Homogeneous and stable (+)-usnic acid loaded liposomes prepared by compressed CO₂

Sara Battista a, Mariana Köber b,c, Guillem Vargas-Nadal b, Jaume Veciana b,c, Luisa Giansanti a,*, Nora Ventosa b,c,**

a Dipartimento di Scienze Fisiche e Chimiche, Università degli Studi dell’Aquila, Via Vetoio 10, 67010 Coppito, AQ, Italy
b Institut de Ciència de Materials de Barcelona, ICMAB-CSIC, Campus UAB, 08193 Bellaterra, Spain
c Networking Research Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Campus UAB, 08193 Bellaterra, Spain

GRAPHICAL ABSTRACT

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ABSTRACT

The administration of hydrophobic actives and drugs for medical or cosmetic purposes generally requires a formulation that ensures adequate water solubility, which can be achieved through the encapsulation in liposomes. For the vehiculation of (+)-usnic acid (UA), a hydrophobic compound with antioxidant activity, we have prepared liposomes in a one-step process using compressed CO₂. The investigated formulations are mainly composed of 1,2-dimyristoyl-sn-glycero-3-phosphocholine and cholesterol, but contain also a small molar fraction (10%) of a synthetic surfactant derived from L-prolinol. In previous investigations liposomes containing L-prolinol derivatives showed a higher efficacy as DNA or drug delivery systems with respect to liposomes of mere phospholipids. As a consequence, they were added to liposomes to make them more suitable UA delivery systems. By testing different surfactant chain lengths and headgroups, we studied how the chemical nature of the surfactant affects the physicochemical vesicle properties and their interaction with UA. Most formulations, especially those containing surfactants with longer alkyl chains (C14 and C16), show a good potentiality as UA delivery systems because they exhibit a higher stability, vesicle-to-vesicle homogeneity and bilayer compaction with respect to analog liposomes prepared by the conventional thin film hydration previously investigated. Our results confirm the advantages of DELOS-SUSP also in the case of mixed liposomes containing phospholipids and synthetic ionic surfactants. Moreover, this study demonstrates that liposomes composed of the same lipids can feature different properties if prepared according to different methodologies. In addition, this investigation

* Corresponding author.
** Corresponding author at: Institut de Ciència de Materials de Barcelona, ICMAB-CSIC, Campus UAB, 08193 Bellaterra, Spain.
E-mail addresses: luisa.giansanti@univaq.it (L. Giansanti), ventosa@icmab.es (N. Ventosa).

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1. Introduction

Liposomes are supramolecular colloidal structures composed of closed bilayers including an internal aqueous compartment that are largely investigated because their properties make them ideal active delivery systems. In fact, they can entrap both hydrophilic and hydrophobic active principles (thus protecting them), they are biocompatible and can influence the clearance rate and the intracellular uptake of the drug [1]. An additional advantage is the possibility to tune their physicochemical properties to optimize their performance in the final application. Of special interest for drug delivery, are unilamellar liposomes featuring a diameter of about 100–200 nm. However, in general, and despite the numerous above-mentioned advantages of liposomes, their use in pharmaceutics is hampered by their relative low long-term stability, due to their tendency to aggregate, with the consequent loss of their structural homogeneity and leakage of their payload upon storage [2–5].

Only few years after their discovery [6], it became evident that the preparation methodology can significantly affect liposome properties, in particular their size, lamellarity and stability, but also the ability to entrap actives [7–9]. Conventional techniques for liposome preparation include thin film hydration (TFH), reverse phase evaporation, injection of lipids dissolved in a proper organic solvent into the aqueous phase; these procedures are often followed by sonication or high pressure membrane extrusion for size reduction and homogenization to achieve the desired small unilamellar vesicles (SUVs) [10]. More recently, different methodologies have been developed based on microfluidics [11] or compressed fluids [12,13] to prepare SUVs in a single operation. Among the latter ones, the DELOS-SUSP (depressurization of an expanded liquid organic solution-suspension) methodology allows to prepare unilamellar liposomes, with large content of cholesterol (more than 40%) that are more homogeneous and stable compared to those prepared by conventional preparation methods [14]. This technique is based on the depressurization of cholesterol (chol) and other desired lipid membrane components dissolved in a CO₂-expanded organic solution into an aqueous phase containing the polar components. If phospholipids are also present in the formulation they can be dissolved together with cholesterol in the organic phase. This one-step procedure has a high batch-to-batch reproducibility and large control of nanovesicles dimensions and morphology, which is difficult to achieve with other solvent-based processes [15]. Moreover, DELOS-SUSP is a scalable methodology that respects the dictates of a green chemistry process and allows working in sterile conditions [16].

Recently, we investigated liposome formulations containing a natural saturated phospholipid, namely 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), cholesterol and a cationic surfactant (CS) or its zwitterionic analog bearing the N-oxide moiety (N-ox) derived from L-prolinol (Chart 1) to vehiculate (+)-unic acid (UA) [1]. The choice of these particular synthetic components is linked to the evidence that liposomes containing surfactants based on the pyrrolidinium skeleton (like L-prolinol ones) showed a higher efficacy in gene therapy with respect to the corresponding open head molecules [18]. Moreover, mixed liposomes containing some of the investigated surfactants or their analogs showed a good ability as drug delivery system in antibacterial therapy (to an extent depending on the molar fraction and/or the molecular structure) [19,20]. Besides the charge, these synthetic compounds also feature alkyl chains of different lengths, other crucial parameters in determining the supramolecular packing behavior to form the liposomes.

UA is a hydrophobic antioxidant [21] derived from lichens with many pharmacological activities [22,23] and strictly related to its antioxidant capacity [24], similarly to many other active principles [25–28]. UA is used for oral care, in topic ointments, and cosmetic formulations [29], but, to the best of our knowledge, it was not included in an aqueous formulation, despite its pharmacological potential. We used the commonly employed TFH to prepare UA loaded liposomes which showed a high potential as UA delivery system, but a relatively low vesicle stability and vesicle-to-vesicle homogeneity [17]. To address this latter deficiency, we used the DELOS-SUSP methodology for the preparation of liposomes of the same composition (Fig. 1). Here we show that this strategy yields significant improvements in vesicle stability and vesicle-to-vesicle homogeneity, allowing the loading of UA with high entrapment efficiencies (E.E.). All these features, together with the absence of leakage of UA, are necessary for the scale up and the development of a pharmaceutical product.

2. Experimental section

2.1. Instrumentation

Liposomes were prepared using a DELOS-SUSP equipment [16]. The water used was pre-treated with the Milli-Q Advantage A10 water purification system (Millipore Iberica, Spain). For dialfiltration we employed a KrosFlo® Diafiltration equipment from Repligen (Rancho Dominguez, CA, USA) with a size-exclusion column of 100 kDa, a surface area of 20 cm² (MicroKros®, Spectrum Labs) and a silicone tubing #14 (with an inner diameter of 1.6 mm). Dynamic light scattering (DLS) and electrophoretic light scattering (ELS) techniques were applied to infer hydrodynamic diameters (Dh), polydispersity index (PDI) and zeta potential of liposomes by using a Zetasizer Nano ZS (Malvern Panalytical, UK) equipped with a 5 mW He-Ne laser operating at 633 nm and combined with non-invasive backscatter technology. Cryogenic transmission electronic microscopy images (Cryo-TEM) were acquired with a JEOL TEM microscope (JEOL, Japan) operating at 120 kV. Images were recorded on a Gatan 724 CCD camera under low-dose conditions using a Digital Micrograph 3.9.2 (Gatan Inc., CA, USA). UV measurements were carried out on a Varian Cary 5000 UV-Vis-NIR Spectrophotometer (Agilent, CA, USA).

2.2. Materials

DMPC was purchased from Avanti Polar Lipids (Alabaster, AL, USA). 5-Cholesten-3β-ol (chol, purity 95%) was purchased from Panrec (Barcelona, Spain). Milli-Q water (Millipore Iberica, Madrid, Spain), ethanol (Teknocroma, Sant Cugat del Vallés, Spain) and DMSO (Sigma Aldrich, Saint Louis, MO, USA) in high purity were used for all the liposomal preparations by DELOS-SUSP. Carbon dioxide (99.9% purity) was purchased from Carburios Metálicos S.A. (Barcelona, Spain). UA,
phosphate-buffered saline tablets (PBS, 0.01 M phosphate buffer; 0.0027 M KCl; 0.137 M NaCl; pH 7.4), dialysis tubing cellulose membrane (cut-off = 14,000 Da), CH₃COONa, H₂O₂ and 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diaminonitrammonium salt (ABTS) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). CS 12, CS 14, CS 16 and N-ox 12, N-ox 14, N-ox 16 were prepared as previously described [30]. All reagents used for the synthesis and solvents were used without further purification.

3. Methods

3.1. Liposomes preparation by DELOS-SUSP methodology

Mixed liposomes were prepared by DELOS-SUSP (depressurization of an expanded liquid organic solution-suspension), a compressed fluids (CFs) based method. Chol (3 mg) and DMPC (10 mg) were first dissolved in 1.2 mL of EtOH at working temperature T_w (T_w = 38 °C). The solution was then added to a high-pressure vessel (V = 7.3 mL) at atmospheric pressure and T_w. After 20 min of equilibration, the vessel was pressurized with CO₂ at the working pressure P_w (P_w = 11.5 MPa) in order to obtain an expanded liquid EtOH solution. The high-pressure vessel was kept at the working condition for 1 h in order to homogenize the system. The organic solution was then depressurized over 24 mL of PBS (warmed at T_w), containing or not around 0.5 mg of one of the CS 12-16 or N-ox 12-16 (molar ratio DMPC/chol/CS(N-ox) 6/3/1). N_w at 11.5 MPa was added to the vessel during the depressurization in order to maintain constant P_w inside. Liposomes were purified by diafiltration adjusting the feed speed and the transmembrane pressure to 10 mL/min and 1 psi, respectively, and applying six cycles of recircularization to ensure the full elimination of all components that were not incorporated in the vesicles; or by dialysis exchanging 4 times the external medium PBS solution (25-fold the liposome dispersion volume) for 1 h in order to remove ethanol.

By incubation: a small amount of UA dissolved in DMSO 37.5 mM was added to preformed liposomes (with a molar ratio of UA/lipids of 1:20) and heated at 40 °C for 1 h. Liposomes were purified by diafiltration or by dialysis to remove, besides ethanol, DMSO and unentrapped UA.

In the high-pressure vessel: UA was entrapped also adding it directly to the high-pressure vessel: chol (3 mg) and DMPC (10 mg) were first dissolved in 1.15 mL of EtOH at working temperature T_w whereas UA was dissolved in 50 µL of DMSO, heated at T_w and then added dropwise to the ethanol solution. The rest of the procedure was the same described above for liposomes without UA. The vessel was equipped with a gas filter, in order to prevent any unsolved compounds present in the CO₂-expanded solution to reach the aqueous solution of the surfactant. Liposomes were purified by diafiltration or by dialysis to remove, besides ethanol, DMSO and unentrapped UA.

E.E. of UA was evaluated by diafiltration or by dialysis to remove, besides ethanol, DMSO and unentrapped UA.

3.2. Inclusion of UA in liposome formulations and evaluation of entrapment efficiency

3.3. Size distribution and zeta potential measurements

DLS and ELS measurements by means of the laser Doppler electrophoresis technique were carried out at 25 °C on liposomal solutions of 1 mM. Helmholtz-Smoluchowski approximation was employed for the determination of the zeta-potential values. The samples were measured without dilution directly just after their preparation and after 1 week. In the latter case measurements were carried out before and after diafiltration or dialysis. The stability of each formulation was checked over time (up to 6 months). All values reported were the average of 3 consecutive measurements of the same samples. Liposome dimensions, polydispersity, and zeta potential were determined one week after liposome preparation to allow some rearrangements of bilayer components.

Fig. 1. Liposome preparation according to the DELOS-SUSP procedure: it consists in (A) the addition of an organic solution of DMPC, cholesterol and the hydrophobic solute UA to a high-pressure vessel, (B) the addition of CO₂ to obtain a CO₂-expanded solution that is equilibrated at working pressure (11.5 MPa) and temperature (311 K), and (C) the depressurization of the CO₂-expanded solution over an aqueous phase containing all polar membrane components, in this case the L-proline derivatives, to produce the desired UA loaded nanovesicles.
3.4. Cryo-TEM measurements

Liposomes morphology was studied by cryo-TEM measurements. The samples were prepared in a controlled environment vitrification system (CEVS) (Leica EM-CPC, Leica, Wetzlar, Germany) in a climate chamber at 23–25 °C keeping the relative humidity close to saturation to avoid evaporation of volatiles from the sample during its preparation. 5 μL of liposomes solution were placed on a carbon-coated holey film supported by a copper standard TEM grid. After about 30 s, the grid was gently blotted with a double layer of filter paper to obtain a thin film (20–400 nm) on the grid before it was plunged into liquid ethane at its freezing temperature (−180 °C) and transferred into liquid nitrogen (−196 °C). The vitrified specimens were stored in liquid nitrogen and transferred to a microscope using a cryotransfer and its workstation (Gatan 626 DH, Gatan, CA, USA). The working temperature was kept below −175 °C, and the acceleration voltage was 200 kV. Images of the nanovesicles in amorphous ice over holes were recorded digitally with a slow-scan camera (Gatan 694 CCD, Gatan, CA, USA) under low-dose conditions using the Digital Micrograph 3.9.2. software package.14. The morphology of the liposomes was also examined using an optical microscope (Olympus BX51, Olympus, UK) by transmitted and polarized light.

3.5. ABTS assay

Aqueous solutions containing (A) CH3COONa 0.4 M and NaCl 150 mM; (B) CH3COONa 30 mM and NaCl 150 mM; (C) glacial acetic acid 0.4 M and NaCl 150 mM; (D) glacial acetic acid 30 mM and NaCl 150 mM were prepared according to a procedure described in literature [31]. Upon the mixing of 235 mL of A solution and 15 mL of D solution a buffer solution (pH = 5.8) was obtained; analogously, mixing 18.75 mL of B solution and 231.25 mL of D solution a buffer solution at lower pH (3.6) was obtained. An acetate buffer at pH 5.5 was prepared adding 28 mL of the buffer at pH 5.8–250 mL of the buffer at pH 3.6. 0.2745 g of ABTS diammonium salt were dissolved in 50 mL of buffer at pH 3.6 to obtain an ABTS solution 10 mM. The amount of liposomes solution (prepared according to a procedure described in literature [31]) was measured. The vitrified specimens were stored in liquid nitrogen and transferred to a microscope using a cryotransfer and its workstation (Gatan 626 DH, Gatan, CA, USA). The working temperature was kept below −175 °C, and the acceleration voltage was 200 kV. Images of the nanovesicles in amorphous ice over holes were recorded digitally with a slow-scan camera (Gatan 694 CCD, Gatan, CA, USA) under low-dose conditions using the Digital Micrograph 3.9.2. software package.14.

4. Results and discussion

4.1. Liposome size and morphology

Almost all formulations obtained by DELOS-SUSP showed a monomodal narrow size distribution with a hydrodynamic diameter Dh centered around 100 nm (in the intensity weighted distributions), independently of the synthetic surfactant or the presence of UA (Fig. 2, Table S1 and Fig. S1). Only the formulation DMPC/chol/N-ox derived surfactants could be removed. Cryo-TEM measurements confirm these observations (Fig. 3): while DMPC/chol/C212 liposomes show a homogeneous morphology one week after the preparation (Fig. 3A), the appearance of large and elongated aggregates is clearly observable after dialfiltration (Fig. 3B). On the other hand, DMPC/chol/C14 liposome morphology is not affected by the dialfiltration process (Fig. 3C and D). Interestingly, vesicles size and polydispersity remained unchanged after dialysis, which indicates that dialysis does not subject the vesicles to the same stress as dialfiltration. This difference could be due to the fact that during dialfiltration washings there is a flow whereas in dialysis the external medium is changed, so it is a static situation and the solution is simply stirred. As a whole, considering the significantly higher stability over time, liposomes prepared by DELOS-SUSP are more suitable as drug delivery systems with respect to the ones prepared by TFH, which tend to aggregate or sediment even two weeks after the preparation. This evidence could be due to a higher lipid packing in the bilayer of liposomes prepared by DELOS-SUSP, entailing stronger van der Waals
interactions among lipid chains that stabilize the supramolecular structure.

Considering that all the formulations prepared by TFH tended to aggregate over time [17], our data confirm that the one-step preparation using compressed \( \text{CO}_2 \) allows to achieve more homogeneous, stable and ordered bilayers with respect to TFH, especially in the presence of \( L \)-prolinol derivatives bearing longer chains. These observations are consistent with previous results [34], which demonstrated a superior compositional homogeneity for vesicles prepared by DELOS-SUSP compared to those prepared by TFH. The advantage of DELOS-SUSP is that lipids remain in solution during the whole preparation process, while hydration methods involve an intermediate solvent-free state that may promote a phase separation of different lipids, leading to a decrease in compositional homogeneity. The high homogeneity obtained with DELOS-SUSP obviates the necessity of any additional processing steps that are generally required for hydration methods [12].

4.2. Zeta-potential of liposomes

Zeta-potentials of diafiltrated liposomes were in the range of 10–30 mV (Fig. 4 and Table SI2), even in the presence of N-ox. Identical results (within experimental uncertainty) were obtained after applying dialysis. Only the formulation containing N-ox 12 showed a very low zeta-potential, which was similarly observed for liposomes prepared by TFH [17]. Evidently the technique used for liposomes preparation does not affect the headgroup exposure to the bulk and the counterion association. The high zeta-potential of zwitterionic formulations (similar to cationic liposomes) containing N-ox could be ascribed to a folding of the pyrrolidinium ring that leads to the exposure of the hydrophilic \( N \)-oxide and hydroxyl groups to the bulk, in analogy with the case of other pyrrolidinium based surfactants [35]. The strong intramolecular hydrogen bond between these two polar moieties is typically observed in \( N \)-oxide derivatives of \( L \)-proline [36] and is maximized by the right orientation and distance. This interaction could promote the folded conformation, inducing a variation of lipid arrangement in the bilayer that can lead to consequent differences in charge exposure, hydration and counterion association.

Fig. 3. Cryo-TEM images of DMPC/chol/CS 12 one week after their preparation (A) before and (B) after diafiltration and of DMPC/chol/CS 14 one week after their preparation (C) before and (D) after diafiltration.

**Fig. 4.** Zeta-potential of the liposome formulations prepared by DELOS-SUSP in the presence or absence of UA in PBS. Measurements were performed 1 week after liposome preparation. Standard deviation over the three measurements < 7%.
The presence of UA in the liposomal formulations caused a decrease of the zeta-potential in all cases, likely due to its position in the headgroup area of the membrane surface. In fact, UA is partially deprotonated at the employed experimental conditions. It is known from literature that UA shows a good lipophilicity also in the anionic form thanks to the possibility to delocalize the charge on the aromatic rings [37]. As a consequence it can partially penetrate the bilayer even in its charged form and, thanks to electrostatic interaction, locate near the polar headgroup.

4.3. Entrapment efficiency of UA

The entrapment efficiencies (E.E.) of UA are reported in Table 1, comparing values that were obtained using dialysis and diafiltration for removing unentrapped UA.

We previously verified that applying the same procedure in samples containing only free UA all the molecules were removed from the solution. Significantly lower E.E. values were obtained using diafiltration, possibly because it entails the pressurization and/or several washings under a continuous flow that all together may lead to a deformation of vesicles during the treatment with a consequent loss of UA (note that zeta potential measurements suggested UA to be located relatively close to the polar headgroup). This effect could also flatten the differences among the observed E.E. values observed and could be more relevant in the case of samples characterized by a higher E.E. Obviously this effect cannot be extended to all solutes included in the vesicles; it is reasonable to hypothesize that in the case of UA it can be particularly relevant considering its location in the bilayer. Nevertheless, a general trend of highest E.E. were observed for liposomes containing surfactants with the longer alkyl chains, as UA is likely more efficiently retained due to more efficient van der Waals interactions and thus a higher lipid packing. Furthermore, E.E. were generally higher when UA was added during, not after, liposomes preparation (this being particularly true for vesicles containing N-ox 12 and N-ox 14). This evidence is probably linked to the high lipid packing that characterizes the bilayer of liposomes prepared according to DELOS-SUSP methodology and that hampers UA penetration in the bilayer after its formation. This trend is not observed in the case of cationic liposomes after dialysis. It seems that the ability of these formulations to retain the drug is affected to a greater extent by the separation technique used to remove unentrapped UA. The observed differences could be ascribed to the highest repulsion among the charged headgroups that favors the penetration of UA when is incubated. On the other hand, when UA was loaded in liposomes prepared according to DELOS-SUSP methodology it did not lead to a relevant absorbance decrease, independently from the liposome composition and the entrapment procedure (Fig. 5).

Table 1
Encapsulation efficiency (E.E.) of UA in the investigated formulations.

<table>
<thead>
<tr>
<th>Formulation (6/3/1)</th>
<th>Dialfiltration</th>
<th>Dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E.E. (%) UA in the vessel</td>
<td>E.E. (%) UA incubated</td>
</tr>
<tr>
<td>DMPC/chol/CS 12</td>
<td>43 ± 3</td>
<td>21 ± 4</td>
</tr>
<tr>
<td>DMPC/chol/CS 14</td>
<td>42 ± 4</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>DMPC/chol/CS 16</td>
<td>42 ± 4</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>DMPC/chol/N-ox 12</td>
<td>28 ± 2</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>DMPC/chol/N-ox 14</td>
<td>36 ± 3</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>DMPC/chol/N-ox 16</td>
<td>42 ± 5</td>
<td>36 ± 3</td>
</tr>
</tbody>
</table>

The reported values are the average of 3 independent measurements and the errors correspond to the standard deviation.

4.4. ABTS assay

The ABTS assay is commonly used to evaluate the antioxidant activity of a compound [31,44]: the presence of an antioxidant should increase the rate of reduction of ABTS radical cation (that imparts a green color to the solution). The extent of the reduction can be assessed by following the decrease of the characteristic absorption peak at 417 nm over time. As expected, the slow degradation rate inherent to the free radical cation was enhanced by the presence of free UA. On the other hand, when UA was loaded in liposomes prepared according to DELOS-SUSP methodology it did not lead to a relevant absorbance decrease, independently from the lipid composition and the entrapment procedure (Fig. 5).

The antioxidant properties of UA are well known [21,24], so it is evident that in this case when UA is included in the liposomal bilayer, the used assay does not allow an evaluation of its antioxidant activity, differently from what occurred when liposomes were prepared according to TFH [17]. Therefore, the obtained results put in evidence that the ABTS radical cation cannot penetrate the bilayer to reach UA. These results confirm the very high lipid packing of the bilayer (in contrast to liposomes prepared by TFH, where UA was indeed accessible by ABTS+) [17], evidence consistent with the high vesicle stability observed and the other parameters investigated in this study. Also in another study the accessibility of ABTS+ to liposomal UA was found to be directly dependent on the rigidity and the compaction of the bilayer [41].

In this experiment ABTS gave no information on the difference in antioxidant activity of liposomal UA, but on its scarce accessibility and, as a consequence, on liposomes tight lipid packing. This peculiarity, together with their stability, makes the investigated formulations suitable as delivery systems for controlled release because of their feasible high drug retention. As a consequence, they would not leak the entrapped payload during storage or before they reach the cells of the target tissue.

Moreover, the results of this assay underline once again the difference with liposomes prepared by TFH and confirm that liposomes of identical components can show different characteristics as a function of the preparation methodology.

5. Conclusion

The physicochemical properties of liposomes containing structurally related L-prololin derivatives prepared by DELOS-SUSP were investigated and compared to those of the same formulations prepared by TFH in the presence or in the absence of UA. Our investigation confirms that liposomes prepared by DELOS-SUSP show long-lasting stability and high homogeneity, differently from what was observed using TFH [29]. Next to the preparation technique, also the procedure used to remove the unentrapped solute (hydrophobic UA in this case) can affect liposome properties such as vesicle stability and the encapsulation efficiency. Moreover, from our analysis the picture of a high compactness of the bilayer of liposomes prepared by DELOS-SUSP clearly emerges, likely contributing to their noticeable stability.

As a whole, the investigated liposomes formulated applying the
DELOS-SUSP methodology, especially those containing surfactants with longer alkyl chains, show a good potentiality as UA delivery systems on the basis of their high stability and the high encapsulation efficiency.

Author contributions
The manuscript was written through contributions of all authors. Sara Battista and Mariana Köber contributed equally to this work. All authors have given approval to the final version of the manuscript.

CRediT authorship contribution statement

Declaration of Competing Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information
Supplementary data associated with this article can be found in the online version at doi:10.1016/j.colsurfa.2021.126749.

References

Fig. 5. Kinetic measurements of ABTS+ degradation, measured through the absorption decrease at 417 nm in the presence or in the absence of free or liposomal UA added (A) during or (B) after liposome production (by incubation).