 855–863.

Oldfield, E., Chapman, D., 1972. Dynamics of lipids in membranes: Heterogenicity and the role of cholesterol. FEBS Lett. 23, 285–297.

Özer, Y., Talsma, H., Crommelin, D.J.A., Hincal, A.A., 1988. Influence of freezing and drying on the stability of liposomes dispersed in aqueous media. Acta Pharm. Technol. 34, 129–139.

Rudt, S., Müller, R.H., 1993. In vitro phagocytosis assay of nano- and microparticles by chemiluminescence. II. Effect of surface modification by coating particles with poloxamer on the phagocytic uptake. J. Controlled Release 25, 51–59.

Talsma, H., Van Steenbergen, M.J., Salemink, P.J.M., Crommelin, D.J.A., 1991. The cryopreservation of liposomes. 1. A study of the thermal behavior of a liposome dispersion containing mannitol during freezing/thawing. Pharm. Res. 8, 1021–1026.

van Winden, E.C.A., Zhang, W., Crommelin, D.J.A., 1997. Effect of freezing rate on the stability of liposomes during freeze–drying and rehydration. Pharm. Res. 14, 1151–1160.