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Physicochemical and toxicological properties of novel amino acid-based amphiphiles and their spontaneously formed catanionic vesicles

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ABSTRACT

The design of efficient liposomal systems for drug delivery is of considerable biomedical interest. In this context, vesicles prepared from cationic/anionic surfactants may offer several advantages, mainly due to their spontaneity in formation and long-term stability. There is also an impending need to produce less toxic, more biocompatible amphiphiles, while maintaining the desirable aggregation properties. In this work, we present data for acute toxicity to *Daphnia magna* (IC_{50}), and potential ocular irritation (HC_{50}) for some newly prepared ionic surfactants with dodecyl chains, derived from the amino acids tyrosine (Tyr), serine (Ser), hydroxyproline (Hyp) and lysine (Lys). The micellization behavior of the compounds, evaluated from surface tension measurements, is presented and compared to more conventional ionic amphiphiles. Two types of spontaneouly formed catanionic vesicles, composed either by a dodecyltrimethylammonium bromide (DTAB)/Lys-derivative and or Ser-/Lys-derivative mixture, have also been tested for their ecotoxicity and hemolytic potential. All the micelle-forming surfactants as well as the vesicle-containing mixtures are found to have lower ecotoxicity than the reference surfactant DTAB. Moreover, the results from hemolysis and hemoglobin denaturation tests show that the Tyr- and Lys-derivatives are moderately irritant, whereas the Hyp- and Ser- ones are just slightly irritant. Even more significantly, the vesicle-containing mixtures exhibit lower hemolytic activity than the neat surfactants, a positive result for their potential use in liposomal formulations.

1. Introduction

Micelles and vesicles are colloidal aggregates of great biomedical relevance, in particular in connection with drug delivery [1–3], gene delivery [4,5] and drug antimicrobial activity [6,7]. In the case of vesicles, they have been classically prepared from polar lipids or their mixtures (liposomes) [1] or from mimicking non-natural double-tailed surfactants, such as dialkyldimethylammonium halides [1,8,9]. Classical liposome dispersions often present drawbacks for applications, mainly due to: (i) chemical degradation of the lipids; (ii) relatively complex preparation procedures and (iii) more significantly, their intrinsic colloidal instability [10]. In fact, these types of liposomes are often metastable aggregates of short-term existence, obtained from the mechanical or chemical disruption of lamellar dispersions, e.g. through dry film hydration, sonication, extrusion, etc. With time, the liposomes flocculate and fuse yielding undesirable phase-separated dispersions, a clear nuisance for practical uses. With the development of surfactant self-assembly studies and cross-interactions from both lipid and surfactant fields, the situation has significantly changed (e.g. see reviews [10,11]). In this context, mixtures of oppositely charged surfactants have emerged in the last 10–15 years as an exciting new class of vesicle-forming systems (catanionic vesicles), as described further [12–16].

Another clear trend in surfactant-related applications has been the strive to design novel amphiphiles that, in addition to the desired performance, have enhanced biocompatibility and biodegradability. Amino acid-based surfactants, consisting of an amino acid moiety as the polar headgroup attached to one or more alkyl chains, have attracted great interest since often they are more biocompatible and environment-friendly than surfactants with conventional headgroups [17]. For instance, cationic amino acid surfactants have been shown to be less toxic and persistent in the environment than the widely used quaternary ammonium

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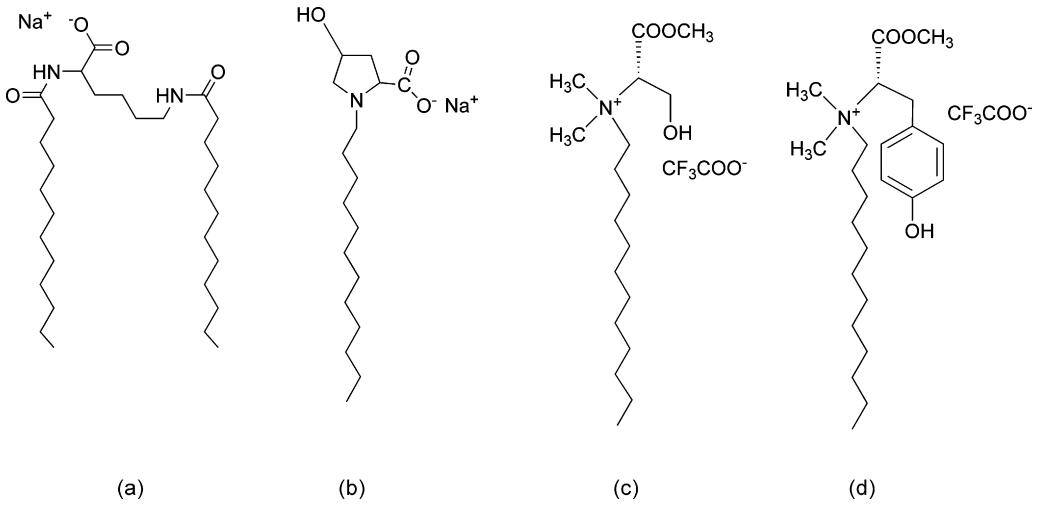

Fig. 1. Chemical structure of the newly synthesized amino acid-derived surfactants, (a) 12Lys12, (b) 12Hyp, (c) 12Ser, (d) 12Tyr. All the molecules are micelle-forming amphiphiles. The cationic/anionic mixture of 12Lys12 with 12Ser forms spontaneous and stable vesicles, at appropriate mixing ratios; 12Lys12 also forms vesicles with commercially available DTAB.

surfactants [18–21]. There is also demand for less irritating surfactants since a large part of skin problems (e.g. irritation, dryness and itching) stems directly from the amphiphiles used in skin-care and cosmetic formulations. By replacing common headgroups by others based on natural raw products, less toxic molecules are expected to be generated.

In this paper, we report basic micellization and toxicological properties of four newly synthesized surfactants, two anionic ones derived from lysine, 12Lys12, and hydroxyproline, 12Hyp, (Fig. 1a and b) and two cationic ones derived from serine, 12Ser, and tyrosine, 12Tyr (Fig. 1c and d). Trifluoracetate has been chosen as counterion for the Ser- and Tyr-derived surfactants, since it is known that larger counterions tend to reduce surfactant toxicity [22,23]. In addition, results are also presented here for novel catanionic vesicles prepared from 12Lys12/DTAB [24] and 12Lys12/12Ser [25] mixtures. From a colloidal standpoint, catanionic vesicles bear several advantages as compared to conventional liposomes: (i) they form spontaneously upon mixing of individual co-solute micellar solutions, requiring no high-energy methods; (ii) they have long-term stability and in many cases exist as equilibrium structures (thus, indefinitely stable); (iii) they often present chain melting transitions well below room temperature; (iv) they offer the possibility of charge regulation, due to variation of the surfactant mixing ratio [9,12–15,26,27].

Though a relatively large number of catanionic systems has been investigated in recent years, the vast majority of reports deal with the structural/equilibrium properties of the aggregates. The vesicles are typically composed by conventional ionic surfactants, known a priori to have relatively high toxicity levels. Still, these "model" vesicles have been reported to possess good encapsulation properties both for probe molecules [28–31] and some currently used therapeutic drugs [29], and have also been employed in the context of non-viral vectors for gene delivery [32–35].

Despite its obvious relevance for potential biomedical uses, the direct assessment of the biological properties of catanionic vesicles has been much less explored. A few recent reports have relevantly addressed the mechanisms behind the toxicity of such vesicles, based on conventional surfactants, towards mammalian cells [36,37]. To our knowledge, the present report is the first to directly address acute toxicity and hemolytic activity/potential ocular irritancy of catanionic vesicles, in particular those synthesized from natural sources (amino acids). Toxicological results are also presented here for the individual surfactants *per se*, using common cationic and anionic surfactants as comparative references.

The *Daphnia magna* acute immobilisation test is part of a wider set of guidelines provided by the OECD to ascertain the ecotoxicity of chemical compounds [38]. Since more advanced tests, namely chronic exposure and effect over the reproduction ability of aquatic organisms, require the knowledge of the IC_{50} – the concentration estimated to immobilize 50% of the Daphnia – this test is mandatory before the former. Further envisioning biomedical applications for the current surfactants, and because the bloodstream is the most common route of *in vivo* inoculation, hemolysis tests are of extreme relevance to assess the initial and immediate damage by the compounds – or their aggregates – to red blood cells (RBC). The protocol used here was specifically designed to assess the adverse effect of surfactants on the cytoplasmic membrane of RBC (hemolysis), as well as the damage caused to the liberated cell proteins, namely hemoglobin [39]. This test leads to the HC_{50} value, the concentration of surfactant at which 50% of the RBC undergo hemolysis and the lysis/denaturation ratio (L/D), which gives the ocular irritation potential of the surfactant. If very high HC_{50} values are obtained, more advanced tests, e.g., cellular compatibility, may be excluded, due to the likely lack of interest of using a highly hemolytic and irritant amphiphile on a formulation for systemic inoculation. Therefore, the combination of aquatic toxicity (IC_{50}), hemolytic activity (HC_{50}) and potential ocular irritation (L/D) assays was chosen considering the certified relevance of the information they provide on biocompatibility, their specificity to surfactants and their celerity.

2. Materials and methods

2.1. Materials

The amino acid-derived surfactants were synthesized in the Organic Chemistry group at Univ. Porto. The 12Lys12 surfactant was prepared by condensation of L-lysine methyl ester with lauric acid followed by saponification, as described in detail in a previous report [24]. The cationic 12Ser and 12Tyr surfactants were prepared by reductive amination of the corresponding "fatty" aldehydes, followed by methylation and deprotection; the anionic 12Hyp was prepared by reductive amination followed by saponification. The detailed methodology for the latter syntheses is described in a separate report [40]. Purity of the surfactants was thoroughly confirmed by means of thin layer chromatography (TLC), mass spectroscopy, FTIR and NMR, as reported elsewhere [40,41], and is also provided as supporting material. DTAB and sodium dodecyl

sulfate (SDS) were supplied by Sigma–Aldrich (99% purity) and used as purchased. High purity Millipore water was used for the preparation of all samples and solutions.

2.2. Sample preparation

All surfactant solutions were freshly prepared before usage. Either high purity Millipore water, for surface tension measurements, or an appropriate buffer solution, for the toxicity and hemolysis tests, were used as solvent. Catanionic vesicle solutions were prepared by direct mixture of the individual surfactant solutions, containing micelles. For the aquatic toxicity tests, the initial stock solutions of catanionic liposomes had a total surfactant concentration of 0.500 wt%. For the hemolysis tests, 0.250 and 0.125 wt% stock solutions were used for the 12Lys12/DTAB and 12Lys12/12Ser mixtures, respectively. The surfactant mixing ratio is defined as $X_S = n_S/(n_S + n_{12Lys12})$, where S stands for the cationic amphiphile, and it has a value of 0.74 for the DTAB/12Lys12 mixture and 0.70 for the 12Ser/12Lys12 mixture. Vesicle formation was checked by Cryo-TEM in a Philips CM 120 Bio-Twin transmission electron microscope, after vitrification, at room temperature, in liquid ethane.

2.3. Surface tension

A DCAT11 surface tensiometer from Dataphysics Instruments GmbH was used. Measurements were done using a Wilhelmy plate and data acquisition was done using the software provided by the manufacturer. Temperature was kept constant at $25.0 \pm 0.2\,^{\circ}C$ using a thermostated Julabo F20 circulating water bath.

2.4. Cryo-TEM imaging

Cryogenic transmission electron microscopy was performed in order to confirm the presence of vesicles in selected mixed cationic/anionic surfactant samples with different compositions. A copper grid with a thin film of the sample was quickly vitrified by immersion in liquid ethane from the temperature of 25 °C [42]. The sample was then carefully transferred to a Philips CM 120 Bio-Twin transmission electron microscope under liquid nitrogen environment.

2.5. Acute toxicity tests

Aquatic toxicity of the surfactants was assessed using the OECD *D. magna* acute immobilization test [38]. The mobility ability of laboratory-bred Daphnia after 24 and 48 h of exposure to the surfactants was used to determine the IC_{50} value, the concentration estimated to immobilize 50% of the Daphnia. The pH of the test medium was 8.0, with total hardness of 250 mg/L (expressed as $CaCO_3$) and a Ca/Mg ratio of 4:1 [38]. A geometric series of 10 concentration values was tested for each amphiphile. Preliminary tests were first carried out to establish the suitable concentration range for each surfactant. Twenty Daphnia, divided in four batches of five animals each, were used at each tested concentration. Incubation took place at 20 °C and in darkness. The percentage of immobilized Daphnia at 24 and at 48 h was plotted against concentration on a logarithmic–probability scale. Normal statistical procedures were used to calculate the IC_{50} values for the appropriate exposure period.

2.6. Red blood cell test assay to predict potential ocular irritation

The method described by Pape et al. [39] was followed, as an alternative to the classical Draize test [43]. Human blood was obtained from the Blood Bank of the Hospital de la Vall d'Hebró (Barcelona, Spain) and used in the same day of the experiments. The erythrocyte suspension was washed three times in PBS buffer (123.3 mM NaCl, 22.2 mM Na_2HPO_4 and 5.5 mM KH_2PO_4, 300 mOsmol/L), with pH 7.4 [39] and then suspended in the same buffer to the appropriate cell density. 25 μL of red blood cells (RBC) suspension in a total vial volume of 1 mL give an extinction of about 2.0 units at 575 nm. Cell counting was based on the extinction of the hemoglobin after lysis of the RBC. The HC_{50} value (defined below) was determined by adding 25 μL aliquots of the RBC suspension to tubes containing a range of different concentrations of the test surfactants, with a final volume of 1 mL. Samples were prepared in triplicate, incubated at room temperature for 10 min and centrifuged for 2 min at 10,000 rpm. Absorbance of the solutions was determined in a dual-beam spectrophotometer and the hemolysis percentage was calculated comparing each individual determination to a situation of 100% hemolysis (erythrocytes in distilled water). A dose–response curve was plotted for the calculation of the HC_{50}, defined as the surfactant concentration inducing 50% of hemolysis.

The irritation index was determined according to the lysis/denaturation ratio (L/D) obtained by dividing the HC_{50} by the denaturation index (DI). The denaturation index of each surfactant was calculated by comparing the denaturation of the hemoglobin induced by the surfactant and SDS effects as positive control. Hemoglobin denaturation was determined after inducing hemolysis by addition of 10 mg mL^{-1} of the surfactant or SDS by measuring the ratio of absorbance at 575 and 540 nm. The ratio between the absorbance value at 575 and 540 is designated as the α/β ratio (R_{Hb}), i.e., the denaturation ratio. For native oxyhemoglobin this value is taken to be 1.05 ± 0.01. SDS is used as an internal standard for the denaturation index, and the calculated α/β ratio will return the R_{SDS}, whereas R_i is the same ratio for a given surfactant i. The difference between R_{Hb}, and R_{SDS} is defined as being equal to 100% denaturation of hemoglobin and is used in eq. 1 to calculate the denaturation index of the studied surfactant:

$$DI = \frac{R_{Hb} - R_i}{R_{Hb} - R_{SDS}} \times 100 \quad (1)$$

The surfactants can be classified according to the L/D ratio (expressed in μg mL^{-1}) as non-irritant (L/D > 100), slightly irritant (L/D > 10), moderately irritant (L/D > 1), irritant (L/D > 0.1) and very irritant (L/D < 0.1) [43].

2.7. Photohemolysis assay

Samples for photohemolysis were prepared in a similar way to the samples used for the simple hemolysis tests. In well plates, 25 μL of RBC suspension was added to 950 μL of surfactant solution in PBS (with a concentration corresponding to a total hemolysis of 75% for a given surfactant). Samples were incubated under an UV lamp (Ultra-vitalux, Osram, UVA 960 μW cm^{-2} and UVB 220 μW cm^{-2}) for 2 h. Absorbance at 525 nm was read immediately after the end of exposure.

The aim of this assay was to study the phototoxic potential of the surfactants and vesicles by their ability to disturb the erythrocyte membrane, oxidize hemoglobin or both under exposure to UV light. The *in vitro* assessment of the photoirritancy of new compounds is essential to evaluate possible damage in skin after sun exposure, providing valuable information on photodynamic effects on cellular proteins and biomembranes [44]. This combined photo–RBC assay has been developed as an alternative to the use of UV-exposed animals and it shows an overall good correlation to *in vivo* data [45].

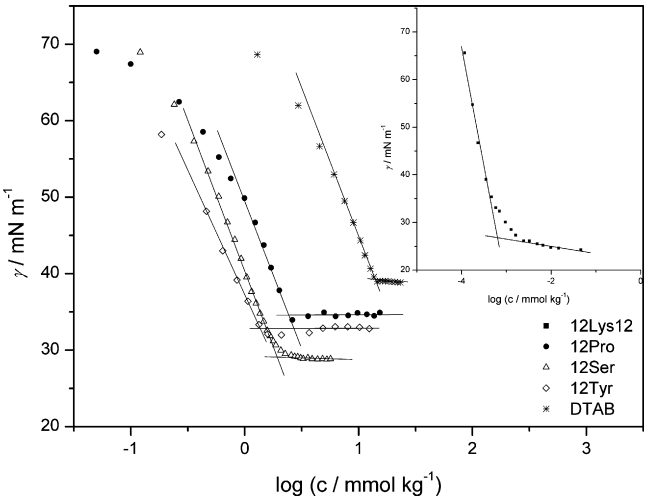

Fig. 2. Surface tension vs. log concentration curves for the different surfactants. The cmc is determined from the intersection point of the straight lines.

3. Results and discussion

3.1. Micellization properties

The critical micellar concentration (cmc) and other interfacial parameters for the surfactants were determined from surface tension measurements. All measurements were done at 25.0 °C, except for 12Lys12, done at 43.0 °C, since the Krafft temperature for this surfactant is 40.6 °C. Also, for the Lys- and Hyp-derived surfactants, both with carboxylate groups, the cmc determination was carried out at a fixed pH 12.0 ± 0.2 (adjusted with NaOH 0.010 mol dm^{-3}), to limit the extent of protonation of these soaps and thus avoid precipitation of their insoluble acidic form. For all the other surfactants, unbuffered aqueous solutions were used. Fig. 1 shows the surface tension vs. log c plots for the four studied surfactants, with the cmc value determined from the inflection point in the curves. The surface excess (Γ) was determined using the Gibbs adsorption equation:

$$\Gamma = -\frac{1}{2.303nRT}\left(\frac{\partial\gamma}{\partial \log c}\right)_T \quad (2)$$

where R is the ideal gas constant, T is the absolute temperature, $(\partial\gamma/\partial \log c)$ is the slope of the surface tension–log c plot below cmc and n is related to the number of species at the interface. The minimum surface area per molecule (A_m) is given by:

$$A_m = 1/N_A\Gamma \quad (3)$$

where N_A is the Avogadro's constant. In the case of the monovalent cationic surfactants n was taken as 2 since the surface tension was measured in neat water [46] while it was taken as 1 for the two anionic surfactants due to the presence of NaOH 0.0100 mol dm^{-3} in solution [47]. Table 1 lists the interfacial parameters determined for the studied surfactants.

Some qualitative considerations between the observed cmc values can be reasonably made, even taking into account the differences in pH. The cmc of the Hyp- and Ser-based amphiphiles are similar and lower than those of surfactants with typical headgroups and identical chain length, e.g. SDS, 8.2 mM and DTAB, 13.6 mM (in unbuffered solutions). The Tyr-derived surfactant has the highest cmc of the group, which is likely related to its bulky headgroup with a hydroxylated aromatic ring and consequent unfavorable packing constraints at the headgroup level in the micelles. The shallow well observed for this surfactant (Fig. 2) could be due to a small amount of an unknown contaminant (despite recristallization and good purity analysis) or to the ionization of the phenolic hydroxyl group.

Table 1
Surface tension at the critical micellar concentration (γ_{CMC}), critical micellar concentration (cmc), surface excess (Γ), surface area per molecule (A_m) for the studied surfactants, at 25 °C (except for 12Lys12, at 43 °C).

Surfactant	γ_{cmc}/mN m^{-1}	cmc/mmol kg^{-1}	10^6 Γ/mol m^{-2}	A_m/nm^2
12Lys12	27.1	0.55×10^{-3}	9.3	0.18
12Hyp	34.2	2.8	5.7	0.29
12Ser	29.2	1.9	3.6	0.46
12Tyr	31.9	13.5	3.3	0.50
DTAB	39.3	13.6	3.1	0.53

The smallest CMC is obtained, as expected, for the 12Lys12 surfactant, due to the fact that it bears two alkyl chains and hence is much more hydrophobic than the other studied compounds. The stronger hydrophobicity of 12Lys12 also explains the large surface excess (9.3 mol m^{-2}), indicating the affinity of the amphiphile to adsorb at the interface, thus efficiently lowering the surface tension. In addition, the existence of groups where intermolecular hydrogen bonding can occur, namely the N–H and C=O groups, can further enhance the surface adsorption [24,48–50]. For instance, comparing this surfactant with a bis(quaternary ammonium) gemini surfactant with four CH_2 spacer groups [46], it can be seen that the lysine derivative attains a much lower surface tension at the cmc (12-4-12: 39.8 mN m^{-1}; 12Lys12: 27.1 mN m^{-1}). With respect to the interfacial molecular areas in Table 1, they also follow a trend coherent with the previous considerations, with the largest and smallest areas observed for the Tyr- and Lys-amphiphiles, respectively.

3.2. Spontaneous formation of catanionic vesicles

Several cationic/anionic pairs from the above set of molecules were tested, for total surfactant concentration of 0.25 and 0.5 wt% and varying mixing ratio, in order to detect the formation of vesicles upon gently mixing the individual micellar solutions. The only mixtures that showed the ability to spontaneously form bluish turbid solutions containing vesicles were 12Lys12/DTAB and 12Lys12/12Ser (Fig. 3), and only for excess cationic surfactant. Cryo-TEM runs made for $X_{DTAB} = 0.74$ and $X_{Ser} = 0.70$ vesicles, both at 0.5 wt% in surfactant confirm that they are unilamellar, with smooth defect-free bilayers (Fig. 3). The vesicle population is relatively polydisperse and the radii distribution can be described by a log–normal distribution (from ca. 500 counts), yielding an average radius of roughly 30 nm Lys/DTAB and about 40 nm for the Lys/Ser system (*note*: here Fig 3b shows the presence of some vesicles larger than the average value). Surfactant hydrocarbon chains in the vesicles are in the fluid state, since differential scanning microcalorimetry runs in the range 2–90 °C show no evidence of a chain melting transition for the bilayers (data not shown). Also, these vesicle solutions remain stable in the course of months, showing no sign of phase separation (flocculation or precipitation).

Other cationic/anionic mixtures investigated for vesicle spontaneous formation were 8Lys8/12Ser, 10Lys10/12Ser, 12Lys12/12Tyr and 16Lys16/12Ser. These mixtures show solid formation (either in the form of crystals or flocs) not only at equimolar composition but over a wide mixing ratio. This fact indicates that the catanionic solid, formed due to stoichiometric electrostatic pairing, is largely the dominant aggregation form to the detriment of vesicles.

3.3. Acute toxicity results

The assessment of the biological properties of the synthesized amino acid-derived surfactants was initially done through the acute toxicity (IC_{50}) test, the details of which have been outlined in Section 2. The percentage of immobilised daphnids was plotted as a

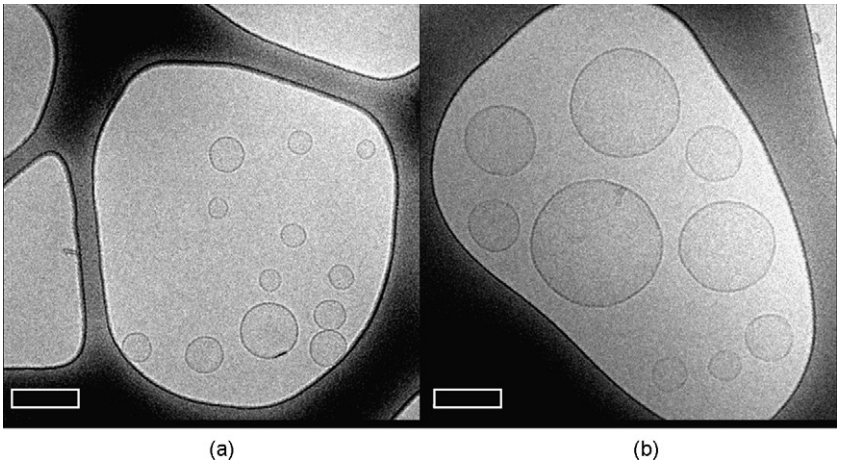

Fig. 3. Cryo-TEM vesicle imaging of (a) 12Lys12/DTAB vesicles, X_{DTAB} = 0.74 and (b) 12Lys12/12Ser vesicles X_{Ser} = 0.70. Total surfactant concentration of 0.5 wt%. Scale bar: 100 nm.

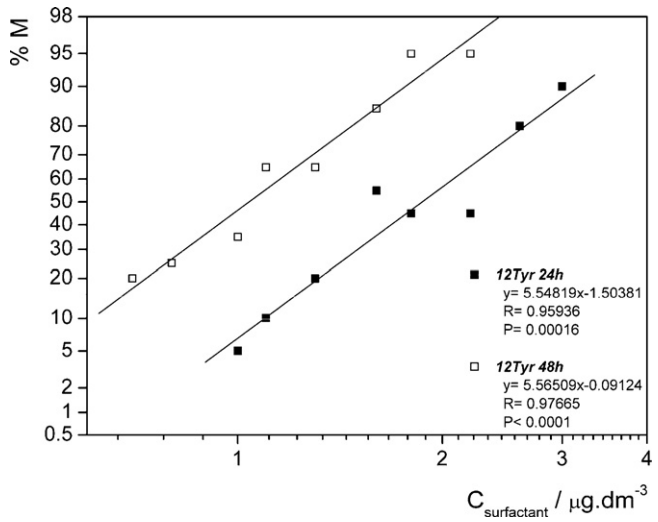

Fig. 4. Representative plot of the percentage of immobilised daphnids as a function of the tested concentration for the 12Tyr surfactant.

function of the logarithm of the tested concentration in a probability scale (Fig. 4). Slopes of the curves were determined and the IC_{50} value is reported with 95% confidence level.

The final results are shown in Table 2 and plotted in Fig. 5, for easier comparison. Inspecting the IC_{50} values in Table 2, it can be seen that all the cationic amino acid-derived surfactants exhibit lower aquatic toxicity for *D. magna* than DTAB, both for 24 and 48 h of exposure, and significantly lower than HTAB (hexadecyltrimethylammonium bromide). The 12Hyp and the double-chained 12Lys12 surfactants were not tested due to their low solubility at 20 °C, but results are also shown for 8Lys8. It can be reasonably assumed that 12Lys12 is more toxic than its C8 analogue, since toxicity typically increases with increasing chain length, as exemplified by DTAB and HTAB [18]. However, the most significant observation in Table 2 is the increase in IC_{50} for the 12Lys12/DTAB vesicle-containing mixture, despite the fact that DTAB corresponds to 74% of the total surfactant amount. Moreover, if DTAB is replaced with the cationic serine-derivative in the mixture, an even higher value of IC_{50} value is obtained, i.e. a lower toxicity is attained. These results, thus, show that the catanionic vesicle-containing solutions herein studied possess a comparatively low level of acute toxicity.

Table 2
Results of the acute toxicity (IC_{50}) for the studied surfactants.

Surfactant	IC_{50} 24 h/µmol dm^{-3} *	IC_{50} 48 h/µmol dm^{-3} *
SDS	80 (69–87)[a]	56 (49–62)[a]
DTAB	1.4 (1.3–1.7)	0.81 (0.71–0.94)
HTAB[b]	0.36 (0.30–0.41)	–
8Lys8[c]	45 (43–50)	38 (33–43)
12Lys12	–**	–**
12Hyp	–**	–**
12Ser	11 (10–12)	11 (10–11)
12Tyr	4.1 (3.7–4.5)	2.2 (2.0–2.4)
12Lys12/DTAB	2.2 (1.9–2.4)	1.3 (0.6–1.6)
12Lys12/12Ser	7.0 (6.3–7.4)	4.8 (4.1–5.2)

* The range of values refers to a 95% confidence level.
** Not tested for aquatic toxicity due to low solubility in water at room temperature.
[a] Ref. [18].
[b] Ref. [19].
[c] Ref. [18].

3.4. Hemolytic activity

The results from the hemolysis assay consist of plots of the percentage of red blood cell lysis as a function of the logarithm of the tested concentration in a probability scale (Fig. 6). The HC_{50}

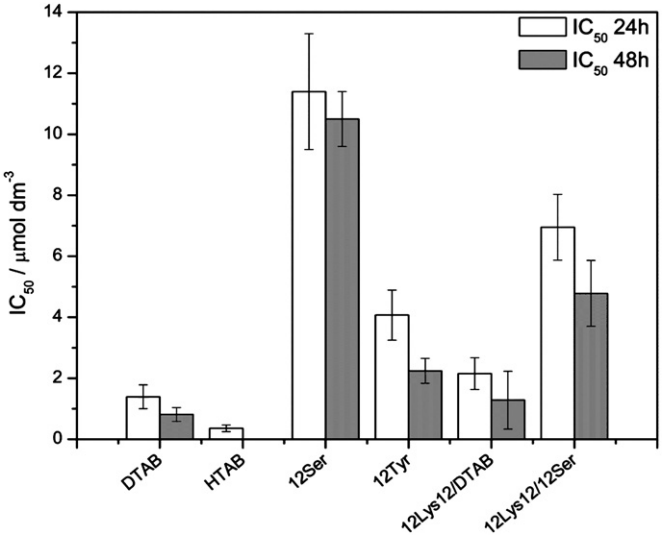

Fig. 5. Graphical representation of the IC_{50} values for the tested cationic surfactants and positively charged catanionic vesicles. HTAB is also represented for comparison purposes (IC_{50} value for 48 h not available).

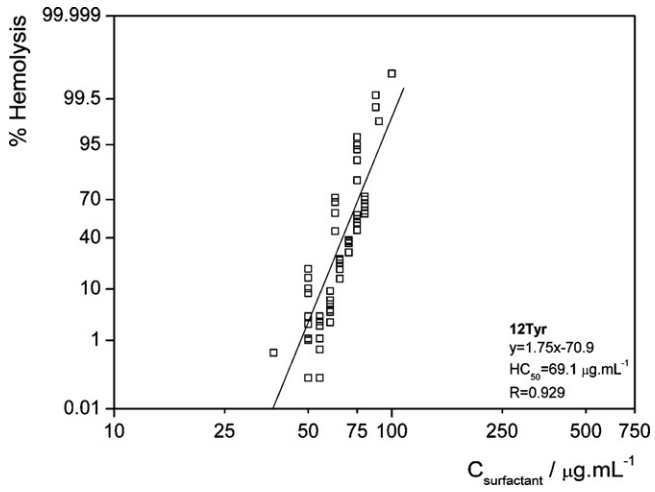

Fig. 6. . Representative plot of the percentage of hemolysed RBC as a function of the tested concentration for the 12Tyr surfactant.

value is determined from the linear fitting of the dose–response curve. The hemolytic activity results can be seen in Table 3. The cationic amino acid-derived surfactants show higher hemolytic activity than DTAB, but lower than HTAB. The anionic 12Lys12 shows higher hemolytic activity than the reference surfactant SDS due to its higher hydrophobicity (two alkyl chains) and significantly higher than 8Lys8 [51,52], indicating that for these Lys-based surfactants the biological activity is highly dependent on chain length. This can be understood if one considers that upon contact the surfactant molecules immediately insert themselves into the erythrocyte lipid bilayer [53,54], likely increasing its permeability or even its lateral expansion ability, and hence initially conferring hypotonic protection. Ultimately, however, they induce hemolysis due to bilayer solubilisation [17,51,55–57] and formation of mixed micelles. Bilayer solubilisation seems to be more efficient if the added surfactants and the bilayer lipids have similar hydrocarbon chain lengths [22]. In the present case, the erythrocyte membrane is essentially rich in C16 and C18 lipids and shows to be more easily disrupted by the C12 than by the C8 lysine surfactant. The influence of hydrophobicity is again observed for the cationic surfactants DTAB and HTAB, where the reduction in four CH_2 units implies a reduction of one order of magnitude in HC_{50}.

Just as for the acute toxicity, the catanionic association leading to vesicle formation dramatically reduces the hemolytic potential of the surfactants. The 12Lys12/12Ser vesicle mixture is ca. twice less hemolytic than 12Ser and both 12Lys12/12ser and 12Lys12/DTAB mixtures are ca. 25 times less hemolytic than 12Lys12. The catanionic solutions prove to be also less hemolytic than HTAB and only slightly more hemolytic than DTAB (Table 3). Again, we highlight the significance of the observed low hemolytic activity for catanionic vesicles. The mechanism behind these effects could lie directly on the fact that the mixed surfactants are self-assembled into vesicles and not micelles.

Table 3
Hemolytic effect (HC_{50}), denaturation index (DI) and lysis/denaturation ratio (L/D) values for the studied surfactants.

Surfactant	HC_{50}/μg mL^{-1}	DI	L/D/μg mL^{-1}	Classification
SDS	46.7 ± 1.2	100	0.49	Irritant
DTAB	449 ± 51	13.1	32.4	Slightly irritant
HTAB[a]	43 ± 2	46.5	0.92	Irritant
8Lys8[a]	261 ± 11	5.56	46.9	Slightly irritant
12Lys12	18.5 ± 0.5	5.29	3.50	Moderately irritant
12Hyp	136 ± 3	6.69	20.2	Slightly irritant
12Ser	213 ± 1	13.2	16.2	Slightly irritant
12Tyr	69.1 ± 5.9	11.5	6.00	Moderately irritant
12Lys12/DTAB	409 ± 24	–[*]	–[*]	–
12Lys12/12Ser	401 ± 9	–[*]	–[*]	–

[*] Not tested due to phase separation at 1 wt% surfactant.
[a] Ref. [52].

The critical packing parameter (CPP) of a surfactant can be defined as the ratio between the cross-section area of the hydrophobic chains and optimal headgroup area at the interface [58,59]. The CPP concept is a simple and convenient way to rationalize and predict the aggregation behavior of a surfactant in a given set of conditions. For a single ionic surfactant film, there are headgroup repulsions due to equal sign charges while attractive hydrophobic interactions keep the chains tightly packed. In mixed cationic/anionic surfactant systems, the headgroup repulsions are weakened due to the presence of opposite charges, leading to much smaller headgroup areas per molecule, whereas the chain cross section areas remain relatively unchanged. Thus, the average effective CPP value (CPP_{eff}) per surfactant for the catanionic mixture is expected to increase, originating an interface with lower curvature [9,15]. Mixtures with a composition closer to equimolarity have a CPP_{eff} approaching 1 and hence a tendency to form bilayer structures (such as vesicles), instead of micelles that have a much higher hemolytic potential toward cell membranes.

Furthermore, cationic/anionic surfactant mixtures are strongly associative (due to headgroup attractions and counterion liberation entropy) and usually present highly non-ideal synergistic effects, manifested as much lower CMCs than any of the constituent surfactants [60,61]. Such synergistic effects are often strongest in the vicinity of equimolarity. At cationic/anionic molar ratios where vesicles occur here (2.5:1 for 12Lys12/DTAB and 2:1 for 12Lys12/12Ser), close to equimolarity, the surfactant monomer concentration in equilibrium with the aggregates, is presumed to be rather low. This implies also that less monomeric surfactant is available in the medium to interact with the membrane and cause its disruption [51,57]. These two effects (catanionic bilayer formation and low monomer concentration) in combination could lie behind the observed decrease in toxicity of the vesicles compared to that of the neat surfactants (in micellar form).

3.5. Potential ocular irritation

The potential ocular irritation of the surfactants was studied using a method based on the use of RBC to quantify adverse effects of surfactants and detergent products on the cytoplasmic membrane (hemolysis) in combination with the damage to liberated cellular proteins (denaturation). The resulting L/D ratio is used instead of the ocular irritancy score in the acute phase of *in vivo* evaluation. The results are also shown in Table 3.

Surfactants can be classified according to their potential eye irritation ability by the lysis/denaturation ratio, L/D (expressed in μg mL^{-1}). The order is: L/D > 100 – non-irritant; L/D > 10 – slightly irritant; L/D > 1 – moderately irritant; L/D > 0.1 – irritant and L/D < 0.1 – very irritant. The Tyr- and 12Lys12 derivatives are thus considered to be moderately irritant, whereas the Hyp- and Ser- ones are just slightly irritant. No data is presented regarding the L/D ratio of the catanionic mixtures due to experimental requirements, namely the need of preparation of 1 wt% surfactant solution, where for both cases vesicles no longer occur as a single solution phase (a multiphase region is attained in the phase diagram).

The assessment of the photoirritancy of new compounds is essential to evaluate possible damage in skin after sun exposure. It has been usually performed in rabbits after application of the test material and exposure to UV light, but also in mice, rats and

guinea-pigs. Thus, a photohemolysis assay has been developed as an alternative to the use of animals. This test revealed an overall good correlation to *in vivo* data [45].

No significant increase in hemolysis and in the hemoglobin oxidation of irradiated samples was observed in comparison with the non-irradiated samples and with the positive control for hemoglobin oxidation, chlorpromazine (data not shown). This finding discards the susceptibility of the studied amino acid-derived surfactants and vesicle-containing mixtures to experience increase in hemolytic activity upon exposure to high levels of UV radiation.

3.6. Overview

One of the main goals of this work has been the assessment of the toxicity and hemolytic activity of the new amino acid-derived surfactants and their catanionic vesicles. A comparison has been done with common ionic surfactants, often used in commercially available formulations, which are in general more toxic and more hemolytic. The new amphiphiles are derived from natural sources (amino acids), but they do not exist as part of living organisms as is the case of alkylphosphatidylcholines and other naturally occurring lipids. Therefore, they are *a priori* expected to be relatively more toxic and exhibit a higher hemolytic activity than lipids. For instance, egg yolk lecithin liposomes show an HC_{50} of 880 μg/mL [62]. By comparison, the values obtained for the vesicles herein studied (409 and 401 μg/mL) indicate still low-toxicity levels. In this respect, the results imply that toxicity should not be an obstacle for their potential use. In addition, one should bear in mind that these vesicles show the superior advantages of being spontaneously formed and having long-term stability (owing to their likely thermodynamic stability), in contrast to classic lipid liposomes [9,10,12,15,63].

4. Concluding remarks

The biological properties, namely ecotoxicity and hemolytic potential, of newly prepared anionic and cationic amino acid-based surfactants, and some of their stable vesicle-forming mixtures, have been evaluated. The micellization studies first show that the compounds exhibit a surface activity that is at least equivalent to that of current commercial surfactants with similar chain lengths. Thus, the inclusion of an amino acid at the headgroup level not only does not hinder any interfacial performance and it can even enhance it. All the single surfactants, as well as the vesicle-containing mixtures, are found to have lower ecotoxicity than DTAB and HTAB. Furthermore, the novel surfactants are either moderately or slightly irritant, with the Ser and Tyr ones proving to be much less irritant than SDS and HTAB. The vesicle mixtures based on 12Lys12/DTAB and 12Lys12/12Ser have lower ecotoxicity and hemolytic activity than the individual micelle-forming surfactants, and comparable hemolytic activity to that of DTAB. The overall good biocompatibility level of the surfactants is thought to derive mainly from the natural origin of the headgroups. The reason behind the comparatively low hemolytic effect of the studied vesicles could be the depleted bulk monomer available for the disruptive interaction with biomembranes. Overall, these results show that catanionic vesicles based on amino acid surfactants could constitute viable agents for molecular delivery.

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