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The biological properties of lysine-derived surfactants

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Summary. We examine the effects of aquatic toxicity on *Daphnia magna*, the antimicrobial activity of new anionic lysine-derivative surfactants, and the influence of different-sized counterions associated with the surfactants. Surfactants with Tris and Lithium had less of a toxic effect on *Daphnia*, while all surfactants proved highly active against yeasts and the gram-negative bacteria *Bordetella bronchiseptica*. Counterion size was found to have no effect on aquatic toxicity or antimicrobial activity.

Keywords: Surfactants – Lysine – CMC – Aquatic toxicity – Antimicrobial activity

Introduction

Surfactants are amongst the most common chemical products, and are employed in large quantities every day on a worldwide scale as constituent elements of many different products (Paulsson and Edsman, 2001).

These compounds are multifunctional substances which, at low concentrations in solutions, are adsorbed onto surfaces and form aggregates known as micelles, at a critical concentration known as critical micellar concentration (CMC). Micellar formation is an important characteristic of surfactants since certain interfacial processes, such as the interaction of surfactants with biological membranes, lytic action, and solubilization, depend on micelles (or aggregates) in solution.

Some surfactants can pose toxicity problems for aquatic organisms due to their high polarity (Ankley and Burkhard, 1992). They play a major role in numerous biological processes and may be used as anti-bacterial agents for biological applications, provided their cell toxicity remains low (Perani et al., 2001). As a result of increasing environmental and toxicological concerns, there is great industrial demand for high-performing surfactants with low toxicity and antimicrobial properties (Infante et al., 1997). One interesting strategy to minimize environmental effects involves the synthesis of new molecules with analogous structures to natural compounds. Amino acid-based surfactants have attracted considerable interest as environmentally friendly surfactants due to their biodegradability, low aquatic toxicity, and low hemolytic activity (Macian et al., 1996). In relation to this, our group has extensive experience in the synthesis of amino acid-based surfactants (Seguer et al., 1994; Infante et al., 1997; Pérez et al., 2002) and to this end we have recently developed a new family of lysine-based surfactants with a non-conventional structure derived from the lysine amino acid. These surfactants have been widely studied in recent years in attempts to evaluate their potential risks in terms of eye and skin irritation. The results revealed low toxicity when compared with conventional surfactants, which led to considerable interest in their use for pharmaceutical and cosmetic preparations as a promising alternative to commercial anionic surfactants (Vives et al., 1997, 1999; Sanchez et al., 2004, 2005).

This article describes the biological properties, including antimicrobial activity and aquatic toxicity, of lysine-derivative surfactants. It also evaluates the influence of counterion size on biological properties of surfactants and CMCs.

Materials and methods

Surfactants

The following five anionic surfactants from the type N^{α},N^{ε}-dioctanoyl lysine were tested: Lysine salt (77KK), Tris(hydroxymethyl)aminomethane

salt (77KT), Sodium salt (77KS), Lithium salt (77KL) and Potassium salt (77KP). They were synthesized as described elsewhere (Vives et al., 1999; Sanchez et al., 2004).

Aquatic toxicity

The aquatic toxicity of surfactants was assessed using the *Daphnia magna* acute immobilization test (OECD, 1984). Laboratory-bred *D. magna* not more than 24 h old were used, with swimming incapability taken as the end point. The pH of the medium was 8.0 and the total hardness was 250 mg/l (as $CaCO_3$), with a Ca/Mg ratio of 4/1. Tests were performed in the dark at 20 °C. Twenty *Daphnia*, divided into four groups of five animals each, were used at each test concentration. For each surfactant, ten concentrations in a geometric series were tested. The percentage of immobile *Daphnia* at 24 and 48 h was plotted against concentration on logarithmic-probability graphs; a linear relationship was then obtained and the corresponding IC_{50} values (the concentration of surfactant causing immobilization in 50% of the *Daphnia*) were calculated.

Antimicrobial activity

Antimicrobial activity was studied by determining the minimal inhibitory concentration (MIC), which was defined as the lowest concentration of antimicrobial agent needed to inhibit the development of visible growth after 24 h of incubation at 37 °C (Woods and Washington, 1995). The following microorganisms were chosen: gram-negative bacteria: *Bordetella bronchiseptica* ATCC 4617, *Enterobacter aerogenes* ATCC 13048, *Escherichia coli* ATCC 10536, *Proteus mirabilis* CECT 170, *Pseudomonas aeruginosa* ATCC 9721, *Salmonella tiphymurium* ATCC 14028, and *Serratia marcenses* ATCC 4563; gram-positive bacteria: *Bacillus subtilis* ATCC 6637, *Enterococcus faecalis* ATCC 10541, *Staphylococcus aureus* ATCC 6538, and *Staphylococcus epidermidis* ATCC 12228; and yeasts: *Candida albicans* ATCC 10231, *Candida tropicalis* ATCC 4563, and *Saccharomyces cerevisiae* ATCC 9763.

Microorganisms, stored on cryobilles (EAS Laboratoire, France) at −20 °C, were streaked on Trypticase Soy Agar (Pronadisa, Barcelona, Spain) and incubated at 37 °C until colony formation occurred.

Using a sterile loop, colonies were collected and dispersed in broth, adjusting to a turbidity of 10^4–10^5 cfu/ml based on the McFarland scale. Antimicrobial activity assessment was conducted on 96 microwell plates (Corning, NY, USA) with Muller-Hinton Broth (ADSA, Barcelona, Spain). A two-fold serial dilution of surfactants (512 to 1 μg/ml) was used, without any active sample solution in the final column. After inoculation, plates were incubated for 24 h at 37 °C. The MICs were determined on the basis of visual observation of turbid and turbid-free wells.

CMC determination

Critical micelle concentrations (CMCs) were determined by measuring surface tension and conductivity values of several surfactant dilutions, all of which were prepared in Milli-Q ultrapure distilled water. Concentrated solutions of individual surfactants of known concentration were progressively diluted.

Surface tension measurements at 25 °C were determined by the Wilhelmy plate method on a Krúss K-12 surface tensiometer. The CMCs were determined by plotting the surface tension against the log of the concentration. The CMC was noted as a sharp change in decreasing surface tension as the concentration of the surface active agent increased.

Conductometry was measured with a Thermo Orion® connected to a water-flow thermostat maintained at 25 °C. The conductivity cell was calibrated with KCl solutions. The CMC values for each surfactant were determined by plotting the values of the specific conductivities against the respective surfactant concentrations. The CMC was calculated from the linear intersection in the dependence conductivity vs. surfactant concentration plots.

Results and discussion

Acute toxicity tests on freshwater crustaceans (*Daphnia magna*) were carried out to assess the aquatic toxicity of the new anionic surfactants (Sandbacka et al., 2000; Cserháti et al., 2002).

To evaluate the relative toxicity of the surfactants, the resulting data were compared with the conventional anionic surfactant, sodium dodecyl sulphate (SDS). Surfactant with potassium as a counterion could not be tested because of lack of solubilization in the assay medium stemming from the high quantity of calcium and magnesium ions.

The results of the *Daphnia magna* 24 and 48 h immobilization tests (IC_{50}) for the surfactants are summarized in Table 1. The higher the value, the lower the toxicity of the compounds. The data indicated that 77KT and 77KL were less toxic to the *Daphnia* population at 24 and 48 h of exposure than the other amino acid-based surfactants studied, and less toxic than SDS. IC_{50} values of surfactants with Lysine and Sodium were similar to that of

Table 1. Acute toxicity on *Daphnia magna* and critical micellar concentrations (CMC)

	CMC (10^3 mg/l)		*D. magna*, IC_{50} (mg/l) 24 h		*D. magna*, IC_{50} (mg/l) 48 h	
	Conductivity	Surface tension	Mean	95% confidence range	Mean	95% confidence range
77KK	1.8	2.6	53	(44–74)	24	(21–28)
77KT	2.3	2.4	316	(301–326)	203	(190–223)
77KS	3	3.1	19	(18–21)	16	(14–18)
77KL	2.9	2.8	309	(298–312)	180	(151–327)
77KP*	1.9	2.2	–	–	–	–
SDS	2.3	2.4	23	(20–25)	16	(14–18)

Concentration values that cause 50% inhibition (IC_{50}) in the crustacean mobility after 24 and 48 h of exposure. * Not tested due to low solubility in the medium

the conventional surfactant. SDS values were similar to those reported in the literature (Emmanuel et al., 2005).

Surfactants associated with Tris and Lithium clearly proved to be less toxic to *D. magna* than those bound to Lysine and Sodium, as was demonstrated by their effective concentrations, which were approximately 6 to 10 times higher than those corresponding to 77KL and 77KS.

Given that biological membranes are essentially non-polar interfaces, evidence indicates that the toxic effect of chemicals on these water-borne species is caused by the ability of molecules to disrupt the integral membrane via a hydrophobic/ionic adsorption phenomenon at the cell membrane/water interface, in a similar way to that of the antimicrobial mode of action. All IC_{50} values were below the CMC, indicating that monomers were responsible for the toxic effect on *Daphnia magna*.

No relation was found between the counterion size associated with surfactants and the aquatic toxicity they induced. A number of studies on quantitative structural activity relationships with regard to the aquatic toxicity of surfactants have been published (Dyer et al., 2000; Uppgard et al., 2000). These authors concluded that toxicity is basically correlated to hydrophobicity and not to surfactant-specific parameters, in a way similar to that observed in our experiments.

In studying the toxicity of surfactants versus *Daphnia magna*, the acute toxic effect of anionic surfactants on aquatic invertebrates was found to vary from 1.7 to 270 mg/l (Verge and Moreno, 2000). The less toxic lysine-derivative surfactants 77KT and 77KL approached the maximum toxicity at 48 h of exposure and exceeded it at 24 h.

All of these lysine-derived surfactants were less toxic to *Daphnia magna* than other amino acid-based surfactants were (Pérez et al., 2002).

The antimicrobial susceptibility test for lysine-derived surfactants was performed, with MIC values subsequently determined (Table 2). An assessment of membrane-disrupting properties was made using bacteria and yeasts as biological membranes. The hydrophobic nature of surfactant tails allows them to interact with and disrupt cytoplasmic membranes, rendering them very useful as general antimicrobial agents (Kanazawa et al., 1995).

The bioactivity of most surfactants is dependent on the concentration of monomers in solution, and is independent of aggregate formations at higher concentrations (Kopecky, 1996). Thus, CMC represents the maximal monomer concentration that will elicit a biological response. Given that the MIC values occur at concentrations below the CMC of surfactants in water (Table 1), it can be inferred that it is surfactant monomers, and not aggregates, that interact with cells (Rosen et al., 1999).

Data on MICs for the selected microorganisms revealed that all surfactants presented the optimum activities when tested against yeasts, particularly against *Candida tropicalis*. All lysine-derived surfactants were inactive (>512 μg/ml) against bacteria, with the exception of the gram-negative bacteria *Bordetella bronchiseptica*, which exhibited MIC values of 128 mg/ml.

No effects of counterion size on lysine-derived surfactants were detected, indicating that differences in the polar head groups do not lead to varied effects on their antimicrobial activity.

In conclusion, all of the amino acid-based surfactants examined in this study showed moderate bacterial activity, as well as high activity against yeasts. Surfactants associated with Tris and Lithium were less toxic to *Daphnia magna*. However, all of the surfactants appeared environmentally friendly given the high effective concentrations.

Table 2. Antimicrobial activity of lysine derivative surfactants

Yeasts	77KK	77KT	77KP	77KS	77KL
Candida tropicalis	64	256	64	128	512
Candida albicans	64	128	2	>512	>512
Saccharomyces cerevisiae	256	512	128	256	256

Minimum inhibitory concentrations (MIC, mg/l)

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