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International Journal of Pharmaceutics 301 (2005) 247–254

international journal of pharmaceutics

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Encapsulation of lipopeptides within liposomes: Effect of number of lipid chains, chain length and method of liposome preparation

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Received 11 January 2005; received in revised form 14 June 2005; accepted 16 June 2005
Available online 28 July 2005

Abstract

The purpose of this study was to systematically investigate the effect of lipid chain length and number of lipid chains present on lipopeptides on their ability to be incorporated within liposomes. The peptide KAVYNFATM was synthesized and conjugated to lipoamino acids having acyl chain lengths of C_8, C_{12} and C_{16}. The C_{12} construct was also prepared in the monomeric, dimeric and trimeric form. Liposomes were prepared by two techniques: hydration of dried lipid films (Bangham method) and hydration of freeze-dried monophase systems. Encapsulation of lipopeptide within liposomes prepared by hydration of dried lipid films was incomplete in all cases ranging from an entrapment efficiency of 70% for monomeric lipoamino acids at a 5% (w/w) loading to less than 20% for di- and trimeric forms at loadings of 20% (w/w). The incomplete entrapment of lipopeptides within liposomes appeared to be a result of the different solubilities of the lipopeptide and the phospholipids in the solvent used for the preparation of the lipid film. In contrast, encapsulation of lipopeptide within liposomes prepared by hydration of freeze-dried monophase systems was high, even up to a loading of 20% (w/w) and was much less affected by the acyl chain length and number than when liposomes were prepared by hydration of dried lipid films. Freeze drying of monophase systems is better at maintaining a molecular dispersion of the lipopeptide within the solid phospholipid matrix compared to preparation of lipid film by evaporation, particularly if the solubility of the lipopeptide in solvents is markedly different from that of the polar lipids used for liposome preparation. Consequently, upon hydration, the lipopeptide is more efficiently intercalated within the phospholipid bilayers.
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Keywords: Liposomes; Lipopeptides; Lipoamino acid conjugates; Sub-unit vaccines

1. Introduction

Synthetic peptides of antigenic epitopes are receiving considerable interest as the basis for 'next generation', highly specific and safe vaccines (Arnon and Ben-Yedidia, 2003). However, synthetic peptide-antigens are typically poorly immunogenic and can

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doi:10.1016/j.ijpharm.2005.06.010

potentially lead to antigen-specific tolerance (Aichele et al., 1995; Le Gal et al., 2002; James et al., 2002). Further, peptides demonstrate poor stability within biological matrices and are poorly absorbed across biological membranes.

These limitations can be overcome to some extent by conjugating peptides to lipids (BenMohamed et al., 2002; Le Gal et al., 2002; McGeary et al., 2003). Lipoamino acids have received considerable attention for peptide conjugation. They are highly versatile, allowing conjugation of peptides through either the N- or C-terminus, and adaptable allowing for the possibility of modification in the length, number and type of lipidic chains (saturated and unsaturated) as well as the number, type and stereochemistry of the amino acids. Conjugation of peptides to lipoamino acids confers increased lipophilicity to the peptides that as a result, increases their membrane permeability (Toth et al., 1994a). Conjugation of peptides to lipoamino acids has also been shown to protect the peptide from enzymatic digestion (Toth, 1994b). Further, lipopeptides are far more immunogenic than unmodified peptides capable of yielding antibody titers greater than those observed when the antigen is administered in Freund's adjuvant (Toth et al., 1994c). Thus, the presence of lipidic moieties appears to overcome the need for additional adjuvant. However, as a result of their increased lipophilicity, the aqueous solubility of the constructs can be dramatically reduced, frequently requiring administration as a suspension. Indeed, in our experience, the nature of the constructs (particularly with short peptides), renders them poorly soluble in a range of solvents including H-bonding solvents such as short chain alcohols and dipolar solvents such as dichloromethane.

To overcome this formulation issue and to further enhance their bioactivity, lipid-conjugated peptides have been encapsulated within liposomes (Haro et al., 2003; Babu et al., 1995). By virtue of the lipid moiety, the lipopeptide is intercalated within the liposome bilayer. However, incorporation of lipopeptides within liposomes is likely to be influenced by the chemical nature of the lipidic anchor used as well as the lipopeptide loading within liposomes and has been reported to be variable (Drouillat et al., 1998; Babu et al., 1995).

The aim of the present study was therefore to investigate the effect of the chemical nature of the lipid (number of acyl chains and chain length) on the efficiency of entrapment of a lipopeptide within liposomes. The $LCMV_{33–41}$ peptide (KAVYNFATM) was used as a model for its immunological relevance. This peptide is expressed by the Lewis lung carcinoma cell line (LL-LCMV), which ultimately will allow for assessment of the efficacy of different formulation and immunization strategies (Hermans et al., 1997).

2. Materials and methods

2.1. Materials

4-Methylbenzhydrylamine (MBHA) resin and *t*-butyloxycarbonyl (Boc) protected amino acids were purchased from REANAL Co. (Budapest, Hungary). Boc protected lipoamino acids were synthesized according to the previously reported method (Gibbons et al., 1990). Trifluoroacetic acid (TFA) and *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate (HBTU) were purchased from Auspep (Melbourne, Australia). *N*,*N*′-Diisopropylethylamine, *tert*-butanol, egg phosphatidylcholine (EPC) and sucrose were purchased from Sigma–Aldrich (Sydney, Australia). All other reagents/solvents were of at least analytical grade.

2.2. Solid phase peptide synthesis

Lipoamino acid peptide conjugates (Fig. 1) were assembled manually using Boc protected chemistry on MBHA resin (1 g, substitution: 0.4 mmol/g). The peptide was first assembled from the C to the N-terminus and the lipoamino acid conjugated to the N-terminus of the formed peptide. Boc-amino acids

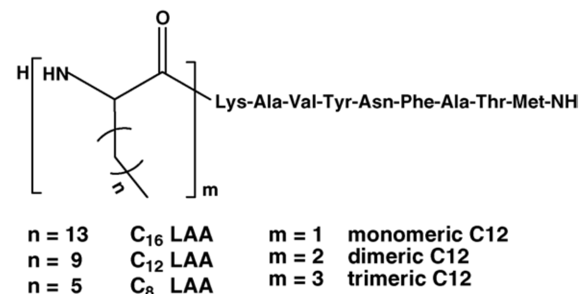

Fig. 1. Chemical structure of synthetic lipopeptides.

and lipoamino acid were activated using equimolar quantities of HBTU and each coupling was achieved using three times excess of activated reagent. All couplings were monitored using a quantitative ninhydrin assay for free amine groups on the resin. Upon completion of the peptide assembly, the peptide-resin was sequentially washed with dimethylformide, dichloromethane and methanol and then dried under vacuum for 24 h. The lipopeptide was cleaved from the resin using high hydrofluoric acid (HF) cleavage (1.5 mL each of cresol and thiocresol as scavengers in 20 mL HF). The crude peptide was precipitated with diethyl ether and then redissolved in aqueous acetic acid solution (20%, v/v, 200 mL). The solution was lyophilised and the crude peptide purified by preparative size exclusion chromatography using a LH20 gel column eluted with 50% methanol in water containing 1% acetic acid. The presence of the peptide in the collected fractions was identified by electrospray mass spectrometry. The fractions containing pure peptide were combined and lyophilised. The purity of the lipopeptide was determined by analytical RP–HPLC using a Vydac C_{18} 22 mm × 4.6 mm column, and a linear gradient from 40% aqueous solution (containing 0.1% TFA) to 100% acetonitrile/water (90/10, v/v, containing 0.1% TFA) over 30 min at a flow rate of 1 mL/min.

2.3. Liposome preparation

Liposomes were prepared following two different procedures: freeze-thawing of liposomes prepared by hydration of dried lipid films and hydration of freeze-dried monophase systems. For all formulations, the total mass of lipid used (EPC and lipopeptide) was 20 mg and liposomes were prepared to contain 5%, 10% and 20% (w/w) lipopeptide.

2.3.1. Freeze-thawing of liposomes prepared by hydration of dried lipid film

The lipopeptides were dissolved (or largely dissolved) in 2 mL of 20% aqueous acetic acid, and the corresponding amount of EPC/chloroform solution (100 mg/mL) was then added. The mixture was found to become clear upon adding *t*-butanol. The mixture was evaporated under vacuum in a rotary evaporator to produce a thin lipid film. To thoroughly remove the residual solvent and acetic acid, the lipid film was stored under vacuum overnight. Two micrilitres of phosphate buffer solution (pH 7.2) was then added and the mixture vortexed for 5 min at room temperature. The mixture was then subjected to seven cycles of freezing (−70 °C) and thawing (60 °C). The final coarse liposome suspension was then extruded five times through 100 nm (or 200 nm for 20% lipopeptide) polycarbonate membranes using an extruder^{TM} (Northern Lipids, Vancouver, Canada).

2.3.2. Hydration of freeze-dried monophase systems

The lipopeptides were dissolved (or largely dissolved) in 2 mL of 20% aqueous acetic acid containing 100 mg sucrose, and the corresponding amount of EPC/chloroform solution (100 mg/mL) was then added. The mixture was found to become clear upon adding *t*-butanol. The mixture was sonicated in water bath for 1 min and snap frozen (acetone/dry ice) prior to being lyophilised at a condenser temperature of −70 °C and a pressure of less than 10^{-1} mbar (Freezone 6, Model 79340, Labconco, MO, USA). The resulting solid matrix was hydrated with 2 mL distilled water and vortexed for 5 min at room temperature. The liposome suspension was subsequently extruded through 100 nm (or 200 nm for 20% lipopeptide) polycarbonate membranes.

2.4. Lipopeptide entrapment efficiency

The entrapment of lipopeptide within liposomes was estimated by measuring the concentration of peptide in the liposome extrudate. The liposomes were dissolved in 2 mL of a mixture of acetic acid, distilled water and *t*-butanol (10:40:50, v/v). This was then diluted as required with a 50% aqueous methanol solution prior to lipopeptide quantification. The amount of peptide remaining on the polycarbonate membrane was also measured to identify where loss of peptide occurred. Any lipopeptide remaining on the membrane was again dissolved in 2 mL of a mixture of acetic acid, distilled water and *t*-butanol (10:40:50, v/v) and diluted as required with a 50% aqueous methanol solution prior to lipopeptide quantification. All entrapment studies were carried out using triplicate liposome preparations.

2.5. Lipopeptide quantification

The solutions of the lipopeptide (either from the liposome extrudate or that extracted from the membrane) were subjected to quantitative analysis using electrospray liquid chromatography coupled to mass spectroscopy (LC–MS). Chromatographic separation was carried out on a Shimadzu HPLC system with C_{18} column (Luna 5 μm, 2.0 mm × 50 mm) using a solvent gradient. The solvent gradient started with 30% B for 0.5 min, increasing to 90% B over 6.5 min. This was maintained for 1 min and then decreased to 30% B over 4 min (solvent A = 0.1% formic acid in water; solvent B = 90% acetonitrile and 0.1% formic acid). Flow rate was 0.3 mL/min. Mass spectra were recorded on a Perkin–Elmer Sciex API 3000 triple quadrupole mass spectrometer, controlled using Sample Control 1.4 software in selected ion monitoring mode (SIM). Data was analysed using Multiview and MacQuan 1.6 software packages (Perkin–Elmer Sciex, Toronto, Canada).

2.6. Liposome characterisation

Size and polydispersity of the extruded liposome aqueous dispersions were measured by photon correlation spectroscopy using a Zetasizer 3000^{TM} (Malvern Instruments, Malvern, UK). Measurements were carried out at room temperature at a 90° detection angle.

3. Results and discussion

Preliminary studies using differential scanning calorimetry to investigate whether the synthesized lipopeptides undergo a phase transition revealed the absence of any thermal events in the temperature range 20–300 °C. This may reflect the uniform and saturated nature of the lipids used for conjugation resulting in highly ordered packing of the lipid chains up to relatively high temperatures. Since none of the lipopeptides exhibited an apparent gel-to-fluid phase transition within this temperature range, egg phosphatidylcholine having a T_m of below zero was chosen as the liposome forming phospholipids. Egg phosphatidylcholine comprises a mixture of choline-based phospholipids having different chain lengths. It was considered that the mixture of phospholipids of different chain lengths may promote intercalation of the shorter chained lipopeptides (C_8–C_{16}) compared to other longer chained and homogeneous C_{16} and C_{18} bilayer-forming phospholipids (e.g. dipalmatoyl and distearyl phosphatidylcholine).

Although not measured, it was apparent that the lipopeptide constructs synthesized were poorly soluble in both aqueous and organic solvents with their aqueous solubility decreasing, as expected with increasing chain length and number of lipid chains. Following subjective solubility evaluation in a wide range of solvents of varying polarities, it was found that a reasonable amount of lipopeptide could be solubilised in an acidified, organically modified aqueous solution. Although the lipoamino acid was conjugated to the N-terminus of the peptide, KAVYNFATM has an amide group at the C-terminus as a result of cleavage from the MBHA resin, which together with the free amino group present on the lipoamino acid allows for ionisation of the construct at low pH. This ionisation inherently increases the solubility of the lipopeptides in aqueous-based solvents. In the present study, a 20% aqueous acetic acid solution was thus chosen as the base solvent for lipopeptides which was modified when required (particularly for the $3 \times C_{12}$ and $1 \times C_{16}$ constructs) with *t*-butanol. This allowed for solutions of lipopeptides to be prepared such that they could be mixed with solutions of phospholipids. It should be noted that mixtures of hydrochloric acid and methanol should be avoided, as this can possibly cause methylation of the tyrosine or threonine residues of the peptide sequence.

Initially, lipopeptide-loaded liposomes were prepared by the traditional "Bangham method", which involves the hydration of dried lipid films. This was followed by a freeze-thawing cycle and size reduction by extrusion. Freeze-thawing is a commonly used procedure to increase incorporation of bioactive molecules within liposomes and has previously been used to increase the entrapment of lipopeptides within liposomes following preparation using a similar protocol to that used in the present study (Yagi et al., 2000). As mentioned, for preparation of liposomes by hydration of dried lipid films, lipopeptides were dissolved in a 20% aqueous acetic acid solution, which was then mixed with a solution of EPC in chloroform. Upon mixing, the mixture was found to become turbid but solubilisation of all components became possible upon addition of small amounts of *t*-butanol. The entrapment efficiency of lipopeptides within liposomes prepared by

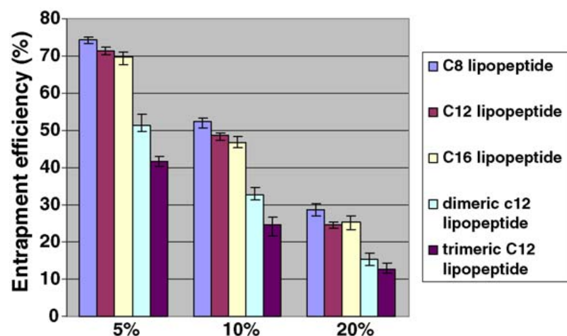

Fig. 2. Entrapment of lipopeptide within liposome prepared by hydration of dried lipid film.

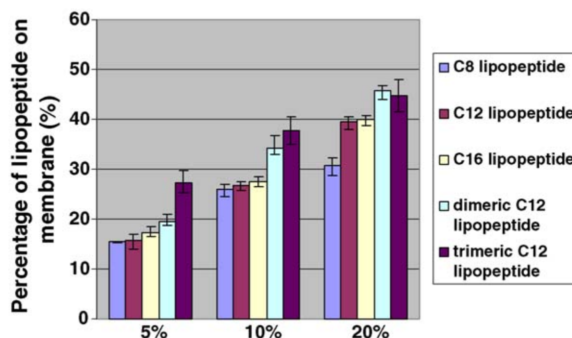

Fig. 3. Percentage of lipopeptide remaining on polycarbonate membrane for liposomes prepared by hydration of dried lipid film.

this procedure is reported in Fig. 2. As can be seen, incorporation was not complete for any of the constructs investigated ranging from around 70% to just over 10%. Encapsulation efficiency was noted to be affected to some extent by the length of the acyl chain but more significantly by the number of acyl chains. For all constructs, encapsulation efficiency decreased dramatically with increasing lipopeptide loading with less than 30% of lipopeptide being incorporated within liposomes when loaded at 20% by weight of phospholipid. To confirm that the lipopeptide was indeed associated with the liposomes in the extrudate, the extrudates were centrifuged (200,000 × g, 45 min, 4 °C, TLX ultracentrifuge, Beckman) following lipopeptide analysis and the supernatants analysed for lipopetide by LC–MS as described. No lipopeptide was detected in the supernatant of any of the centrifuged liposome extrudates confirming the association of lipopeptide with the liposomes. This is in agreement with other in-house studies (not reported) investigating the micelle forming properties of the lipopeptides using the change in absorptivity of 1,6-diphenyl-1,3,5-hexatriene, which occurs in aqueous systems upon its disposition within the hydrophobic domain of micelles (Zhang et al., 1996). In these studies, no change in absorptivity was observed when lipopeptide was dispersed in distilled water and equilibrated for 24 h at room temperature suggesting that under these conditions the lipopeptides did not spontaneously form micelles.

To elucidate as to where loss of peptide occurred and why entrapment was not complete, the amount of lipopeptide remaining on the polycarbonate membrane (used to size-reduce liposomes in addition to acting as a filter to separate liposome associated lipopeptide from undissolved lipopeptide) was measured. The amount of lipopeptide remaining on the membrane is reported in Fig. 3. The results concur with the results for lipopeptide entrapment within liposomes (Fig. 2). That is, as entrapment within liposomes decreases, more lipopeptide is deposited on the polycarbonate membrane reflecting that portion which is not entrapped and is present undissolved in solution. It was also apparent that upon hydration of the dried lipid films, lipid deposits were present on the flask wall, which proved difficult to disperse, and the presence of these deposits increased in systems where entrapment was poor. Thus, it would appear that incomplete entrapment of lipopeptides within liposomes prepared by the traditional and commonly used "hydration of dried lipid films" method is a result of the different solubility profiles of lipopeptides and phospholipids. As a result, upon evaporation one component precipitates out of solution faster than the other resulting in the formation of a lipid film, which is not homogeneous. Upon hydration of such a film, the phospholipids and some of the presumably molecularly dispersed lipopeptide spontaneously form liposomes in which the lipopeptide becomes intercalated within the phospholipids bilayer. Concurrently, the ability of the lipopeptide precipitate to hydrate and interact with the hydrating phospholipid bilayer depends to a large extent on its ability to become hydrated and solubilised. Thus, entrapment efficiency of the lipopeptide within liposomes prepared by hydration of dried lipid films reflects to some extent the aqueous solubility of the lipopeptide and as observed decreases as with the aqueous solubility of the lipopeptide.

The entrapment efficiency of lipopeptides within liposomes reported herein is in contrast to some other reports on the entrapment of lipopeptides within liposomes prepared by the hydration of dried lipid films (Roth et al., 2004; Haro et al., 2003; Nicolau et al., 2002; Boeckler et al., 1998; Fernandes et al., 1997; Tosi et al., 1995). In these studies, lipopeptides were also dissolved with the phospholipids in an organic solvent, typically chloroform-based, prior to film formation. Although not always clearly specified and sometimes not investigated, entrapment of lipopeptide within liposomes in these studies did not appear to be an issue with entrapment in one case reportedly being almost complete (Fernandes et al., 1997). None of the reports comment neither on problems of lipopeptide solubility within the solvent used for lipid film formation nor on poor entrapment. However, it must be borne in mind that the solubility profile of these lipopeptide constructs will depend on the nature of both the peptide and the lipids, and as such, each case is different. Further, these previous studies have only investigated lipopeptide loadings up to a maximum of 5 mol%.

In view of these findings, the method of liposome preparation was modified in an attempt to maintain a molecular dispersion of the lipopeptide within the phospholipid matrix. Liposomes were thus also prepared by the method of Li and Deng (2004) in which a monophase solution of phospholipid, lipopeptide and sucrose was freeze dried and subsequently hydrated with distilled water. Isotropic monophase solutions were obtained by adding *t*-butanol to mixtures of lipopeptide dissolved in 20% acetic acid (containing sucrose) and phospholipid dissolved in chloroform. Solid dispersions of these components were then instantaneously produced by freezing in a dry ice–acetone bath followed by lyophilisation. Hydration of these matrices spontaneously produced liposome suspensions having a size of between 150 nm and 200 nm with a narrow size distribution (as measured by photon correlation spectroscopy). Entrapment efficiency of lipopeptides within liposomes prepared by this method is reported in Fig. 4. As can be seen, entrapment of lipopeptide within liposomes prepared by this method is far superior to when liposomes are prepared by hydration of dried lipid films. Indeed, entrapment is high for all constructs (>85%) when prepared by this method with entrapment efficiency only decreasing slightly at lipopeptide loading of 20% (w/w). Further,

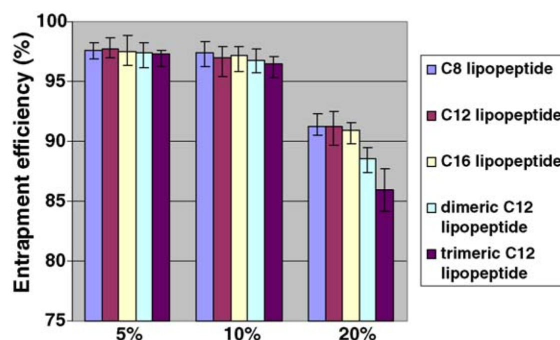

Fig. 4. Entrapment of lipopeptide within liposome prepared by hydration of freeze-dried monophase systems.

entrapment of lipopetides within liposomes prepared by this method was much less influenced by the length and number of the acyl chains The efficient entrapment of lipopeptides within liposomes prepared by this method was further confirmed by the greatly reduced amount of lipopeptide detected on the polycarbonate membrane used for liposome extrusion and the absence of noticeable deposits on the container used for freeze drying (Fig. 5).

Of interest was also the marked difference in the physical stability of the liposome preparations produced by hydration of the freeze-dried monophase solutions as compared to those produced by hydration of dried lipid films. Lipopeptide-loaded liposomes prepared by hydration of dried lipid films were observed to aggregate within days of preparation. In contrast, no aggregation of lipopeptide-loaded liposomes prepared by hydration of freeze-dried monophase solu-

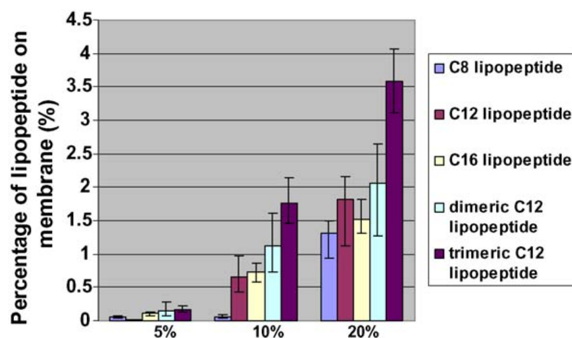

Fig. 5. Percentage of lipopeptide remaining on polycarbonate membrane for liposomes prepared by hydration of freeze-dried monophase systems.

tions containing sucrose was observed following 1 week of storage. It has previously been proposed that this increased physical stability is the result of the presence of the sugar in the system. Disaccharides can hydrogen bond with the phosphate and ester groups of the phospholipids, and presumably with functional groups on the peptide, thereby stabilizing the bilayer and preventing bilayer fusion and collapse (Sum et al., 2003).

4. Conclusion

An increasing number of papers are appearing in the literature in which lipopeptides are being formulated within liposomes with a view of either increasing their bioactivity or to overcome formulation issues relating to their poor solubility. To date, nearly all papers have prepared liposome dispersions by the method of hydration of dried lipid films as produced by evaporation of an organic solution of the polar lipid with or without slight modification (Riche et al., 2004; Roth et al., 2004; Yagi et al., 2000; Fernandes et al., 1997). Only a couple of reports however have raised the issues of variable entrapment of lipopeptides within liposomes or poor solubility of the lipopeptide in the organic solvents typically used for lipid film formation (Yagi et al., 2000; Babu et al., 1995). In the present study, we have systematically investigated the effect of lipid chain length and number on the entrapment efficiency of lipopeptides within liposomes prepared by this method and also an alternative method of preparation (hydration of freeze-dried monophase systems). When liposomes are prepared by hydration of dried lipid films, the differential solubility of lipopeptides and phospholipids results in differing rates of precipitation upon solvent evaporation and the formation of a lipid film in which the lipopeptide is not homogeneously dispersed. As a consequence, lipopeptide entrapment within liposomes prepared by this method is incomplete and dependent on the structure of the lipopeptide and loading. In contrast, when liposomes are prepared by hydration of freeze-dried monophase systems, entrapment is high and is less affected by lipopeptide structure and higher loadings can be achieved. This is a result of the more homogeneous dispersion of the lipopeptide in the lipid matrix produced by snap freezing the monophase system and drying the matrix in the solid state. This research would therefore strongly suggest the use of this latter method of preparation to efficiently entrap lipopeptides within liposomes particularly if the lipopeptide has a different solubility in volatile organic solvents compared to the phospholipid and if high lipopeptide loadings are required. This method of hydration of freeze-dried monophase systems containing disaccharides would also seem to have an additional benefit of enhanced physical stability of the liposome dispersion as compared to lipopeptide loaded liposomes prepared by hydration of dried lipid films.

Acknowledgements

M.T. Liang gratefully acknowledges the Australian Government and The University of Queensland for granting the Endeavour IPRS and UQILAS scholarships to support his PhD study. The authors also wish to thank The University of Queensland Research Development Grants Scheme for funding aspects of this work.

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