Pharmaceutical Nanotechnology

Avoiding failed reconstitution of ultradeformable liposomes upon dehydration

J. Montanari, D.I. Roncaglia, L.A. Lado, M.J. Morilla, E.L. Romero *

Programa de Nanomedicinas (PNM), Laboratorio de Diseño de estrategias de Targeting de Drogas (LDTD), Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Roque Saenz Peña 352, Bernal B1876BXD, Buenos Aires, Argentina

ARTICLE INFO

ABSTRACT

Although freeze-drying is an ordinarily used technique to dehydrate conventional liposomes, we have found that ultradeformable liposomes (UDLs) suffered irreversible aggregation when rehydrated upon freeze-drying (99.4% water elimination), even in high sugar content (4/1 sucrose/lipid mass ratio). When dehydrated by speed vac and vacuum drying, two alternative techniques that rendered less pronounced dehydration (94.27 and 96.2% water elimination, respectively) and avoid ice formation, however, UDL could only be successfully rehydrated when vacuum dried in 4/1 sucrose/lipid mass ratios. Conventional liposomes, on the other hand, were successfully reconstituted upon dehydrated by the three methods in lower sugar content (2/1 sucrose/lipid mass ratio). These results indicated that the 27% mole sodium cholate within the UDL lipid matrix was responsible for a greater and differential mechanical sensitivity of the bilayers to the different dehydration stress, as compared to conventional liposomes.

© 2009 Published by Elsevier B.V.

1. Introduction

Ultradeformable liposomes (UDLs) are highly hydrophilic liposomes containing border activators such as bile salts, non-ionic detergents or ethanol within the phospholipid matrix so as to drastically reduce the value of its elastic module (Cevc, 1995b). Applied on the skin surface, UDL are capable of experience deformation across thin channels without aggregation or coalesce, and spontaneously penetrate to the deeper strata of the epidermis (Cevc and Blume, 1992; Cevc and Gebauer, 2003). Any liposome with diameter below the 100 nm has been included in the recently launched definition of nano-object (ISO/TS 27687:2008 nanotechnologies – terminology and definitions for nano-objects – nanoparticle, nanofibre and nanoplate). A deeper insight on the use of different type of vesicular structures within the drug delivery field is strongly recommended for instance, at the European Technology Platform Nanomedicine, in its Strategic Research Agenda (http://cordis.europa.eu/nanotechnology/nanomedicine:htm), and therefore the entering of this special type of liposomes to the clinical area is probably quite close. However, in order to enable its future industrialization, several current drawbacks such as extension of shelf life and proper handling must be overcome.

The conventional method for liposomal preservation is the dehydration by freeze-drying (Crowe and Crowe, 1988; Crowe et al., 1994; Van Winden and Crommelin, 1999; Glavas-Dodov et al., 2005; Mohammed et al., 2006). In the presence of the proper lyoprotectant disaccharide such as trehalose or sucrose (a minimum 1–2 g disaccharide/g lipid) both inside and outside of vesicles (Crowe and Crowe, 1993), and accomplishing standard freeze-drying procedures (Fransen et al., 1986; Bedu-Addo, 2004) liposomes can be rehydrated while maintaining the mean size distribution, and minimizing solute leakage. However, since liposomes containing fatty acids or lysoderivatives in their phospholipid matrices are known to be highly unstable upon freeze-drying (Crowe and Crowe, 1993), we wondered if the presence of border activators such as the tensioactive sodium cholate in UDL should be a structural constraint, absent in conventional liposomes, that could difficult their further reconstitution upon drying. Up to the moment, no reports on freeze-drying feasibility for UDL are available. Because of this, we have dehydrated UDL by freeze-drying but also by alternative softer drying techniques such as vacuum drying and speed vac. The resultant solid residua were rehydrated and a series of properties were determined in order to define successful reconstitution: (1) absence of aggregation, (2) mean size distribution maintenance, (3) aqueous content retention and (4) ultradeformability maintenance. Not surprisingly, we found that UDL did not resist freeze-drying and that could only be reconstituted if dehydrated by vacuum drying, but not by speed vac, even in the presence of high amounts of sugars.

2. Materials and methods

2.1. Materials

Soybean phosphatidylcholine (SPC) (phospholipon 90 G, purity >90%) was a gift from Phospholipid/Natterman, Germany.

Sodium cholate (NaChol), sucrose, and Sephadex G-50 were purchased from Sigma. Phthalocyanine [Tetrakis(*N,N,N*-trimethy-lammoniumetoxi)-phthalocyaninate]zinc(II)tetraiodide (ZnPcMet) was synthesized as stated in Montanari et al., 2007. Other reagents were analytic grade from Anedra, Argentina.

2.2. Liposomal preparation

UDL composed of SPC and NaChol at 6:1 (w/w) ratio (26.6 mole% NaChol), were prepared by mixing lipids from $CHCl_3$ and $CHCl_3$:CH_3OH (1:1, v/v) solutions, respectively, that were further rotary evaporated at $40\,°C$ in round bottom flask until organic solvent elimination. The thin lipid film was flushed with N_2, and hydrated either with 0, 10 or 20% (w/v) sucrose in 10 mM Tris–HCl buffer plus 0.9% (w/v) NaCl, pH 7.4 (Tris buffer), up to a final concentration of 43 mg SPC ml^{-1}, in order to obtain liposomal suspensions of 0, 2 and 4 sucrose/lipid mass ratios, respectively. Those suspensions were sonicated (45 min with a bath type sonicator 80 W, 40 kHz) and extruded 15 times through two stacked 0.2 and 0.1 μm pore size polycarbonate filters using a 100 ml Thermobarrel extruder (Northern Lipids, Canada).

Conventional, non-ultradeformable, liposomes (without NaChol) were prepared by the same procedure with 0, 10 and 20% (w/v) sucrose.

2.3. Drying conditions

2.3.1. Freeze-drying

Four hundred fifty microliters aliquots of freshly prepared liposomes were freeze dried in Eppendorf tubes. The tubes were frozen either at $-18\,°C$ to maximize ice formation (Fennema, 1996) or at $-120\,°C$ for 24 h. After that, samples were incubated at $-80\,°C$ for 2 h and placed into the drying chamber of a Labconco Freeze Dry System/Freezone 4.5 (Kansas City, MO, USA) pre-cooled at $-40\,°C$. Drying was performed at a pressure form 37.10^{-3} to 62.10^{-3} mbar for 24 h. The chamber was removed and the tubes closed and stored at $-4\,°C$ until further analyses.

2.3.2. Vacuum drying

Fifty microliters aliquots of freshly prepared liposomes were vacuum dried at $25\,°C$ in Eppendorf tubes or in 96-well microplates. Tubes or plate were placed in a desiccator with recently dry silica connected to an oil vacuum pump for 72 h, until sample weight remained unchanged. The relative humidity during drying was kept at 20%.

2.3.3. Speed vac drying

Fifty microliters aliquots of freshly prepared liposomes were dried in Eppendorf tubes on a Savant Speed Vac system AES 1010 (GMI, Inc. Ramsey, MN, USA) equipped with a RH 40-11 rotor, in full vacuum (oil-free diaphragm vacuum pump, 10 Torr maximum vacuum) at medium drying rate along 90 min, until sample weight remained unchanged.

Residual water content in dry liposomal samples obtained by the three methods was estimated gravimetrically after heating the dry samples at $70\,°C$ for 72 h. A minimum of 20 Eppendorf tubes was employed, so as to the total mass loss was high enough to be accurately measured by means of an analytical balance.

2.4. Liposomal characterization

Dried liposomal residua were rehydrated to initial volume with distilled water and submitted to the following structural characterizations.

2.4.1. Turbidity

Turbidity of liposomal suspensions before and after drying/rehydration was measured by absorbance at 400 nm upon dilution in Tris buffer as an indicator of lamellarity (Rowe, 1982).

2.4.2. Size

Size before and after drying/rehydration was measured by dynamic light scattering (DLS) with a nanoZ sizer (ZEN 3600, Malvern, UK).

2.4.3. Leakage

Leakage of soluble molecules form liposomes after drying/rehydration was estimated by measuring the aqueous soluble phthalocyanine–ZnPcMet-entrapment before and after the drying/rehydration process.

Conventional liposomes and UDL-containing ZnPcMet (UDL-ZnPcMet) were prepared as stated before (Section 2.2) excepting that ZnPcMet was dissolved in the Tris buffer containing 20% (w/v) sucrose (2.8 mg ZnPcMet/g SPC) to hydrate the thin lipid film. Non-incorporated ZnPcMet was separated from UDL-ZnPcMet by gel permeation chromatography in a Shepadex G-50 column using the minicolumn centrifugation method (Fry et al., 1978). Sucrose was added to each eluted fraction in order to replace the fraction lost in the column.

Conventional liposomes and UDL-ZnPcMet were vacuum dried as state in Section 2.3.2, and upon rehydration the samples were submitted to gel permeation chromatography to remove leakaged free ZnPcMet.

Liposomal phospholipids and ZnPcMet were quantified before and after drying/rehydration, by a colorimetric phosphate micro-assay (Bötcher et al., 1961) and absorbance at 702 nm (Montanari et al., 2007), respectively.

2.4.4. Deformability test

The flux of 3.5 ml of conventional liposomes and UDL in 20% (w/v) sucrose vacuum dried and rehydrated and also not submitted to drying, driven by an external pressure of 0.8 MPa through two stacked 50 nm pore size membranes (Thermobarrel extruder) was measured in order to test deformability (Cevc, 1995a). Extruded volume was collected every minute along 15 min.

2.5. Differential scanning calorimetry and scanning electron microscopy

Portions of solid residua of UDL dried by the three methods in 20% (w/v) sucrose were placed on carbon tapes and analyzed by a Zeiss DSM 982 Gemini microscope equipped with a field emission barrel. Other solid portions of UDL were analyzed form -50 to $80\,°C$ at $3\,°C$/min rate in a DSC Q 100 (TA Instruments). First derivative were determined using the Universal Analysis 2000 software, from Thermal Advantage.

2.6. Fourier transform infrared spectroscopy (FTIR)

FTIR spectra were acquired with a Nicolet 8700 FTIR spectrometer. Attenuated total reflectance (ATR) spectra (64 scans, between 4000 and 554 cm^{-1}, with 4 cm^{-1} resolution) were obtained with an ATR Smart Orbit accessory at $25\,°C$. Transmittance spectra (64 scans, between 2500 and 1800 cm^{-1} with 2 cm^{-1} resolution), were obtained by two methods depending on the sample: a portion of solid residua obtained by vacuum dry or speed vac was directly place on CaF_2 windows meanwhile freeze dried cakes were included in KBr by vacuum press.

Deconvolution were done by Omnic software and fitted by Origin. The water sub-band NaChol associated, from 2000

to $2400\,cm^{-1}$ and the OH stretching vibration, from 3600 to $3000\,cm^{-1}$, were analyzed.

3. Results

3.1. Drying, rehydration and characterization

The aspect of the white and porous cakes obtained upon freezes drying of conventional and UDL liposomes did not differ from each other. However, upon being rehydrated, conventional liposomes in 10% (w/v) sucrose did not change their mean size distribution, whereas UDL in 10% (w/v) sucrose immediately aggregated upon water addition, with remarkably increased turbidity, size and polydispersity. This also happened with UDL freeze dried in 20% (w/v) sucrose. No differences were observed on the behavior of samples frozen at −18 or −120 °C.

On the other hand, a number of differences between the two alternative drying methods deserve to be highlighted. Vacuum drying produced a slow drying (72 h) at room temperature, in a sealed chamber with vacuum provided by an oil pump, on aliquots of 50 μl in steady Eppendorf tubes. Speed vac, on the other hand, produced 40-fold faster drying rate; samples were submitted to a continuous vacuum at 25 °C and the process was developed under centrifugation in a fixed angle rotor. In the current experimental design, for a given volume distributed in Eppendorf tubes, this last contributed to a higher surface exposed to evaporation per tube, as compared to vacuum drying.

Conventional liposomes dried by speed vac in 10 or 20% (w/v) sucrose, rendered translucent liposomal dispersions upon rehydration, of the same size and lamellarity than non-dried liposomes. However UDL dried in the same conditions, increased their turbidity, size and polydispersity when rehydrated.

Finally, conventional liposomes vacuum dried in 10 or 20% (w/v) sucrose were also successfully rehydrated, as observed by size and turbidity measurements. On the other hand, UDL vacuum dried in 10% (w/v) sucrose increased their turbidity, size and polydispersity when rehydrated excepting those in 20% (w/v) sucrose (Fig. 1). All the vacuum dried samples showed a hard and translucent aspect.

The measurement of residual water content showed that freeze-drying was the most efficient method for water removal (99.39%), rendering samples of 0.05 g water/g dry weight. Vacuum drying and speed vac eliminated only 96.22 and 94.27% water, rendering 0.14 and 0.22 g residual water/g dry weight, respectively. In all cases the results showed are an average of ten batch preparations.

3.2. Leakage

Although UDL vacuum dried in 20% (w/v) sucrose could be rehydrated conserving the size of UDL before drying, the leak-

Fig. 1. Mean size and turbidity upon rehydration of UDL dried in 20% (w/v) sucrose by the three methods.
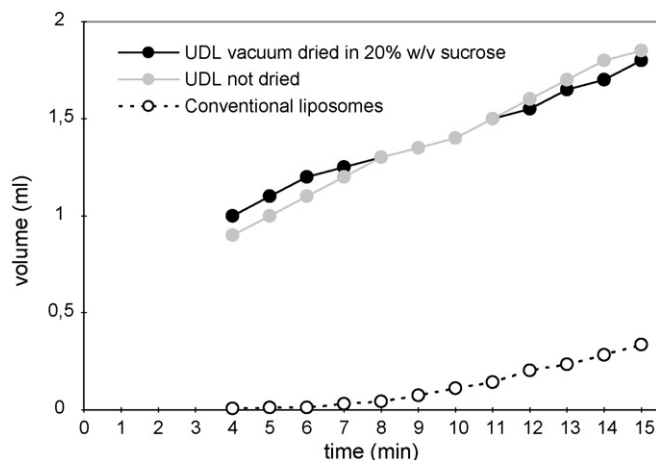

Fig. 2. Profile of liposomes passage (volume) through 50 nm pore size vs. time.

age of liposomal aqueous content was reported to occur even in the absence of vesicle aggregation or fusion in the presence of sugars (Cacela and Hincha, 2006). Because of this, the leakage of the water-soluble ZnPcMet phthalocyanine was determined. The results showed that 85% of ZnPcMet was retained into UDL (15% leakage) upon vacuum drying and rehydration. On the other hand, upon rehydration conventional liposomes dried in 10% (w/v) sucrose by the three methods, no significant leakage was detected (<10%).

3.3. Deformability test

UDL vacuum dried in 20% sucrose and rehydrated without aggregation nor significant leakage and size conservation, were submitted to deformability test. Liposomal passage across 50 nm pore size membrane under an external pressure of 0.8 MPa is shown in Fig. 2. The volume profiles of vacuum dried/rehydrated UDL and not dried UDL were similar, with most of the sample flux occurring during the first 15 min; meanwhile no flux was registered for conventional liposomes during the first 10 min.

These results confirmed that NaChol and water associated molecules remained in the membrane after vacuum drying in 20% (w/v) sucrose and rehydration.

3.4. Scanning electron microscopy and calorimetric measurements

As observed in Fig. 3, the morphology of the sucrose matrix of dried UDL in 20% (w/v) sucrose was different according to the drying method. Upon freeze-drying UDL, it was not possible to detect any intact vesicular structure, and the field showed 1 μm × 0.5 μm pieces of amorphous sucrose (Fig. 3a). Those pieces were absent when UDL were dried by speed vac in 20% (w/v) sucrose (Fig. 3b), but some vesicular structures of nearly 100 nm diameter (probably UDL) could be observed (Fig. 3insert b-i). Finally, when UDL were vacuum dried in 20% (w/v) sucrose, the fields was dominated by high number of vesicular structures, similar to those observed in the speed vac samples, both protruding or superimposed to the underlying sucrose matrix (Fig. 3c).

The thermotropic profiles of UDL dried in 20% (w/v) sucrose and its first derivative (suitable to improve weakly manifested transition in the presence of residual hydration (Sircar and Chartoff, 1994; Makar et al., 2007; Chartoff, 2008), showed in Fig. 4, were roughly similar. Independently of the drying method, two transitions, one around −28 °C and the other at 2.5 °C, were detected upon freeze dried and vacuum dried (Fig. 4a and b, respectively, the same pro-
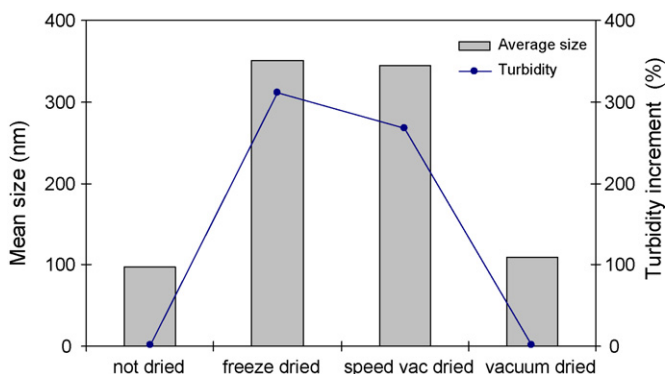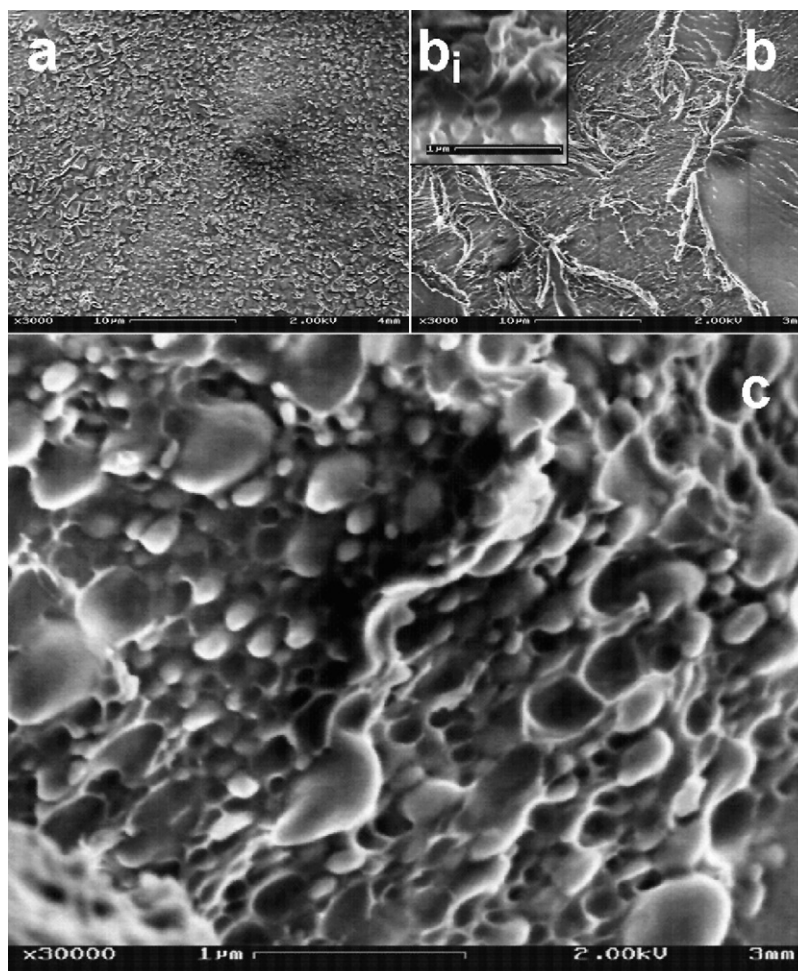
Fig. 3. Scanning electron microscopy of UDL in 20% (w/v) sucrose (a) freeze dried, (b) dried by speed vac or (c) vacuum dried.

file as vacuum dry was registered after drying by speed vac). The former transition could probably be associated with UDL phase transition temperature (Tm), previously reported at −22.81 °C in fully hydrated media in the absence of sugars (Montanari et al., 2007), and the latter transition to the fusion of residual water. These findings meant that sugar matrices could efficiently interact with phospholipid headgroups so as to avoid the transition to the gel state along the drying process, but also that the drying was incomplete in all cases. Finally, UDL vacuum dried and dried by speed vac showed an exothermic peak in DSC around 60–80 °C, which could result from re-crystallized sucrose.

3.5. FTIR study

Firstly the FTIR spectra allowed to survey the presence of water associated to NaChol upon each drying process. The water association band which appears in the region 2000–2400 cm^{-1} is assigned to a combination of water molecules bending modes with intermolecular vibrational modes (Eisenberg, 1969) that under conditions of very low water content (Giuffrida et al., 2006) can also involve non-water hydrogen-bond-forming groups, such as in the surrounding water–saccharide matrix. NaChol-containing but not NaChol-free liposomes show a small sub-band at 2025 cm^{-1}. This band is due to the presence of water molecules since, the same as the whole water association band, it is absent in dry samples of phosphatidylcholine and/or cholic acid (Chiantia et al., 2005). The thermal evolution of water sub band is tightly coupled to that of the spectrum of the CH_2 peak frequencies revealing such strong associ-

ation between hydrated CholNa and the lipid bilayers. This sub band is absent when the CholNa is not inserted within the lipid matrix, as occurring when CholNa is added from the outside to the pure SoyPC bilayers (Chiantia et al., 2005). Transmission FTIR spectra of UDL vacuum dried in 0, 10 and 20% (w/v) sucrose, fitted to Gaussian components, showed the water sub-band at 2025 cm^{-1} (Fig. 5a–c). These components were also present in UDL dried in 20% (w/v) sucrose by speed vac as well as freeze dried (not shown). In both, conventional liposomes and conventional liposomes added with NaChol and vacuum dried in 20% (w/v), the water sub-band was absent (Fig. 6a and b, respectively). The presence of nearly 27% mole NaChol within the lipid bilayer is the main difference between conventional liposomes and UDL. Water molecules associate to NaChol that is inserted within the lipidic structure, in the region located at the border between the hydrophilic and the hydrophobic moieties of phospholipids. Those water molecules act as lubricant at the contacts between the lipid polar heads and could be responsible for flexibility, hydrophilicity (Schubert et al., 1986; Barry, 2001), and also for the spontaneous locomotion of UDL following water gradients under conditions of low water (Cevc, 1995a; Chiantia et al., 2005). The fact that UDL vacuum dried in 20% (w/v) sucrose could be reconstituted without loosing the ultradeformability, indicated that the drying stress did not increase the bilayer instability caused by the NaChol and at the same time that the hydrated NaChol remained associated to the lipid matrix upon dehydration stress. This last was not unexpected, since the successfully reconstituted UDL after vacuum drying maintained their ultradeformability (which exclusively depends on the presence of hydrated NaChol).
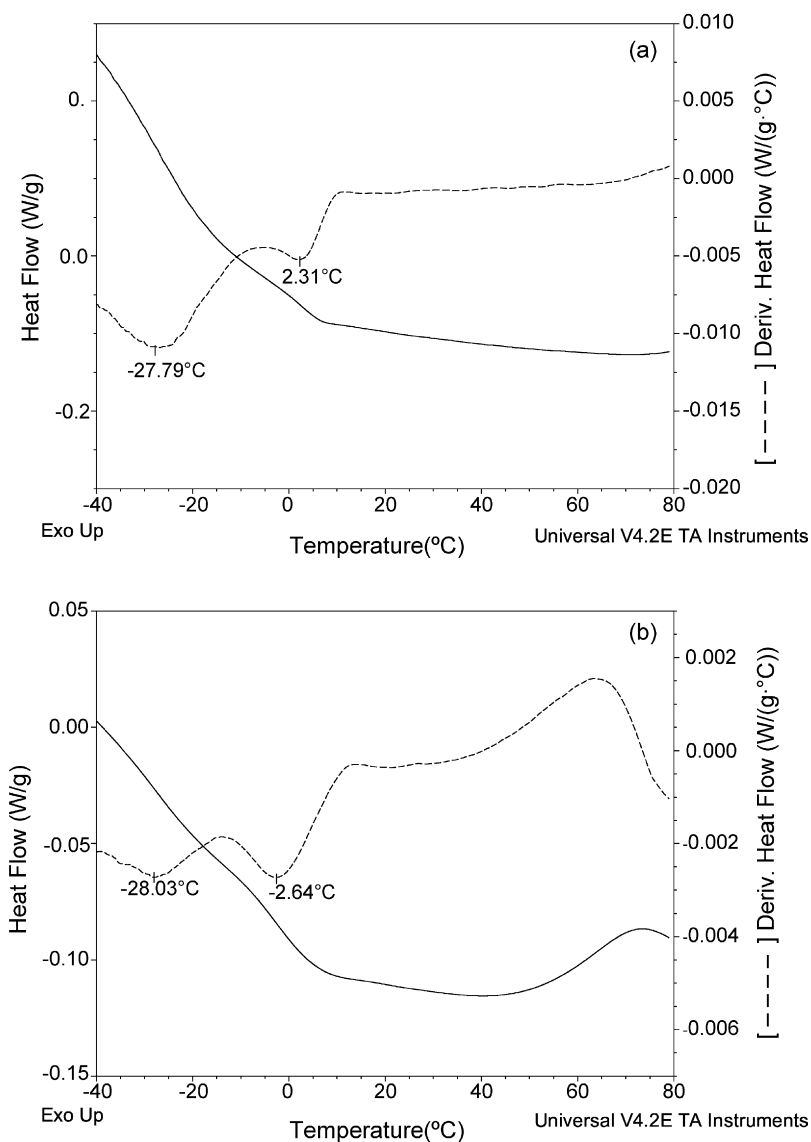
Fig. 4. Differential scanning calorimetry (solid line) and first derivative (dashed line) of UDL: (a) freeze dried and (b) vacuum drying in 20% (w/v) sucrose.

Second, the spectra allowed to estimate the dehydration degree of the sugar matrix (rubber or glassy) upon vacuum drying. UDL and conventional liposomes vacuum dried in 20% (w/v) sucrose, showed the same peak corresponding to OH stretching of the sugar matrix at 3288 cm^{-1} (Fig. 7) (data shown corresponded only to UDL). Note that the broad stretching band of OH from the two UDL dehydrated by vacuum drying at high sugar ratios (4/1 and 2/1 sucrose/lipid mass ratio, corresponding to 20 and 10%, w/v sucrose) could be ascribed to H-bonds between the sugar matrixes, that masked the sugar lipids interactions (Buitink et al., 2000). Since amorphous dry sucrose peak is reported at 3370 cm^{-1} (Sun et al., 1996), the shift to lower wavenumers revealed the existence of sugar–water H-bonding interactions and therefore an incomplete dehydration and probably a rubbery state of the matrix. Finally, the peaks corresponding to the residual water remaining post dehydration both for UDL and conventional liposomes vacuum dried in the absence of sugar were observed at 3345 cm^{-1}.

4. Discussion

In this work the conventional technique of freeze-drying, and speed vac and vacuum drying, two alternative drying techniques

where the ice formation is avoided, were used for drying sugar-containing aqueous suspensions of conventional and UD liposomes. It was found that conventional liposomes freeze dried in 10% (w/v) sucrose could be successfully rehydrated, but freeze dried UDL cakes could not, even in 20% (w/v) sucrose. Conventional liposomes could also be successfully rehydrated upon dried in 10% (w/v) sucrose by the two alternative techniques, while UDL could not, neither if dried by speed vac in 20% (w/v) sucrose, nor by vacuum drying in 10% (w/v) sucrose. Successful reconstitution of UDL was only achieved after vacuum drying in 20% (w/v) sucrose. Those facts showed that when dried and rehydrated, UDL behaved in a radical different manner than conventional liposomes, being strongly dependent on the drying method and on the sugar/lipid mass ratio employed.

It is possible to state that freeze-drying produced the formation of extra-vesicular ice responsible for mechanical and/or osmotic damage on the quasi-unstable UDL structure (Siow et al., 2007; and references therein), or alternatively that freeze-drying produced an excessive drying stress. However, if those were the only reasons for the failed reconstitutions, the two alternative drying methods that avoid ice formation and provide a less intense dehydration should have equally succeeded. Different phenomena occurring
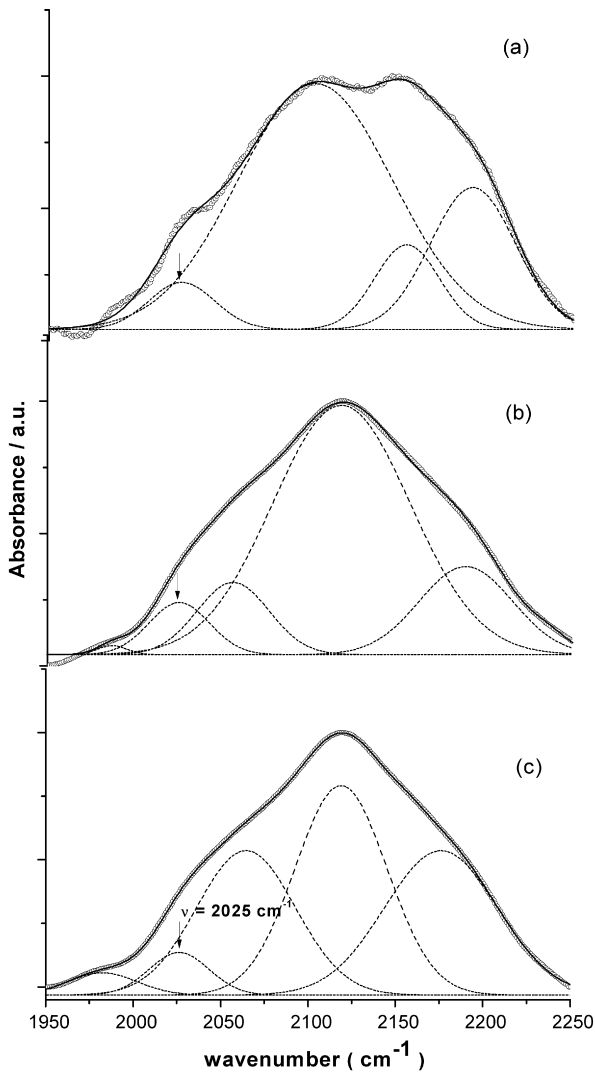
Fig. 5. FTIR spectra of UDL vacuum dried (a) without sucrose, (b) in 10% (w/v) sucrose and (c) in 20% (w/v) sucrose (after subtraction and normalized), where the water association sub-band at 2025 cm^{-1} is indicated by arrows. The open circles represent the experimental data, the continuous line the overall fitting and the dashed lines represent the fitting in terms of Gaussian components of the water association band. All fitted with $r^2 > 0.99$.

Fig. 6. FTIR spectra of conventional liposomes in 20% (w/v) sucrose (a) dried by speed vac and (b) vacuum dried with NaChol added to suspension medium. The water association sub-band at 2025 cm^{-1} is absent. The open circles represent the experimental data, the continuous line the overall fitting and the dashed lines represent the fitting in terms of Gaussian components of the water association band. All fitted with $r^2 > 0.99$.

When submitted to centrifugal forces, rubber matrices obtained by speed vac were unable to impair the UDL aggregation/fusion. When employing vacuum drying, a method where the centrifugation stress is absent, only the more viscous rubber matrix (20%, w/v, assuming equivalent water loss/dry weigh) could impair the UDL aggregation/fusion. The higher elastic module of conventional liposomes could make them less sensitive to the viscosity of rubber matrices.

along the drying process should account for the failure of the speed vac.

When sugar-containing samples are dehydrated at less than 0.2 g residual water/g dry weight, a glassy matrix is formed (Williams and Leopold, 1989). Conventional liposomes are ordinarily protected against dehydration and during storage, when the temperature of glass transition (Tg) of the sugar matrix – inversely proportional to its water content – is above room temperature. In this way, the highly viscous glassy matrices impair the vesicles fusion and the raise of Tm of bilayers upon submission to dehydration forces, eliminating the transient increase of permeability (Crowe et al., 1984; Crowe et al., 1989). Even in high sucrose contents, UDL are sensitive to the freeze-drying, which paradoxically is the only dehydration method capable of producing glassy matrices. Sucrose glasses were formerly regarded as sufficient to protect conventional liposomes, but were found unsuitable to protect UDL submitted to freeze-drying. We have found that neither the vacuum drying nor the speed vac produced glassy states at room temperature, since glassy matrices with Tg above 0 °C are only produced at 0.02–0.05 g residual water/g dry weight (Sun et al., 1996).
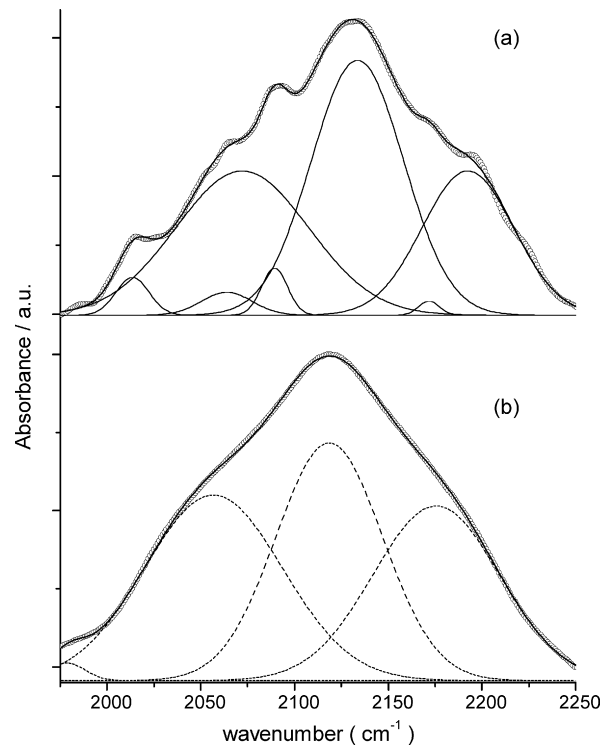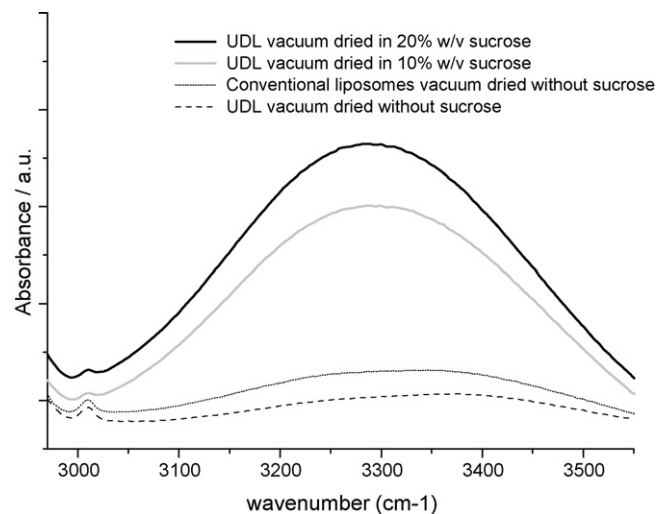

Fig. 7. FTIR spectra of UDL vacuum dried with out or in 10 and 20% (w/v) sucrose, and conventional liposomes without sucrose; the OH stretching vibration region is shown.

In sum, rubbers produced by vacuum drying are suitable to stabilize both conventional liposomes and UDL upon dehydration, but at the expense of a higher sensitivity against mechanical stress for UDL and probably lower shelf life for the two types of vesicles. We have surveyed the successful reconstitutions of UDL up to 15 days upon storage at 4 °C, but the dilemma presented by rubber matrices in UDL conservation must be faced in further deeper investigations.

Acknowledgements

References

Barry, B.W., 2001. Novel mechanisms and devices to enable successful transdermal drug delivery. Eur. J. Pharm. Sci. 14, 101–114.

Bedu-Addo, F.K., 2004. Understanding lyophilization formulation development. Pharmaceut. Technol. 28, 10–19.

Bötcher, C.J.F., Van Gent, C.M., Pries, C., 1961. A rapid and sensitive sub-microphosphorus determination. Anal. Chim. Acta 24, 203–204.

Buitink, J., Van Den Dries, I.J., Hoekstra, F.A., Alberda, M., Hemminga, M.A., 2000. High critical temperature above T(g) may contribute to the stability of biological systems. Biophys. J. 79, 1119–1128.

Cacela, C., Hincha, D.K., 2006. Low amounts of sucrose are sufficient to depress the phase transition temperature of dry phosphatidylcholine, but not for lyoprotection of liposomes. Biophys. J. 90, 2831–2842.

Cevc, G., 1995a. In: Lipowsky, R.S.E. (Ed.), Handbook of Biological Physics. Elsevier, Amsterdam.

Cevc, G., 1995b. Material transport across permeability barriers by means of lipid vesicles. In: Lipowsky, R.S.E. (Ed.), Handbook of Physics of Biological Systems. Elsevier Science, Amsterdam.

Cevc, G., Blume, G., 1992. Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force. Biochim. Biophys. Acta 1104, 226–232.

Cevc, G., Gebauer, D., 2003. Hydration-driven transport of deformable lipid vesicles through fine pores and the skin barrier. Biophys. J. 84, 1010–1024.

Chartoff, R.P., 2008. Thermal analysis of polymers. In: Characterization and Analysis of Polymers. John Wiley & Sons, pp. 817–818.

Crowe, J.H., Crowe, L.M., 1988. Factors affecting the stability of dry liposomes. Biochim. Biophys. Acta 939, 327–334.

Crowe, J.H., Crowe, L.M., 1993. Preservation of liposomes by freeze-drying. In: Gregoriadis, G. (Ed.), Liposome Technology: Liposome Preparation and Related Techniques. CRC Press, Boca Raton, FL.

Crowe, J.H., Crowe, L.M., Chapman, D., 1984. Preservation of membranes in anhydrobiotic organisms: the role of trehalose. Science 223, 701–703.

Crowe, J.H., Hoekstra, F.A., Crowe, L.M., 1989. Membrane phase transitions are responsible for imbibitional damage in dry pollen. Proc. Natl. Acad. Sci. U.S.A. 86, 520–523.

Crowe, J.H., Leslie, S.B., Crowe, L.M., 1994. Is vitrification sufficient to preserve liposomes during freeze-drying? Cryobiology 31, 355–366.

Chiantia, S., Giannola, L.I., Cordone, L., 2005. Lipid phase transition in saccharide-coated cholate-containing liposomes: coupling to the surrounding matrix. Langmuir 21, 4108–4116.

Eisenberg, D.K.W., 1969. The Structure and Properties of Water. Oxford University Press, London.

Fennema, O.R., 1996. Food Chemistry. Marcel-Dekker, Basel.

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

Fry, D.W., White, J.C., Goldman, I.D., 1978. Rapid separation of low molecular weight solutes from liposomes without dilution. Anal. Biochem. 90, 809–815.

Giuffrida, S., Cottone, G., Cordone, L., 2006. Role of solvent on protein–matrix coupling in MbCO embedded in water–saccharide systems: a Fourier transform infrared spectroscopy study. Biophys. J. 91, 968–980.

Glavas-Dodov, M., Fredro-Kumbaradzi, E., Goracinova, K., Simonoska, M., Calis, S., Trajkovic-Jolevska, S., Hincal, A.A., 2005. The effects of lyophilization on the stability of liposomes containing 5-FU. Int. J. Pharm. 291, 79–86.

Makar, J.M., Chan, G.W., Esseghaier, K.Y., 2007. A peak in the hydration reaction at the end of the cement induction period. J. Mater. Sci. 42, 1388–1392.

Mohammed, A.R., Bramwell, V.W., Coombes, A.G., Perrie, Y., 2006. Lyophilisation and sterilisation of liposomal vaccines to produce stable and sterile products. Methods 40, 30–38.

Montanari, J., Perez, A.P., Di Salvo, F., Diz, V., Barnadas, R., Dicelio, L., Doctorovich, F., Morilla, M.J., Romero, E.L., 2007. Photodynamic ultradeformable liposomes: design and characterization. Int. J. Pharm. 330, 183–194.

Rowe, E.S., 1982. The effects of ethanol on the thermotropic properties of dipalmitoylphosphatidylcholine. Mol. Pharmacol. 22, 133–139.

Schubert, R., Beyer, K., Wolburg, H., Schmidt, K.H., 1986. Structural changes in membranes of large unilamellar vesicles after binding of sodium cholate. Biochemistry 25, 5263–5269.

Siow, L.F., Rades, T., Lim, M.H., 2007. Characterizing the freezing behavior of liposomes as a tool to understand the cryopreservation procedures. Cryobiology 55, 210–221.

Sircar, A.K., Chartoff, R.P., 1994. Measurement of the glass transition temperature of elastomer systems. In: Seyler, R.J. (Ed.), Assignment of the Glass Transition. American Society for Testing and Materials, Philadelphia, pp. 226–238.

Sun, W.Q., Leopold, A.C., Crowe, L.M., Crowe, J.H., 1996. Stability of dry liposomes in sugar glasses. Biophys. J. 70, 1769–1776.

Van Winden, E.C., Crommelin, D.J., 1999. Short term stability of freeze-dried, lyoprotected liposomes. J. Control. Release 58, 69–86.

Williams, R.J., Leopold, A.C., 1989. The glassy state in corn embryos. Plant Physiol. 89, 977–981.