Avoiding failed reconstitution of ultradeformable liposomes upon dehydration


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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 Gebauer, 2003). Any liposome with diameter below the 100 nm has been included in the recently launched terminology and definitions for nano-objects – nanoparticle, nanofibre and nanoplate (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 freeze-drying and speed vac. The resultant solid residua were rehydrated 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 but also by alternative softer drying techniques such as vacuum drying and speed vac. The resultant solid residua were rehydrated 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-trimethyl-lammoniummetoxi)-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 CHCl3 and CHCl3:CH3OH (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 N2, 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 Sephadex 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 after drying/rehydration, the samples were submitted to gel permeation chromatography to remove leaked free ZnPcMet.

Liposomal phospholipids and ZnPcMet were quantified before and after drying/rehydration, by a colorimetric phosphate microassay (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 electron microscope. Other solid portions of UDL were analyzed by differential scanning calorimetry (DSC) at 3 °C/min rate in a DSC Q 100 (TA Instruments). First derivative was measured 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 CaF2 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 $2400\,\text{cm}^{-1}$ and the OH stretching vibration, from $3600$ to $3000\,\text{cm}^{-1}$, were analyzed.

### 3. Results

#### 3.1. Drying, rehydration and characterization

The aspect of the white and porous cakes obtained upon freeze-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 $-120\,^{\circ}\text{C}$.

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 $\mu\text{l}$ in steady Eppendorf tubes. Speed vac, on the other hand, produced 40-fold faster drying rate; samples were submitted to a continuous vacuum at $25\,^{\circ}\text{C}$ 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 freezedrying 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 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 leakage 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 $1\,\mu\text{m} \times 0.5\,\mu\text{m}$ 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 $100\,\text{nm}$ diameter (probably UDL) could be observed (Fig. 3c insert 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 thermotropics 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 $-28\,^{\circ}\text{C}$ and the other at $2.5\,^{\circ}\text{C}$, were detected upon freeze dried and vacuum dried (Fig. 4a and b, respectively, the same pro-
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 incom-
plete 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 asso-
ciation 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 con-
ditions 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 CH₂ 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 con-
ventional 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 con-
tacts 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 gra-
dients 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 ultra deformability, 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 vac-
uum drying maintained their ultra deformability (which exclusively
depends on the presence of hydrated NaChol).

![Fig. 3. Scanning electron microscopy of UDL in 20% (w/v) sucrose (a) freeze dried, (b) dried by speed vac or (c) vacuum dried.](image_url)
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 wavenumbers 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 radically 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...
When sugar-containing samples are dehydrated at less than 0.2 g residual water/g dry weight, a glassy matrix is formed \((\text{Williams and Leopold, 1989})\). Conventional liposomes are ordinarily protected against dehydration and during storage, when the temperature of glass transition (\(T_g\)) 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 \(T_m\) of bilayers upon submission to dehydration forces, eliminating the transient increase of permeability \((\text{Crowe et al., 1984; Crowe et al., 1989})\). 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 \(T_g\) above 0 ºC are only produced at 0.02–0.05 g residual water/g dry weight \((\text{Sun et al., 1996})\).
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.

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