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Use of *N*-oxide and cationic surfactants to enhance antioxidant properties of (+)-usnic acid loaded liposomes

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ABSTRACT

The influence of the presence of synthetic structurally related *N*-oxide and corresponding cationic surfactants with different chain lengths on the properties of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine liposomes was investigated. Their potentiality as delivery systems of (+)-usnic acid was also evaluated by studying its entrapment efficiency and its antioxidant activity. In fact, (+)-usnic acid has many pharmacological properties that, as many natural substances, are often strictly linked to their antioxidant power. *N*-oxide surfactants can enhance this property and improve the efficacy of the system. Based on this premise, we verified how and to what extent the molecular structure of liposomes components affects this effect: the best antioxidant effectiveness was observed when (+)-usnic acid was included in liposomes containing the *N*-oxide surfactant with C14 alkyl chain . Our results underline the importance of the hydrophilic/hydrophobic balance of the monomer in determining the properties of the aggregates in which it is included.

1. Introduction

N-Oxide surfactants (N-ox) are amphiphilic molecules with zwitterionic character at neutral pH. They are mostly employed in the production of dish and laundry detergents or as components of hair and body care products due to their foaming, wetting and thickening properties [1]. N-ox can be easily prepared, show low toxicity, emulsifying capacity and are often called soft surfactants due to the fact that they are biodegradable and environmentally friendly [2,3]. Their pH-sensitivity implies that their charge can be modified as a function of pH thus allowing the control of their properties and those of the aggregates in which they are included. Literature reports show that they can confer antimicrobial [4], antioxidant [5] and immunomodulatory [6] properties to the aggregates they form. Moreover, *N*-oxide moiety mimes the activity of superoxide dismutase removing superoxide anions and neutralizes carbon radicals stopping the radical chain reactions [7]. Based on these premises, it is evident that N-ox are very attractive and could be employed in many different research fields. In addition, the potential therapeutic uses of lipid and surfactant assemblies in general are well described in literature [8–12]. It is well known that the physicochemical properties and the performance features (as for example the ability to solubilize or entrap solutes) of surfactant assemblies are strongly affected by the molecular structure of the monomers (*e.g.* chain length, size and charge of the headgroup, unsaturation, substituents).

Here we report an investigation on mixed liposomes composed of the natural phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), cholesterol (chol) and one among *L*-prolinol derived surfactants reported in Chart 1. In particular, the effect of different chain lengths (C12, C14, and C16) as well as the presence of an *N*-oxide or quaternary ammonium moiety were taken into account.

In general, quaternary ammonium surfactants are largely investigated because of their ability to impart to the aggregates in which are included different pharmacological activity such as anti-bacterial [13,14], anti-fungal [15,16] and anti-viral [17] activity. The extent of these induced features has been observed to depend on the molecular structure such as chain length [18] or counterion effect [19]. Compared to the open-head analogues, the presence of the pyrroli-

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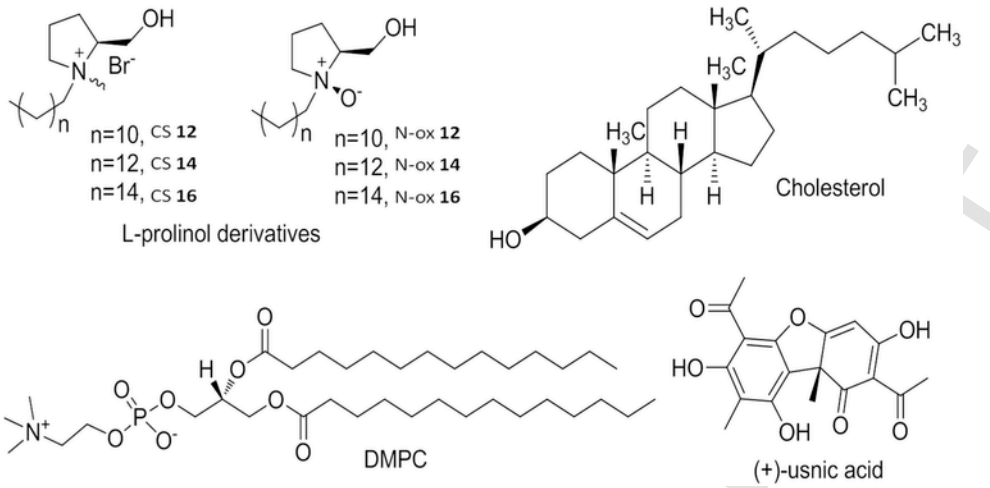

Chart 1. Liposomes components and UA.

Table 1
D_H of the investigated formulations in the presence or in the absence of UA. Reported D_H values correspond to the average values over 3 measurements and are obtained from intensity weighted distributions. PDI is lower than 0.2.

Formulation	Without UA (nm)	With UA (nm)
DMPC/chol	92 ± 3	110 ± 4
DMPC/chol/CS **12**	90 ± 5	122 ± 6
DMPC/chol/CS **14**	84 ± 3	114 ± 3
DMPC/chol/CS **16**	75 ± 2	87 ± 2
DMPC/chol/N-ox **12**	80 ± 6	106 ± 4
DMPC/chol/N-ox **14**	85 ± 2	96 ± 4
DMPC/chol/N-ox **16**	92 ± 4	100 ± 5

Table 2
Zeta potential of the investigated formulations in the presence or in the absence of UA in PBS or in water (data in brackets). All values reported were obtained by the average of 3 consecutive measurements of the same samples.

Formulation	Without UA (mV)	With UA (mV)
DMPC/chol	−2 ± 3 (-2 ± 2)	−4 ± 2 (-5 ± 2)
DMPC/chol/ CS **12**	24 ± 4 (44 ± 8)	17 ± 4 (37 ± 8)
DMPC/chol/ CS **14**	27 ± 6 (47 ± 13)	22 ± 5 (42 ± 10)
DMPC/chol/ CS **16**	33 ± 4 (52 ± 6)	15 ± 3 (35 ± 7)
DMPC/chol/ N-ox **12**	4 ± 2 (4 ± 7)	1 ± 2 (1 ± 6)
DMPC/chol/ N-ox **14**	28 ± 4 (43 ± 7)	17 ± 6 (37 ± 8)
DMPC/chol/ N-ox **16**	19 ± 4 (29 ± 6)	18 ± 5 (28 ± 5)

dinium ring reduces the degrees of freedom of the molecule with a consequent impact on hydration, volume and topology of the polar headgroup and obviously of the entire molecule [20]. Liposomes containing surfactants bearing a pyrrolidinium ring exert a higher ability to deliver DNA segments if compared with those formulated with the same amount of the acyclic counterparts [21]. We previously demonstrated that mixed liposomes formulated with DMPC and other structurally related cationic surfactants (CSs) derived from *L*-prolinol showed physicochemical properties and a drug delivery efficiency strictly dependent on the molecular structure of their synthetic components [22]. Also the presence of the *N*-oxide moiety was observed to influence the aggregates properties such catalytic activity [1] or their ability to entrap solutes [23] to an extent depending on their number [24,25], and on the molecular scaffold of the surfactant (conventional, gemini or twin tailed) [26].

Besides the aggregation and the physicochemical properties, the entrapment efficiency (E.E.) of (+)-usnic acid (UA) in mixed liposomes and the evaluation of its antioxidant activity when included into

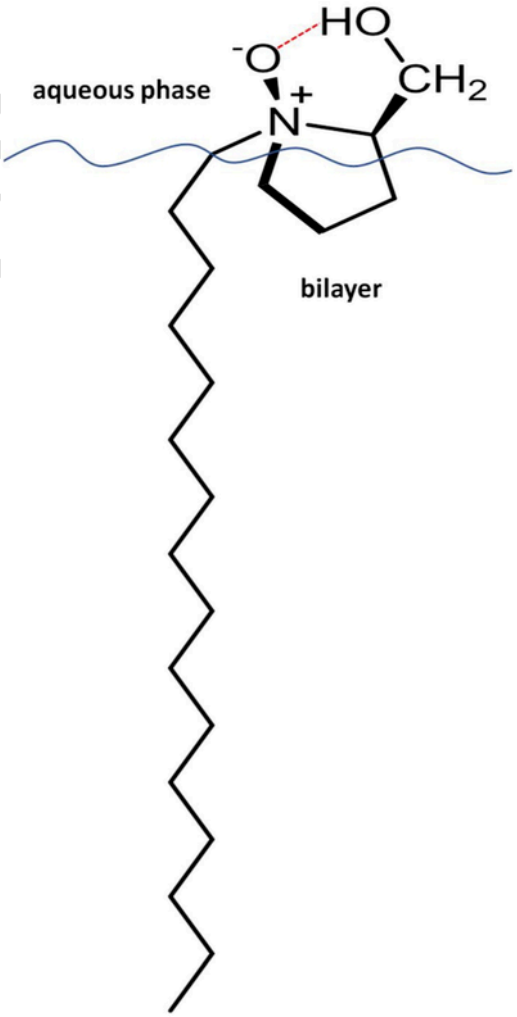

Fig. 1. Possible topology of the pyrrolidinium ring of the N-ox in lipid bilayer.

the aggregates were also evaluated. UA is a natural compound produced by lichens that displays many pharmacological properties and shows good antioxidant activity [27,28]. This property is related to its ability to behave as a peroxyl radical scavenger, to neutralize hydroxyl radicals and to reduce the production of nitrite. In addition, UA inhibits the formation of reactive species, reduces lipid peroxidation and increases

Table 3
E.E. of UA in the investigated formulations obtained by incubation. The reported values are the average of 3 independent measurements and the errors correspond to standard error of the mean.

Formulation (6/3/1)	E.E. %
DMPC/chol	77 ± 2
DMPC/chol/CS **12**	52 ± 4
DMPC/chol/CS **14**	56 ± 2
DMPC/chol/CS **16**	58 ± 3
DMPC/chol/N-ox **12**	52 ± 2
DMPC/chol/N-ox **14**	55 ± 4
DMPC/chol/N-ox **16**	66 ± 5

the enzymatic antioxidant activity of glutathione peroxidase and superoxide dismutase [29]. Unfortunately its low solubility in water (less than 10 mg/100 mL at 25 °C) [30], and its dose-dependent hepatotoxicity [31] limits its potential therapeutic applications. The aim of this investigation is to study the effect of the charge and/or of the length of the alkyl chain of the included N-ox or cationic *L*-prolinol derivatives on the physicochemical properties of the mixed liposomes in which they are included and on the antioxidant ability of liposomal UA to evaluate their potentiality as drug delivery system and their interaction with the solute. This peculiar aspect, commonly neglected in the investigations on the physicochemical properties of liposomes, is particularly important because the antioxidant activity is often related to the biological properties of many active principles [28,32–34]. Literature reports demonstrate that aggregates of surfactants bearing the *N*-oxide moiety can affect the antioxidant activity of a molecule they include (such as UA) [35] or with which they strictly interact (such as *L*-ascorbic acid) [1].

2. Experimental section

2.1. Materials

DMPC, chol, 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), CH_3COONa, H_2O_2 and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma-Aldrich. The synthetic surfactants were prepared as previously described [35]. All reagents used for the synthesis and solvents were used without further purification.

2.2. Instrumentation

Liposomes were prepared using a Hielscher UP100-H ultrasonic processor with microtip probe (7 mm). Dynamic light scattering (DLS) and electrophoretic mobility measurements were performed to infer hydrodynamic diameters and zeta potential of liposomes by using a Malvern Zetasizer Nano ZS, equipped with a 5 mW He-Ne laser operating at 633 nm. UV measurements were carried out on a Varian Cary 50 UV–vis double beam spectrophotometer (Agilent).

2.2.1. Liposomes preparation

Lipid films were prepared on the inside wall of a round-bottom flask by evaporation of solutions containing the proper amount of DMPC, cholesterol one *L*-prolinol derivatives (molar ratio: 6/3/1) dissolved in $CHCl_3$. The obtained films were stored overnight under reduced pressure (0.4 mbar), then PBS was added to obtain a 1 mM lipid dispersion. The solutions were heated at 50 °C and vortex-mixed, then the suspensions were sonicated for 4 min at 72 W (cycles: 0.5 s) under cooling condition of an ice-water bath.

3. Methods

3.1. Inclusion of UA in liposome formulations and evaluation of E.E

A small amount of UA dissolved in DMSO was added to preformed liposomes (molar ratio UA/lipids 1/20) and the solution was heated and left under stirring at 40 °C for 1 h. The unentrapped UA was removed by dialysis exchanging 4 times the external medium PBS solution (25 fold the liposome dispersion volume) during an hour. The E.E. of UA was evaluated by UV measurements at 290 nm of solutions composed of 1.5 mL of methanol and 1.5 mL of the liposome suspension before and after removal of unentrapped UA. Dialysis and UV measurements were repeated one week after liposomes preparation to evaluate UA leakage upon storage.

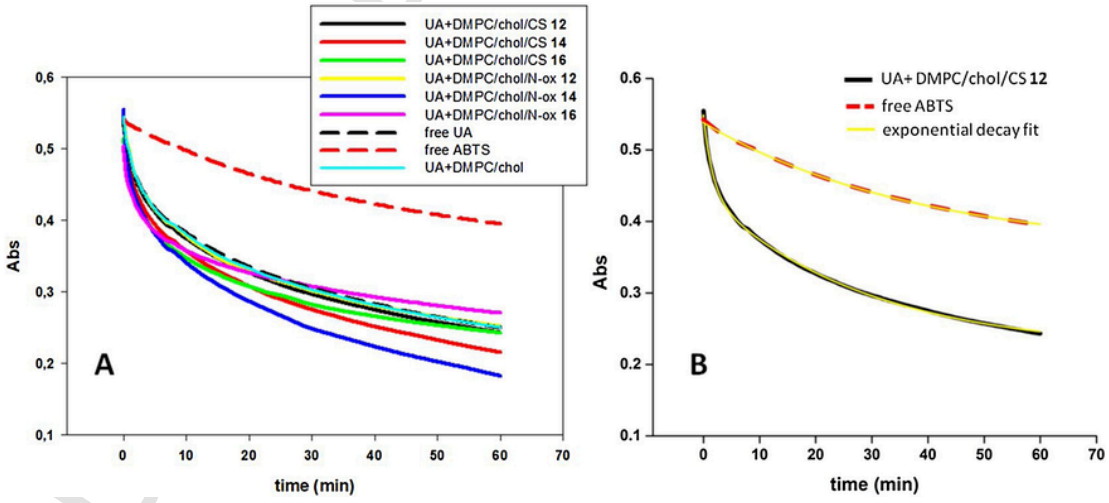

Fig. 2. (A) Kinetic measurements of $ABTS^{\cdot}+$ degradation determined by monitoring the absorbance at 417 nm in the presence or in the absence of free or liposomal UA. The reported curves are the average of at least 3 independent measurements. (B) Fit (yellow traces) to the absorbance curves in the absence (red dashed trace) or in the presence of UA entrapped in DMPC/chol/CS **12** liposomes (black trace) reported as an example. The degradation of $ABTS^{\cdot}+$ itself leads to a simple exponential decay ($y = y_0 + A_{ABTS} \cdot \exp(-x/\tau_{ABTS})$, while all samples containing UA show two processes ($y = y_0 + A_{UA} \cdot \exp(-x/\tau_{UA}) + A_{ABTS} \cdot \exp(-x/\tau_{ABTS})$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

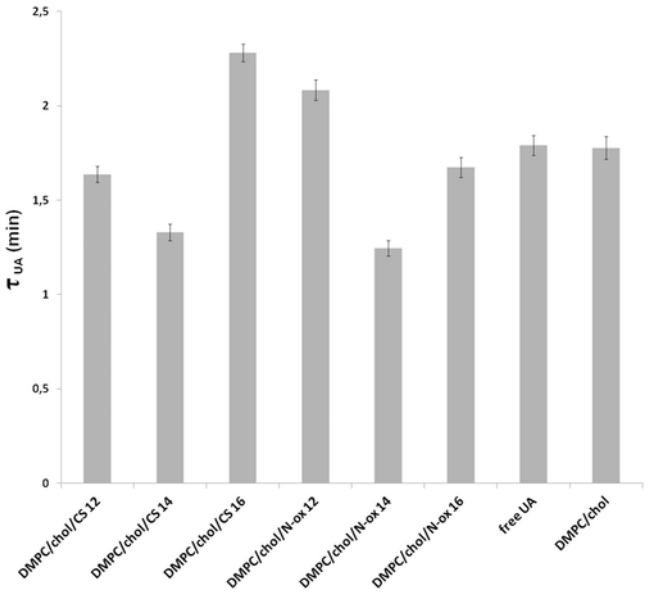

Fig. 3. Comparison of τ_{UA} relative to of ABTS$^{\cdot}$+ degradation curve in the presence of free UA or liposomal UA. The reported errors correspond to the standard error obtained from the fit.

3.2. DLS and zeta potential measurements

DLS and electrophoretic mobility measurements were carried out at 25 °C on liposomes solutions 1 mM soon after their preparation and after two weeks. To obtain the size distribution the measured autocorrelation functions were analyzed by means of the non-negative least square (NNLS) algorithm. The distribution of the diffusion coefficients D of the particles was converted in a distribution of apparent hydrodynamic diameters D_H using the Stokes–Einstein relationship $D_H = kT/3\pi\eta D$, where kT is the thermal energy and η the solvent viscosity. Reported D_H values correspond to the average values over several measurements and are obtained from intensity weighted distributions. The measurements of the electrophoretic mobility to determine zeta potential were carried out by means of the laser Doppler electrophoresis technique. Analysis of the Doppler shift in the Zetasizer Nano series was done by using phase analysis light scattering (PALS) implemented with M3 (mixed mode measurement). Zeta potential was inferred from the electrophoretic mobility under the Smoluchowsky approximation. Low applied voltages were used to avoid the risk of effects due to Joule heating. All values reported were the average of 3 consecutive measurements of the same samples.

3.3. Preparation of ABTS$^{\cdot+}$ reagent solution

Four aqueous solutions were prepared as reported in literature [36]: (A) CH_3COONa 0.4 M and NaCl 150 mM; (B) CH_3COONa 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. An acetate buffer at pH 5.8 was obtained mixing 235 mL of A solution and 15 mL of C solution; an acetate buffer at pH 3.6 was obtained mixing 18.75 mL of B solution and 231.25 mL of D solution. A solution at pH 5.5, was prepared by mixing 28 mL of the buffer at pH 5.8 and 250 mL of the buffer at pH 3.6. An ABTS$^{\cdot+}$ solution 10 mM was prepared solubilizing 0.2745 g of ABTS diammonium salt in 50 mL acetate buffer at pH 3.6: the radical cation was generated when 14 μL of H_2O_2 35% (w/v, final concentration 2 mM) were added and left stirring in the dark for one hour and stored in the dark at 4 °C.

3.4. Evaluation of antioxidant activity of UA by ABTS$^{\cdot+}$ methodology

25 μL of ABTS$^{\cdot+}$ reagent solution were rapidly added to 2450 μL of acetate buffer at pH 5.5 prepared as described above containing a proper amount of dialysed liposomes, depending on the E.E. of each system, and PBS (total PBS volume 250 μL, final concentration in cuvette of UA: $1.38\cdot10^{-6}$ M and of ABTS$^{\cdot+}$ $9.17\cdot10^{-5}$ M). If liposomes entrap 100% of incubated UA, 82 μL of liposomes solution would be added in cuvette together with 168 μL of PBS to have final PBS volume of 250 μL. This means that in the case of UA E.E. equal to 50%, 123 (82 + 41) μL of liposomes solution would be added in cuvette together with 127 μL of PBS. We followed the variation of the maximal absorbance at 417 nm over 60 min. All the measurements were repeated at least 3 times and then averaged. The temporal evolution of the absorption at 417 nm was then fitted to obtain the time constants that describe the degradation rate of ABTS$^{\cdot+}$. ABTS$^{\cdot+}$ itself showed an inherent decay of the absorbance that could be properly fitted with a simple exponential decay ($y = y_0 + A_{ABTS} \cdot \exp(-x/\tau_{ABTS})$), with a time constant of τ_{ABTS} = 28.2 min. All preparations containing UA, however, show a decay by two processes and cannot be fitted with a simple exponential decay. Therefore, these curves were fitted according to the following equation

$$y = y_0 + A_{UA}\cdot\exp(-x/\tau_{UA}) + A_{ABTS}\cdot\exp(-x/\tau_{ABTS}) \quad (1)$$

and fixing the parameter τ_{ABTS} = 28.2 min to obtain the values of the time constant τ_{UA}. The relative change in absorbance, A_{UA}/A_{tot}, was calculated with $A_{tot} = y\ (t = 0) - y_0$.

4. Results and discussion

4.1. DLS and zeta potential

Size and stability of liposomes were evaluated by DLS measurements soon after their preparation and over time. Freshly prepared, all formulations, both in presence or in the absence of UA, showed a bimodal distribution of the diameters with a main peak around 100 nm and a minor population (less than 10% in intensity weighted distribution) with dimensions ranging from 300 nm to 600 nm. This is not surprising because it is known from literature that usually a small population of large vesicles is still present in solution after sonication [37,38].

As expected, liposomes dimensions did not show any dependence on the molecular structure of the minority component: formulations freshly prepared, both in presence or in the absence of UA, showed dimensions around 100 nm as reported in Table 1. In all cases only a slight increase of liposomes dimensions (about 10%) was observed after UA loading, as observed in other cases after UA inclusion [39].

All the samples tended to aggregate over time suggesting a low stability of these systems: in two weeks the dimensions of each population tended to increase and the largest population became the most abundant with a diameter of about 1 μm. Only in the presence of N-ox **12** precipitate was observed after one week.

Zeta potential values are reported in Table 2.

As expected, we observed a potential around zero for DMPC/chol liposomes and a positive one for DMPC/CS liposomes. Surprisingly, in N-ox containing liposomes, even if they are zwitterionic as DMPC, potentials were positive and quite high, with the exception of liposomes formulated with CS **12**. The low potential observed in the latter case could explain the fact that this formulation showed the lowest stability. The highest zeta potential (similar to cationic liposomes) for N-ox containing liposomes was observed in the case of liposomes formulated with N-ox **14**. The variability in zeta potential values of N-ox containing liposomes demonstrates as the subtle hydrophobic/hydrophilic balance in surfactant structure can significantly influence their properties and confirms that chain length can play a crucial role in determin-

ing liposomes properties [40–42], in analogy with what observed for micelles of pure synthetic surfactants.[35] In particular, in the literature it is reported that properties of the bilayer such as thickness, surface area, *trans-gauche* chain isomerization and bilayer fluctuations in mixed liposomes containing *N*-oxide surfactants depend on chain length mismatch with the other lipid components [43]. The unexpected zeta potential of DMPC/chol/N-ox liposomes could result from the location at the same side of the pyrrolidinium ring of both the hydrophilic *N*-oxide and hydroxyl moieties. This characteristic could induce a folding of the pyrrolidinium ring that entails the exposure of these two polar moieties to the bulk (Fig. 1), as hypothesized in the case of other pyrrolidinium based surfactants [44]. The presence of the strong intramolecular hydrogen bond between the polar *N*-oxide and the hydroxyl groups (favoured by the proximity due to the pyrrolidinium ring and typically observed in *N*-oxide derivatives of *L*-proline [45]) could stabilize this folded conformation. This peculiar topology could in turn cause a variation of lipid bilayer organization and of charge exposure and/or hydration and/or counterion association with a logical consequent influence on the potential of the aggregates in which they are included.

Another aspect that could contribute to explain this anomalous result could be the high concentration of the sodium cation (abundant in the buffer) in the region of the polar headgroups and its strong interaction with the carbonyl region of the phospholipid membrane [46]. It is reasonable to hypothesize that Na^+ could also interact electrostatically with the oxygen of the *N*-oxide moiety, thus partially shielding its negative charge and bringing to a higher potential than the expected one. To verify the veracity of this hypothesis we carried out the same measurement in water. It is evident that the same trend in PBS and in water was observed, thus demonstrating that this explanation is not realistic.

In all cases the presence of UA caused a decrease of the zeta potential in the case of DMPC/CS liposomes and of DMPC/ N-ox **12** liposomes. This shielding effect (UA is partially deprotonated in the experimental conditions) indicates that in these formulations it is located in the polar headgroup region, in good agreement with what observed in glucosylated cationic liposomes [39]. The fact that UA can penetrate the bilayer (even if not deeply) being negatively charged is not surprising considering that also its deprotonated form is highly lipophilic because it can delocalize the charge on the aromatic rings [47]. The fact that the inclusion of UA in DMPC/ N-ox **12** and DMPC/ N-ox **16** liposomes does not interfere with zeta potential values indicates that UA is located in a deeper region of the bilayer, confirming once more that either the nature of the headgroup and chain length can control liposomes properties.

4.2. UA liposomal entrapment

UA was added to preformed liposomes by incubation even if this molecule is a weak acidic compound that could be entrapped exploiting an active loading methodology based on a pH gradient. We used passive loading because we previously demonstrated that the highest E.E. was observed applying this technique [39]. The E.E. values of UA in the investigated formulations are reported in Table 3. In all cases similar E.E. values ranging around 50–60% were observed. The fact that DMPC/chol liposomes showed the highest E.E. could be due to their higher bilayer compaction with respect to the other formulations: in fact, the investigated synthetic surfactants, featuring a very different molecular structure with respect to DMPC, could reduce lipid packing (especially in the case of folding of the pyrrolidinium ring) thus lowering the ability of the liposomes membrane to retain the UA molecule. UA release upon storage (1 week) showed that all liposomes (with exception of N-ox **12** containing liposomes that were not evaluated because they precipitate) feature a good ability to retain entrapped UA. In fact, only about 10% of UA passes into the bulk without significant differences changing the synthetic component. This high stability of UA entrapment likely results from the high bilayer rigidity brought about by the presence of the chol.

4.3. Evaluation of antioxidant activity of UA by $ABTS^{\cdot+}$ methodology

The antioxidant activity of UA entrapped in liposomes was evaluated according to a procedure described in the literature by using $ABTS^{\cdot+}$ [36], which is reduced (with a consequent fading of the solution from green to transparent) at a rate that depends on the antioxidant effectiveness of the system. The degradation of $ABTS^{\cdot+}$ as a function of time was followed by monitoring the absorbance at 417 nm in the presence or in the absence of UA entrapped in liposomes or free UA (Fig. 2A and SI7). The acetate buffer also contains NaCl 150 mM to avoid liposomes rupture due to osmotic shock because for their preparation we used PBS 150 mM. The UV measurements were performed at pH 5.5 in order to obtain a low (but valuable) free $ABTS^{\cdot+}$ degradation rate (red dashed line).

As expected, the presence of free UA (black dashed trace) strongly increases the degradation rate of $ABTS^{\cdot+}$ due to the well-known antioxidant properties of UA [27,28]. When UA was formulated in liposomes containing the surfactants bearing the alkyl chain with 14 carbons, mostly N-ox **14**, the antioxidant activity was increased significantly. On the other hand, if UA is included in liposomes containing CS **16** or N-ox **16**, after an initial burst common to the other samples, its antioxidant activity is slightly slowed down, and in the case of DMPC/ chol and DMPC/chol/ CS **12**(N-ox **12**) liposomes the antioxidant activity is comparable to that of free UA.

Our data indicates that the length of the chain has a greater impact on the antioxidant efficacy of liposomal UA than the charge of the polar headgroup. This influence of the chain length could be ascribed to a different accessibility of $ABTS^{\cdot+}$ to UA and/or to a different location of UA in the bilayer. These results could also be partially explained considering that both DMPC (the major liposomes component) and the surfactants CS **14** and N-ox **14** bear chains of 14 carbon atoms. The mismatch in chain length liposomes components that occur in the other cases can affect lipid organization [48] and/or the exposure of the charged polar headgroups [49–51], confirming the influence of this feature, and in general of monomer chain length, on liposome properties. The differences of zeta potential values of N-ox containing liposomes indicate that the polarity of the microenvironment of the headgroup region can vary among the different formulations. As reported in literature, the antioxidant effectiveness is strictly dependent on the microenvironment [52,53]. In particular, the fact that in the case of UA the oxidation rate increases as a function of the polarity of the medium [54] could contribute to the highest antioxidant activity of UA observed in DMPC/ chol/ N-ox **14** liposomes. It is interesting that in the case of micelles of pure synthetic surfactants the nature of the polar headgroup rather than chain length seems to play a major role in affecting the antioxidant activity of UA, indicating the complex crucial effect of the incorporating aggregates [35]. To deepen our understanding of the investigated process, kinetics curves were fitted with an exponential decay by two processes (Eq. (1) reported in the experimental section), related to the spontaneous degradation of free $ABTS^{\cdot+}$ and the reaction between $ABTS^{\cdot+}$ and our systems containing UA. The time constant τ_{ABTS} was determined by fitting the temporal evolution of the absorption of free $ABTS^{\cdot+}$ with a simple exponential decay ($y = y_0 + A_{ABTS} \bullet \exp(-(x)/\tau_{ABTS})$). In Figure SI2 the curve relative to free UA was fitted using both a simple and a double exponential decay (as an example) to confirm that in the presence of UA in the latter case the best fit parameters are obtained.

This analysis allows exploring more in the detail the initial phases of the oxidation process in which most part of the oxidation occurs. The fit to the data of free $ABTS^{\cdot+}$ and of UA entrapped in DMPC/chol/

CS **12** liposomes are reported as an example in Fig. 2B (all the curves are reported in the SI) and the obtained τ_{UA} values are shown in Fig. 3.

In agreement with the results obtained by kinetic curves, the fastest initial catalytic effect was obtained using liposomes containing CS **14** and N-ox **14** (the time constant τ_{UA} is reduced of ≈ 30% compared with free UA). On the other hand, the slowest oxidation occurred with liposomes including surfactants containing 16 methylenes: considering that in these cases the highest value of absorbance measured is lower than the one observed for the other samples (this evidence is confirmed in repeated experiments), the initial rate of $ABTS^{\cdot+}$ reduction is very fast (see Fig. 2A and Figure SI7). As a consequence, it is reasonable to hypothesize that τ_{UA} is overestimated and is real value is lower than the observed one. As previously mentioned, it is possible that in this case UA is differently accessible (and then disposable) to react with $ABTS^{\cdot+}$. These results confirm once again that chain length can exert a significant effect on liposomes properties. Also in the case of DMPC/chol/ N-ox **12** a slow reaction rate was observed, but reasonably this result can be ascribed to the low potential that reduces the electrostatic interactions with anionic UA. The analysis of the amplitude of the curves puts in evidence that liposomal UA contributes to $ABTS^{\cdot+}$ reduction at ≈ 30% in all cases similarly to what observed with free UA (see Figure SI8).

5. Conclusion

Our investigation points out that, more than the charge *or* the chain length, the combination of both these parameters is fundamental in determining liposomes physicochemical properties and their ability of influencing the antioxidant effectiveness of UA. In particular, a synergistic effect between C14 chain length and the *N*-oxide moiety confirm the potentiality of N-ox in enhancing the antioxidant properties of the included solute [35]. As a consequence, this formulation shows the best potentiality as UA delivery systems, despite its relatively low stability.

Our results confirm that also subtle variations of molecular structure of liposomes components, even the less abundant ones, can palpably affect their physicochemical properties. As a consequence, even considering an analogous series-based scaffold, it is possible to modulate the properties of the aggregates they form or in which they are included to optimize them for specific application areas by changing the length of the hydrophobic chains or functionalizing the hydrophilic moiety.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.colsurfa.2019.124154.

References

[1] K. Niedziółka, M. Szymula, A. Lewińska, K.A. Wilk, J. Narkiewicz-Michałek, Studies of vitamin C antioxidative activity in the N-oxide surfactant solutions, Colloids Surf. A Physicochem. Eng. Asp. 413 (2012) 33–37.
[2] D. Bernier, U.K. Wefelscheid, S. Woodward, Properties, preparation and synthetic uses of Amine N-Oxides. An update, Org. Prep. Proced. Int. 41 (2009) 173–210.
[3] M.T. Garcìa, E. Campos, I. Ribosa, Biodegradability and ecotoxicity of amineoxide based surfactants, Chemosphere 69 (2007) 1574–1578.
[4] J. Subik, G. Takacsova, M. Psenak, F. Devinsky, Antimicrobial activity of amine oxides: mode of action and structure-activity correlation, Antimicrob. Agents Chemother. 12 (2) (1977) 139–146.
[5] A. Pisecki, D. Pilakowska-Pietras, A. Baran, A. Krasowska, Synthesis and properties of surface chemically pure alkylamidoamine-N-oxides at the air/water interface, J. Surfact. Deterg. 11 (2008) 187–194.
[6] M. Bukovský, D. Mlynarcík, V. Ondrácková, Immunomodulatory activity of amphiphilic antimicrobials on mouse macrophages, Int. J. Immunopharmacol. 18 (6-7) (1996) 423–426.
[7] A. Krasowska, A. Piasecki, A. Murzyn, K. Sigler, Assaying the antioxidant and radical scavenging properties of aliphatic mono- and di-N-oxides in superoxide dismutase-deficient yeast and in a chemiluminescence test, Folia Microbiol. 52 (1) (2007) 45–51.
[8] A. Angelova, B. Angelov, Dual and multi-drug delivery nanoparticles towards neuronal survival and synaptic repair, Neural Regen. Res. 12 (2017) 886–889.
[9] A. Angelova, M. Drechsler, V.M. Garamus, B. Angelov, Liquid crystalline nanostructures as PEGylated reservoirs of omega-3 polyunsaturated fatty acids: structural insights toward delivery formulations against neurodegenerative disorders, ACS Omega 33 (2018) 3235–3247.
[10] A. Angelova, C. Fajolles, C. Hocquelet, F. Djedaïni-Pilard, S. Lesieur, V. Bonnet, B. Perly, G. Lebas, L. Mauclaire, Physico-chemical investigation of asymmetrical peptidolipidyl-cyclodextrins, J. Colloid Interface Sci. 322 (1) (2008) 304–314.
[11] M. Rakotoarisoa, B. Angelov, V.M. Garamus, A. Angelova, Curcumin- and fish oil-loaded spongosome and cubosome nanoparticles with neuroprotective potential against H_2O_2-induced oxidative stress in differentiated human SH-SY5Y cells, ACS Omega 42 (2019) 3061–3073.
[12] B. Angelov, V.M. Garamus, M. Drechsler, A. Angelova, Structural analysis of nanoparticulate carriers for encapsulation of macromolecular drugs, J. Mol. Liq. 235 (2017) 83–89.
[13] A. King, S. Chakrabarty, W. Zhang, X. Zeng, D.E. Ohman, L.F. Wood, S. Abraham, R. Rao, K.J. Wynne, High antimicrobial effectiveness with low hemolytic and cytotoxic activity for PEG/quaternary copolyoxetanes, Biomacromolecules 15 (2014) 456–467.
[14] M. Lukáč, I. Lacko, M. Bukovský, Z. Kyselová, J. Karlovská, B. Horváth, F. Devínsky, Synthesis and antimicrobial activity of a series of optically active quaternary ammonium salts derived from phenylalanine, Cent. Eur. J. Chem. 8 (2010) 194–201.
[15] S.J. Hiom, J.R. Furr, A.D. Russell, J.R. Dickinson, Effects of chlorhexidine diacetate and cetylpyridinium chloride on whole cells and protoplasts of Saccharomyces cerevisiae, Microbios74 (1993) 111–120.
[16] A.K. Gupta, I. Ahmad, R.C. Summerbell, Fungicidal activities of commonly used disinfectants and antifungal pharmaceutical spray preparations against clinical strains of Aspergillus and Candida species, Med. Mycol. 40 (2002) 201–208.
[17] D.L. Prince, H.N. Prince, O. Thraenhart, E. Muchmore, E. Bonder, J. Pugh, Methodological approaches to disinfection of human hepatitis B virus, J. Clin. Microbiol. 31 (1993) 3296–3304.
[18] E. Obłąk, T.M. Lachowicz, J. Łuczyński, S. Witek, Comparative studies of biological activities of the lysosomotropic aminoesters and quaternary ammonium salts on yeast Saccharomyces cerevisiae, Cell. Mol. Biol. Lett. 6 (2001) 871–880.
[19] E. Obłąk, A. Piecuch, E. Dworniczek, T. Olejniczak, The influence of biodegradable gemini surfactants, N,N'-bis(1-decyloxy-1-oxopronan-2-yl)-N,N,N',N'-tetramethylpropane-1,3-diammonium dibromide and N,N'-bis(1-dodecyloxy-1-oxopronan-2-yl)-N,N,N',N'-tetramethylethane-1,2-diammonium dibromide, on fungal biofilm and adhesion, J. Oleo Sci. 64 (2015) 527–537.
[20] K.K. Karukstis, J.R. McDonough, Characterization of the aggregates of N-Alkyl-N-methylpyrrolidinium bromide surfactants in aqueous solution, Langmuir 21 (2005) 5716–5721.
[21] B.K. Majeti, R.S. Singh, S.K. Yadav, S.R. Bathula, S. Ramakrishna, P.V. Diwan, S.S. Madhavendra, A. Chaudhuri, Enhanced intravenous transgene expression in mouse lung using cyclic-head cationic lipids, Chem. Biol. 11 (2004) 427–437.
[22] C. Bombelli, F. Bordi, S. Ferro, L. Giansanti, G. Jori, G. Mancini, C. Mazzuca, D. Monti, F. Ricchelli, S. Sennato, M. Venanzi, New cationic liposomes As vehicles

of m-tetrahydroxyphenylchlorin in photodynamic therapy of infectious diseases, Mol. Pharmaceut. 5 (4) (2008) 672–679.
[23] S. Huláková, B. Fulier, J. Gallová, P. Balgavý, Effect of N-dodecyl-N,N-dimethylamine N-oxide on unilamellar liposomes, Acta Fac. Pharm. Univ. Comen. LX 2 (2013) 7–13.
[24] A. Lewińska, M. Witwicki, U. Bazylinska, A. Jezierski, K.A. Wilk, Aggregation behavior of dicephalic di-N-oxide surfactants in aqueous solution: experimental and computational approaches, Colloids Surf. A Physicochem. Eng. Asp. 442 (2014) 34–41.
[25] A. Piasecki, B. Wójcik, J. Łuczyński, D. Piłakowska-Pietras, S. Witek, A. Krasowska, Bifunctional N-oxides of alkyldiamidoamines, J. Surfact. Deterg. 12 (2009) 201–207.
[26] F. Bordi, G. Cerichelli, N. De Berardinis, M. Diociaiuti, L. Giansanti, G. Mancini, S. Sennato, Synthesis and physicochemical characterization of new twin-tailed N-oxide based gemini surfactants, Langmuir 26 (9) (2010), 6177-6173.
[27] O.A. Luzina, N.F. Salakhutdinov, Usnic acid and its derivatives for pharmaceutical use: a patent review (2000-2017), Expert Opin. Ther. Pat. 28 (2018) 477–491.
[28] M. Suwalsky, M. Jemiola-Rzeminska, C. Astudillo, M.J. Gallardo, J.P. Staforelli, F. Villena, K. Strzalka, An in vitro study on the antioxidant capacity of usnic acid on human erythrocytes and molecular models of its membrane, Biochim. Biophys. Acta 1848 (11A) (2015) 2829–2838.
[29] F. Odabasoglua, A. Aslan, A. Cakir, H. Suleyman, Y. Karagoza, M. Halici, Antioxidant activity, reducing power and total phenolic content of some lichen species, Fitoterapia 76 (2005) 216–219.
[30] T. Kristmundsdóttir, H.A. Aradóttir, K. Ingólfsdóttir, H.M. Ögmundsdóttir, Solubilization of the lichen metabolite (+)-usnic acid for testing in tissue culture, J. Pharm. Pharmacol. 54 (2002) 1447–1452.
[31] P. Pramyothin, W. Janthasoot, Hepatotoxic effect of (+)-usnic acid from Usnea siamensis Wainio in rats, isolated rat hepatocytes and isolated rat liver mitochondria, J. Ethnopharmacol. 90 (2004) 381–387.
[32] L. Saso, O. Firuzi, Pharmacological applications of antioxidants: lights and shadows, Curr. Drug Targets 15 (13) (2014) 1177–1199.
[33] H.L. Lin, T.C. Chang, S.T. Chang, A review of antioxidant and pharmacological properties of phenolic compounds in Acacia confusa, J. Tradit. Complement. Med. 8 (4) (2018) 443–450.
[34] A.M. Bast, G.R. Haenen, Ten misconceptions about antioxidants, Trends Pharmacol. Sci. 34 (8) (2013) 430–436.
[35] S. Battista, P. Campitelli, A. Carlone, L. Giansanti, Influence of structurally related micelle forming surfactants on the antioxidant activity of natural substances, Chem. Phys. Lip. 225 (2019) 104818.
[36] O. Erel, A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation, Clin. Biochem. 37 (4) (2004) 277–285.
[37] A.N. Joseph, J.A. Zasadzinski, Transmission Electron microscopy observations of sonication-induced changes in liposome structure, Biophys. J. 49 (1986) 1119–1130.
[38] R.L. Hamilton, J. Goerke, L.S.S. Guo, M.C. Williams, R.J. Havel, Unilamellar liposomes made with the French pressure cell: a simple preparative and semiquantitative technique, J. Lipid Res. 21 (1980) 981–992.
[39] I. Francolini, L. Giansanti, A. Piozzi, B. Altieri, A. Mauceri, G. Mancini, Glucosylated liposomes as drug delivery systems of usnic acid to address bacterial infections, Colloids Surf. B Biointerfaces 181 (2019) 632–638.
[40] R. Nagarajan, Molecular packing parameter and surfactant self-assembly: the neglected role of the surfactant tail, Lanmguir 18 (2002) 31–38.
[41] S. Svenson, Controlling surfactant self-assembly, Curr. Opin. Colloid Interface Sci. 9 (3-4) (2004) 201–212.
[42] D. Jurašin, I. Habuš, N. Filipović-Vinceković, Role of the alkyl chain number and head groups location on surfactants self-assembly in aqueous solutions, Colloids Surf. A Physicochem. Eng. Asp. 368 (1-3) (2010) 119–128.
[43] J. Karlovská, K. Lohner, G. Degovics, I. Lacko, F. Devínsky, P. Balgavý, Effects of non-ionic surfactants N-Alkyl-N,N-dimethylamine-N-oxides on the structure of a phospholipid bilayer: small-angle X-ray diffraction study, Chem. Phys. Lip 129 (2004) 31–41.
[44] A. Bartoloni, C. Bombelli, S. Borocci, M.G. Bonicelli, L. Galantini, L. Giansanti, M. Ierino, G. Mancini, A. Muschietti, C. Sperduto, Synthesis and physicochemical characterization of pyrrolidinium based surfactants, J. Colloid Interface Sci. 392 (2013) 297–303.
[45] I.A. O'Neil, A.J. Potter, Simple azetidine N-oxides: synthesis, structure and reactivity, Chem. Commun. (Camb.) 14 (1998) 1487–1488.
[46] A.A. Gurtovenko, M. Miettinen, M. Karttunen, I. Vattulainen, Effect of monovalent salt on cationic lipid membranes as revealed by molecular dynamics simulations, J. Phys. Chem. B 109 (2005) 21126–21134.
[47] L. Guo, Q. Shi, J.L. Fang, N. Mei, A. Afshan Ali, S.M. Lewis, J.E.A. Leakey, V.H. Frankos, Review of usnic acid and Usnea barbata Toxicity, J. Environ. Sci. Health C26 (2008) 317–338.
[48] R.L. Biltonen, D. Lichtenberg, The use of differential calorimetry as a tool to characterize liposomes preparation, Chem. Phys. Lipids 64 (1993) 129–142.
[49] D. Gradella Villalva, L. Giansanti, A. Mauceri, F. Ceccacci, G. Mancini, Influence of the state of phase of lipid bilayer on the exposure of glucose residues on the surface of liposomes, Colloids Surf. B Biointerfaces 159 (2017) 557–563.
[50] G.C. Goodwin, K. Hammond, I.G. Lyle, M.N. Jones, Lectin-mediated agglutination of liposomes containing glycophorin effects of acyl chain length, Biochim. Biophys. Acta 689 (1982) 80–88.
[51] H. Yun, Y.W. Choi, N.J. Kim, D. Sohn, Physicochemical properties of phosphatidylcholine (PC) monolayers with different alkyl chains, at the air/water interface, Bull. Korean Chem. Soc. 24 (3) (2003) 377–383.
[52] M. Dracha, J. Narkiewicz-Michałek, A. Sienkiewicz, M. Szymula, C. Bravo-Díaz, Antioxidative properties of vitamins C and E in micellar systems and in Microemulsions, Colloids Surf. A Physicochem. Eng. Asp. 379 (2011) 79–85.
[53] H. Bae, G.K. Jayaprakasha, K. Crosby, J.L. Jifon, B.S. Patil, Influence of extraction solvents on antioxidant activity and the content of bioactive compounds in non-pungent peppers, Plant Foods Hum. Nutr. 67 (2) (2012) 120–128.
[54] E.E. Iorgulescu, E. Varzaru, D. Bala, C. Mihailciuc, Electrochemical study of the oxidation of usnic acid in different organic solvents, Rev. Roum. Chim. 57 (2012) 699–705.