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# Influencia del contraión en las propiedades biológicas de tensioactivos aniónicos derivados de la $N^{\alpha}$,$N^{\varepsilon}$-dioctanoil lisina: citotoxicidad y ecotoxicidad *in vitro*

Memòria presentada per Lourdes Sánchez Molina per optar al títol de doctor per la Universitat de Barcelona

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# 3. ARTÍCULOS

# ARTÍCULO 1

## Evaluación del potencial efecto irritante dérmico de los tensioactivos en fibroblastos y queratinocitos

## Assessment of the potencial skin irritation of lisine-derivative anionic surfactants using mouse fibroblasts and human keratinocytes as an alternative to animal testing

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### Resumen

Los tensioactivos constituyen una clase de compuestos químicos que están presentes en la mayoría de preparaciones farmacéuticas, cosméticas y productos de limpieza. Debido a sus múltiples aplicaciones, el uso de estos productos puede comportar riesgos derivados de su efecto irritante.

El objetivo de este trabajo ha sido estudiar el potencial efecto irritante dérmico de una familia de tensioactivos aniónicos derivados del aminoácido lisina con diferentes contraiones en su estructura mediante técnicas de cultivo celular como alternativa al ensayo de Draize dérmico. Se han utilizado dos líneas celulares: una de fibroblastos (3T6) y otra de queratinocitos (NCTC 2544) para realizar los ensayos de citotoxicidad de captación de rojo neutro (NRU) y reducción de la sal de tetrazolio (MTT) y también se han incluido tres tensioactivos comerciales con propósitos comparativos. Los resultados de $CI_{50}$ obtenidos sugieren que estos tensioactivos son menos citotóxicos y por tanto, menos irritantes que el tensioactivo aniónico SDS y el catiónico HTAB y presentan un potencial efecto irritante similar al de la tegobetaína. Los tensioactivos con contraión orgánico (77KT y 77KK) mostraron una tendencia a ser menos irritantes que los de contraión inorgánico.

Por tanto, podemos concluir que los tensioactivos derivados de lisina presentan un potencial efecto irritante dérmico menor que los tensioactivos comerciales, por lo que podrían formar parte de formulaciones farmacéuticas o cosméticas sin comprometer la seguridad tras su aplicación.

# Assessment of the Potential Skin Irritation of Lysine-Derivative Anionic Surfactants Using Mouse Fibroblasts and Human Keratinocytes as an Alternative to Animal Testing

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***Purpose.*** The aim of this study was to identify new surfactants with low skin irritant properties for use in pharmaceutical and cosmetic formulations, employing cell culture as an alternative method to *in vivo* testing. In addition, we sought to establish whether potential cytotoxic properties were related to the size of the counterions bound to the surfactants.

***Methods.*** Cytotoxicity was assessed in the mouse fibroblast cell line 3T6 and the human keratinocyte cell line NCTC 2544 using the MTT assay and uptake of the vital dye neutral red 24 h after dosing (NRU).

***Results.*** Lysine-derivative surfactants showed higher $IC_{50}$s than did commercial anionic irritant compounds such as sodium dodecyl sulfate, proving to be no more harmful than amphoteric betaines. The aggressiveness of the surfactants depended on the size of their constituent counterions: surfactants associated with lighter counterions showed a proportionally higher aggressivity than those with heavier ones.

***Conclusions.*** Synthetic lysine-derivative anionic surfactants are less irritant than commercial surfactants such as sodium dodecyl sulfate and hexadecyltrimethylammonium bromide and are similar to betaines. These surfactants may offer promising applications in pharmaceutical and cosmetic preparations, representing a potential alternative to commercial anionic surfactants as a result of their low irritancy potential.

**KEY WORDS:** cytotoxicity; fibroblast; keratinocyte; lysine-derivative surfactants; skin irritation.

## INTRODUCTION

Surfactants are common constituents in many topical drugs and cosmetics. They are often used as additives in pharmaceutical and dermatological preparations, cleansers, soaps and shampoos due to their surface and interface properties (1).

Application of active ingredients and pharmaceutical additives may cause skin irritation; the majority of adverse skin reactions to personal-care products are presumed to be caused by surfactants (2). As a result, it is of great interest to identify surfactants with low irritant properties (3), and it is necessary to develop rapid assays to assess potentially damaging effects.

Evaluation of the potential for an ingredient or product to cause skin irritation is one of the various studies undertaken in the overall safety assessment process. Testing for skin corrosion or irritation has traditionally been conducted in animals, particularly in rabbits via the Draize test method (4). However, due to increasing concern over animal experimentation and its potential prohibition in the near future (5), alongside the obvious ethical implications of the use of human subjects, *in vitro* alternatives must now be developed.

*In vitro* toxicity testing systems also offer several advantages over *in vivo* systems, an obvious advantage being their immediate availability and reproducibility (6). Cell culture has been gradually introduced as an *in vitro* technique for the assessment of skin irritancy (7,8). Skin cultures are useful for the design of safer, more efficient and cost effective human skin irritation tests, in certain cases, eliminating the need for human or animal skin and *in vitro* cytotoxicity has generally been found to be a useful predictor of skin irritation potential (5).

Skin irritation is a reversible inflammatory reaction produced by the arachidonic acid cascade and cytokines in the viable keratinocytes and fibroblasts of the skin (9). Because of the increasing appreciation of the complex and dynamic regulatory role played by these cells in terms of the inflammatory responses to irritants and sensitizers, we chose to use both 3T6 and NCTC 2544 cells as model cell systems: the use of keratinocyte and fibroblast cultures offering an appropriate *in vitro* model for skin irritation (10–12).

Previous studies have suggested that cultured normal human keratinocytes may be predictive of irritancy caused by various surfactants in human subjects (13) and these monolayer cultures have been compared with *in vivo* skin irritation data (14,15). A good correlation with *in vivo* human skin data has been demonstrated for surfactants of different chemical types and irritation potential (16). However, in spite of the advantages of *in vitro* models, cell culture lacks some of the properties of intact skin, such as its selective barrier role or the interaction between different cell types. Thus, although irritation potential may be overestimated, it can nevertheless function as a useful prescreening tool (11).

Cell cytotoxicity assays are amongst the most common *in vitro* bioassay methods used to predict the toxicity of substances in various tissues and the potential of a chemical to elicit a corrosive response can be successfully predicted using appropriate endpoints because they demonstrate the degree of damage caused by the chemical. Thus,to assess the *in vitro* cytotoxic effects of surfactants, we measured cell viability through neutral red uptake and the MTT assay.

We have investigated the cytotoxic effects of five anionic lysine-derivative surfactants on human keratinocyte NCTC 2544 and Swiss albino 3T6 mouse fibroblast cultures in order to predict their skin irritation potential. We have also evaluated the relationship between potential cytotoxic properties and the size of the counterions bound to the surfactants.

## MATERIALS AND METHODS

### Chemicals

L-Lysine monohydrochloride, L-lysine, Tris, and the bases NaOH, LiOH, KOH, and sodium dodecyl sulfate (SDS) were purchased from Merck (Darmstadt, Germany), TEGO betaine T-50 (TGB) was obtained from Goldschmidt Ltd (Essen, Germany). RPMI 1640 medium without glutamine, L-glutamine, phosphate-buffered saline (PBS), and fetal calf se-

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rum (FCS) were supplied by Reactiva (Beit Haemek, Israel). Neutral red (NR) dye, MTT salt, dimethylsulfoxide (DMSO), and hexadecyltrimethylammonium bromide (HTAB) were supplied by Sigma-Aldrich (St Louis, MO, USA).

Penicillin (10,000 U/ml), streptomycin (10,000 μg/ml) mixture and fetal bovine serum (FBS) were purchased from Bio-Whittaker (Verviers, Belgium).

### Surfactants

Five types of salts were tested in this study: lysine salt (77KK); tris(hydroxymethyl)aminomethane salt (77KT); sodium salt (77KS); lithium salt (77KL), and potassium salt (77KP) (Fig. 1). Anionic surfactants of the salts derived from $N^{\alpha}$,$N^{\varepsilon}$-dioctanoyl lysine were synthesized in our laboratory according to the procedure described previously (18). The potassium salt is a new lysine-derivative surfactant synthesized for first time to perform this study according to the same procedure described previously.

### Cell Cultures

The normal human keratinocyte cell line, NCTC 2544, (provided by Interlab Cell Line Collection of Genoa, Italy) and the mouse cell line, 3T6, were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 1% penicillin (10,000 U/ml)/streptomycin (10,000 μg/ml) mixture at 37°C, 5%$CO_2$.

When 75 $cm^2$ culture flasks were approximately 80% confluent, the cells were seeded ($5 \times 10^4$ cells/ml for NCTC 2544 and $4 \times 10^4$ cells/ml for 3T6) into the central 60 wells of 96-well plates and then incubated at 37°C, 5%$CO_2$ for 24 h.

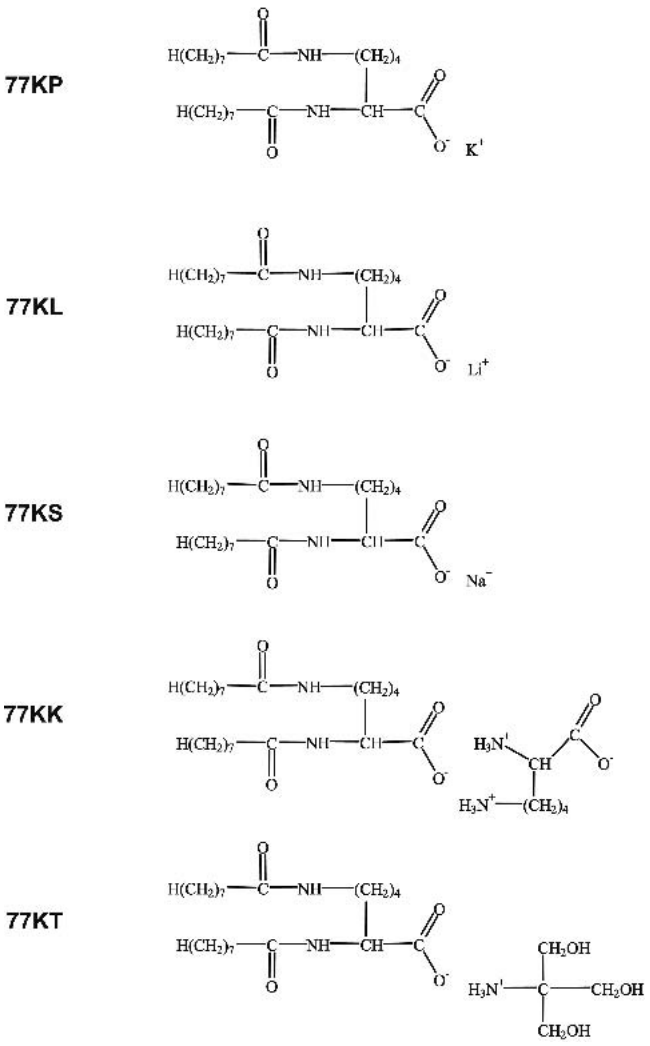


**Fig. 1.** Chemical structure and code of the lysine-derivative surfactants. K represents lysine in the international abbreviation.

### Experimental Treatments

Twenty-four hours after seeding in 96-well plates, cultures were exposed to several concentrations (from 7 μg/ml to 500 μg/ml of the surfactants (sterilized by filtration) dissolved in RPMI medium supplemented with 5%FCS, 2 mM glutamine, and 1% antibiotic mixture. Controls, containing culture medium only, were included in each plate. Plates were then incubated at 37°C, 5% $CO_2$ for 24 h.

### NRU Assay

The NRU assay was performed according to the method described previously (17). After treatment with the surfactants, medium was aspirated and replaced with 100 μl per well of NR solution (50 μg/ml in culture medium). After 3 h incubation at 37°C, 5% $CO_2$, medium was aspirated, cells were washed twice with PBS and a solution containing 50% absolute ethanol, 1% acetic acid in distilled water (100 μl/well) was added to extract the dye.

After 10 min shaking on a microtitre-plate shaker, the absorbance of the solutions was read at a wavelength of 550 nm in a Bio-Rad 550 microplate reader.

### MTT Assay

The MTT assay was performed according to the method of Mosmann (18). After treatment with the surfactants, medium was aspirated and replaced with 100 μl per well of the MTT solution (dissolved at 5 mg/ml in PBS and diluted 1:10 in cell culture medium without phenol red). At the end of the 3 h incubation, cultures were washed once with PBS and 100μl/well of dimethylsulphoxide (DMSO) was added to dissolve the purple formazan product while shaking for 10 min at room temperature. The absorbance of the resulting solutions was read at a wavelength of 550 nm in a Bio-Rad 550 microplate reader.

### Statistical Analysis

Both NRU and MTT experiments were performed at least 3 times using three wells for each concentration of the surfactant.

The cytotoxicity of each surfactant was expressed as the percentage viability compared to controls in terms of its $IC_{50}$ (concentration of surfactant that causes 50% inhibition of growth), calculated from the dose-response curves by linear regression analysis. The NRU assay is expressed as percentage uptake of neutral red dye by the lysosomes and the MTT assay as percentage reduction of tetrazolium salt by the mitochondrial enzyme.

## RESULTS AND DISCUSSION

In the present work, we studied the cytotoxicity of five anionic lysine-derivative surfactants, compared with three commercial surfactants: an irritant anionic surfactant, sodium dodecyl sulphate (SDS); a slight irritant amphoteric surfactant, TEGO betaine (TGB); and a highly irritant cationic surfactant, hexadecyltrimethylammonium bromide (HTAB).

The effects of the surfactants on cell membrane integrity were measured using two different endpoint assays for *in vitro* cytotoxicity: measurement of mitochondrial dehydrogenase activity (MTT) and a colorimetric assay of the ability of live cells to take up neutral red (NRU).

Results of NRU and MTT tests, carried out in keratinocytes and fibroblasts, were obtained at different concentrations for the surfactants tested and are presented as dose-response curves (Figs. 2 and 3). Uptake of neutral red dye by lysosomes and mitochondrial viability decreased with increasing surfactant concentration. Although similar curve profiles were obtained in both tests, with the exception of HTAB, in which the results were similar, the $IC_{50}$ values obtained by NRU analysis were higher than those with MTT (Table I). A possible explanation for this is that the cytotoxic effect of the majority of compounds was greater in the MTT reduction than in the neutral red uptake because mitochondria might generally be a more prominent site of surfactant cytotoxicity in the cells while high NRU values show an increase of the NR dye into the cells and thus a decrease in lysosomal damage.

Both the NRU and MTT methods have been used as indirect measures of cell vialbility (19,20) The $IC_{50}$ values of the surfactants studied (Table I) reveal that the lysine-derivative compounds are less cytotoxic, and thus predicted to be less irritant, than the commercial surfactants HTAB and SDS. The lysine-derivative surfactants showed higher $IC_{50}$s than SDS, one of the most widely used surfactants, which has been shown to damage barrier function by denaturation of the corneocytes and alteration intercellular lipids (19), whilst the commercial cationic surfactant HTAB was the most cytotoxic compound in all experiments, its $IC_{50}$ value being two hundred orders of magnitude lower than that of the other compounds tested. Although the nature of TEGO betaine is not the same as that of the five anionic surfactants tested here, the results suggest that these compounds are as harmless as amphoteric betaines. In agreement with this, a number of studies have shown that betaines cause less irritation both in skin and oral personal products than SDS (2).

No significant differences in the cytoxicicty, and thus the predicted dermal irritation of the lysine-derivative surfactants were observed in our study. Nevertheless, there was a clear trend toward the surfactants bound to the heavy counterions, lysine (77KK) and tris (77KT), being less irritant than those bound to light counterions. Neither surfactants associated with heavy counterions nor surfactants associated with light counterions were found significant differences in the cytotoxicity. In agreement with these data, we may conclude that there was a relationship between the size of the counterion and the cytotoxic properties of these surfactants: the heavy counterion they are bound to, the lower cytotoxicity they induce. In light of this observation, it is interesting to note that similar results have been reported for the induction of

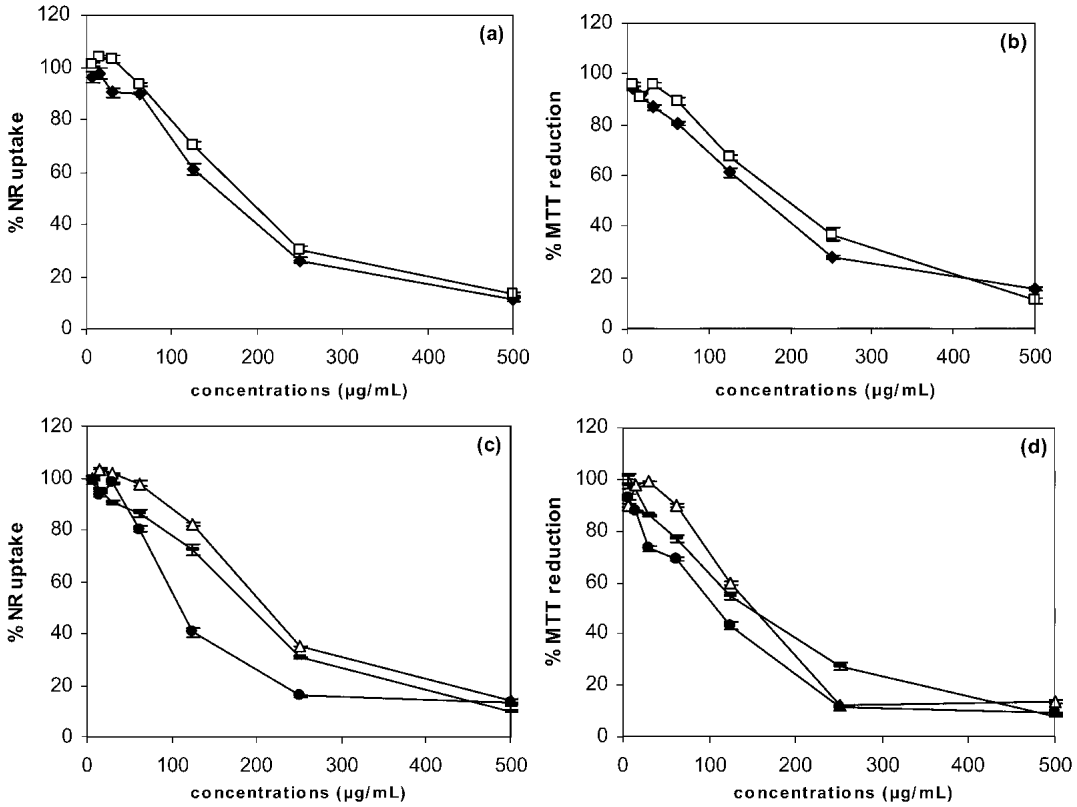


**Fig. 2.** Comparative cytotoxicity of compounds 77KK (♦) and 77KT (□) (a, b); 77KS (△), 77KP (–), and 77KL (●) (c, d) in NCTC 2544 human keratinocytes as detected with NRU and MTT assays. Results are expressed as mean ± SEM of three experiments.

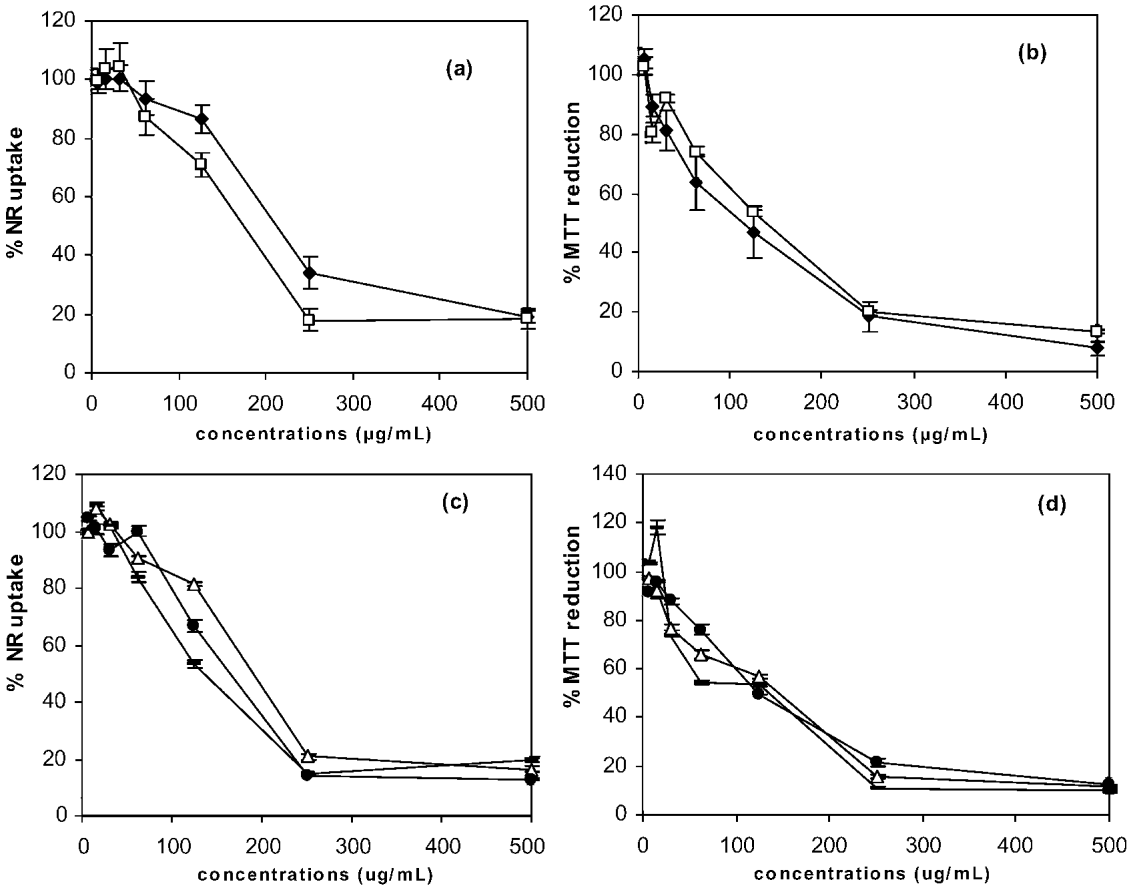


**Fig. 3.** Comparative cytotoxicity of compounds 77KK (♦) and 77KT (□) (a, b); 77KS (△), 77KP (–), and 77KL (●) (c, d) in Swiss Albino 3T6 mouse fibroblasts as detected with NRU and MTT assays. Results are expressed as mean ± SEM of three experiments.

necrosis or apoptosis in a mammalian cell line: of the surfactants studied, the 77KT surfactant showed significantly lower apoptotic and necrotic activity (20). However, we have not found significant differences between both surfactants associated with heavy counterions (77KT and 77KK. The physicochemical properties of anionic surfactants depend on the counterion they were bound to. To further research of the irritation potential toxicity of the lysine-derived surfactants (21), the results obtained in this study can be related to previous works performed in our laboratory to test these compounds on human and rat erythrocytes (22,23).

By comparing the $IC_{50}$s (concentration killing 50% of the cell population), it is clear that, with the exception of SDS, the cytotoxicity of the compounds in the 3T6 fibroblasts was greater than in the NCTC 2544 keratinocytes, as evaluated by both NRU and MTT methods. The irritancy classification of the compounds was different in both cellular models, due to morphologic and physiologic differences between the cell lines. These results indicate that keratinocytes might be more resistant to surfactant exposure because of their ultrastructure. Keratinocytes have been described as epithelial-like cells (24), while the fibroblasts have a dermal origin (25).

**Table I.** Cytotoxicity of Surfactants and Three Commercial Surfactants in NCTC 2544 Human Keratinocytes and 3T6 Mouse Fibroblasts Evaluated as $IC_{50}$ (the Dose to Inhibiting Viability to 50%) (Mean ± SEM)

| | 3T6 Fibroblasts ($IC_{50}$ µg/ml) | | NCTC 2544 Keratinocytes ($IC_{50}$ µg/ml) | |
|---|---|---|---|---|
| Surfactant | NRU test | MTT test | NRU test | MTT test |
| 77KK | 206.1 ± 4.25 | 129.07 ± 14.09 | 184.2 ± 9.99 | 166.45 ± 5.75 |
| 77KT | 172.53 ± 17.57 | 137.68 ± 11.21 | 191.4 ± 10.63 | 175.63 ± 9.77 |
| 77KP | 149.65 ± 6.85 | 114.92 ± 13.17 | 159.5 ± 10.45 | 133.63 ± 7.39 |
| 77KS | 159.63 ± 14.21 | 116.12 ± 8.35 | 182.7 ± 7.95 | 138.14 ± 8.39 |
| 77KL | 143.72 ± 2.21 | 104.4 ± 2.2 | 149.2 ± 16.9 | 108.47 ± 6.29 |
| SDS | 71.11 ± 5.11 | 63.86 ± 4.57 | 53.53 ± 0.69 | 44.67 ± 1.71 |
| HTAB | 0.46 ± 0.22 | 0.78 ± 0.13 | 2.07 ± 0.18 | 2.17 ± 0.15 |
| TGB | 165.66 ± 19.75 | 102.6 ± 3.96 | 203.23 ± 16.23 | 117.87 ± 13.70 |

Thus, the use of human keratinocytes may be of greater human relevance in the prediction of skin irritation.

## CONCLUSIONS

According to the results of the present work, we conclude that the synthetic lysine-derived anionic surfactants are less irritating than the commercial surfactants tested (HTAB and SDS) and similar to betaines.

Our results also show that the aggressiveness of the surfactants depends on the size of their constituent counterions: higher in surfactants associated with light counterions than in those carrying heavier ones.

In conclusion, these surfactants may be of interest for use in pharmaceutical and cosmetic preparations and represent an alternative to commercial anionic surfactants as a result of their low irritancy potential.

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# ARTÍCULO 2

## Evaluación del potencial irritante de los tensioactivos mediante hemólisis y viabilidad celular en células HaCaT

## Potential irritation of lysine derivative surfactants by hemolysis and HaCaT cell viability

Artículo publicado en *Toxicology Letters*

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### Resumen

Los tensioactivos son uno de los componentes más habituales en preparaciones farmacéuticas y cosméticas de aplicación tópica y también forman parte de detergentes y productos de higiene personal que se utilizan habitualmente. Por ese motivo, resulta primordial disponer de tensioactivos no irritantes que minimicen las reacciones irritantes derivadas de la aplicación de estos productos.

Se han estudiado las propiedades toxicológicas *in vitro* de un grupo de tensioactivos aniónicos derivados de lisina mediante el ensayo de hemólisis (determinación del potencial efecto irritante ocular), fotohemólisis (evaluación de las propiedades fotoirritantes) y viabilidad celular en células HaCaT (estimación del potencial efecto irritante dérmico). Los resultados muestran que los tensioactivos fueron menos hemolíticos y por tanto, menos irritantes oculares que los tensioactivos de referencia. Ninguno de los tensioactivos presentó efectos fototóxicos en comparación con la clorpromacina. Los resultados de $CI_{50}$ obtenidos a partir de los ensayos de citotoxicidad en cultivo celular mostraron que los derivados de lisina presentan un potencial efecto irritante inferior al de los tensioactivos comerciales y además, los de contraión orgánico fueron significativamente los menos citotóxicos.

Estos resultados confirman el bajo potencial irritante de los tensioactivos derivados de lisina y apoya la idea de su posible aplicación en preparaciones de aplicación tópica.

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# Potential irritation of lysine derivative surfactants by hemolysis and HaCaT cell viability

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**Abstract**

Surfactants represent one of the most common constituents in topical pharmaceutical and cosmetic applications or cleansers. Since adverse skin and ocular reactions can be caused by them, it is important to evaluate damaging effects. Amino acid-based surfactants deserve particular attention because of their low toxicity and environmental friendly properties.

New lysine derivative surfactants associated with heavy and light counterions were tested. The ocular irritancy was assessed by hemolysis, and photohemolysis was employed to evaluate their phototoxicity. Cytotoxicity on HaCaT cells was determined by neutral red uptake and MTT assay to predict skin irritation. All lysine derivative surfactants were less hemolytic and thus less eye-irritating than the commercial surfactants used as model irritants. No phototoxic effects were found. All surfactants presented cytotoxic effects as demonstrated by decrease of neutral red uptake and reduction of MTT salt, with clear concentration–effect profiles. However, the rates of cytotoxicity on HaCaT for the new surfactants suggested that they were less cytotoxic and then, less skin-irritating than the reference ones; surfactants with heavy counterions were the less cytotoxic. The anionic surfactants investigated in the present work may constitute a promising class of surfactants given their low irritancy potential for pharmaceutical and cosmetic preparations.


*Keywords:* Lysine derivative surfactants; Hemolysis; Photohemolysis; Cytotoxicity; Irritation

## 1. Introduction

Surfactants are among the most common and versatile constituents of many topical drugs, cosmetic products and cleansers, soaps and shampoos, and they are applied as additives because of their surface and interface activities. Thus, the concentrations used in commercial formulations should avoid adverse reactions like skin or eye irritation and damage. For this reason, the search for new non-irritant surfactants is of great interest and requires pre-formulation development trials (Benassi et al., 2003; Paulsson and Edsman, 2001). To ensure that dermal and ocular preparations are innocuous, it is necessary to study the potential irritation of surfactants by determining their toxicity.

There is a pressing need to develop surfactants with biodegradable and biocompatible properties, low toxicity, excellent emulsifying properties and antimicrobial activity because of the increasing concern about

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energy savings and environmental and toxicological trends (Richter and Kanaut, 1998). Therefore, it is necessary to use renewable, low-cost raw materials and design compounds with an inert structure. An interesting and useful strategy is the synthesis of surfactants based on the design of molecular mimics of natural compounds, such as lipoaminoacids, phospholipids and glycolipids. These surfactants are characterized by a non-conventional structure derived from amino acids, oligosaccharides and glycerols, respectively.

Among amino acid-based surfactants, we focused on the diacyl lysine derivative surfactants prepared as mimics of lecithins (Seguer et al., 1994).

New surfactants derived from amino acids and with low irritancy potential have been extensively studied in the last years (Benavides et al., 2004; Mitjans et al., 2003). Several studies have evaluated the effects of lysine derivative surfactants on human and rat erythrocytes in isotonic and hypotonic conditions through in vitro methods (Vives et al., 1997, 1999). $N^{\alpha}$,$N^{\varepsilon}$-dioctanoyl lysine derivatives were selected for the present study because homologues with eight carbon atom chains were the least hemolytic and irritant, and thus the most suitable for practical applications (Macian et al., 1996). We have also recently shown that these surfactants present low potential skin irritation compared to widely used commercial surfactants (Sanchez et al., 2004).

In addition, the evaluation of irritation potential of new products or ingredients is mandatory in most EU countries prior to human exposure and it has traditionally been conducted in animals, particularly via Draize test, to assess ocular and dermal irritancy (Draize et al., 1944). Moreover, the photoirritancy of new compounds has also been performed in rabbits after application of the test material and exposure to UV light, as well as in mice, rats and guinea-pigs (Spielmann et al., 1994a). However, due to increasing concern about animal experimentation and its potential prohibition in the near future, alongside the obvious ethical implications of the use of human subjects, in vitro alternatives must now be developed. Nevertheless, no single in vitro model or in vitro assay can mimic all the events that occur in vivo in the human eye or skin. Thus, a battery of complementary in vitro methods based on various mechanisms, as in the present study, may be useful (Benavides et al., 2004; Fischer et al., 2003) and necessary to complete the toxicological study of these surfactants.

The aim of this study was to investigate the toxicological effects of five anionic lysine derivative surfactants with heavy and light counterions in their structure and thus predict their dermal and ocular irritation potential using several in vitro methods. They were compared to reference surfactants to predict their toxicity for future applications. We also evaluated the possible effects of the counterion size associated with the surfactants to correlate counterion size and surfactant toxicity.

The potential ocular irritation was evaluated by the red blood cell assay and by the photohemolysis assay, which can be regarded as a second in vitro test, to obtain information about photodynamic effects on cellular proteins and membranes (Pape et al., 2001). One human keratinocyte cell line (HaCaT) was used as in vitro model to predict the potential skin irritation, and uptake of the vital dye neutral red (NRU) and the MTT reduction assay were used as endpoints to evaluate cell viability.

## 2. Materials and methods

### 2.1. Materials

L-lysine monohydrochloride, L-lysine, Tris, the bases NaOH, LiOH and KOH, sodium dodecyl sulphate (SDS), NaCl, $Na_2HPO_4$ and $KH_2PO_4$ were purchased from Merck (Darmstadt, Germany). TEGO® Betaine T-50 (TGB) was obtained from Goldschmidt Ltd. (Essen, Germany). L-glutamine, trypsin/EDTA, phosphate buffer solution (PBS, without calcium and magnesium) and RPMI 1640 without phenol red and glutamine were supplied by Reactiva (Beit Haemek, Israel). Neutral red dye (NR), MTT salt, dimethylsulphoxide (DMSO), hexadecyl trimethyl ammonium bromide (HTAB) and chlorpromazine hydrochloride were from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), Hepes buffer, penicillin (10,000 U/ml)–streptomycin (10,000 μg/ml) mixture and fetal bovine serum (FBS) were purchased from Bio-Whittaker (Verviers, Belgium). The 75 $cm^2$ flasks and 96-well plates were obtained from TPP (Trasadingen, Switzerland).

### 2.2. Synthesis of surfactants

Five types of surfactants were synthesized and tested in this study: lysine salt (77KK), Tris(hydroxymethyl)aminomethane salt (77KT), sodium salt (77KS), lithium salt (77KL) and potassium salt (77KP) (Fig. 1).

The anionic surfactants of the salts derived from the $N^{\alpha}$,$N^{\varepsilon}$-dioctanoyl lysine type were synthesized in our laboratory as described elsewhere and their surface activity was determined (Vives et al., 1999). Potassium salt is a new lysine derivative surfactant synthesized for first time following the same procedure.

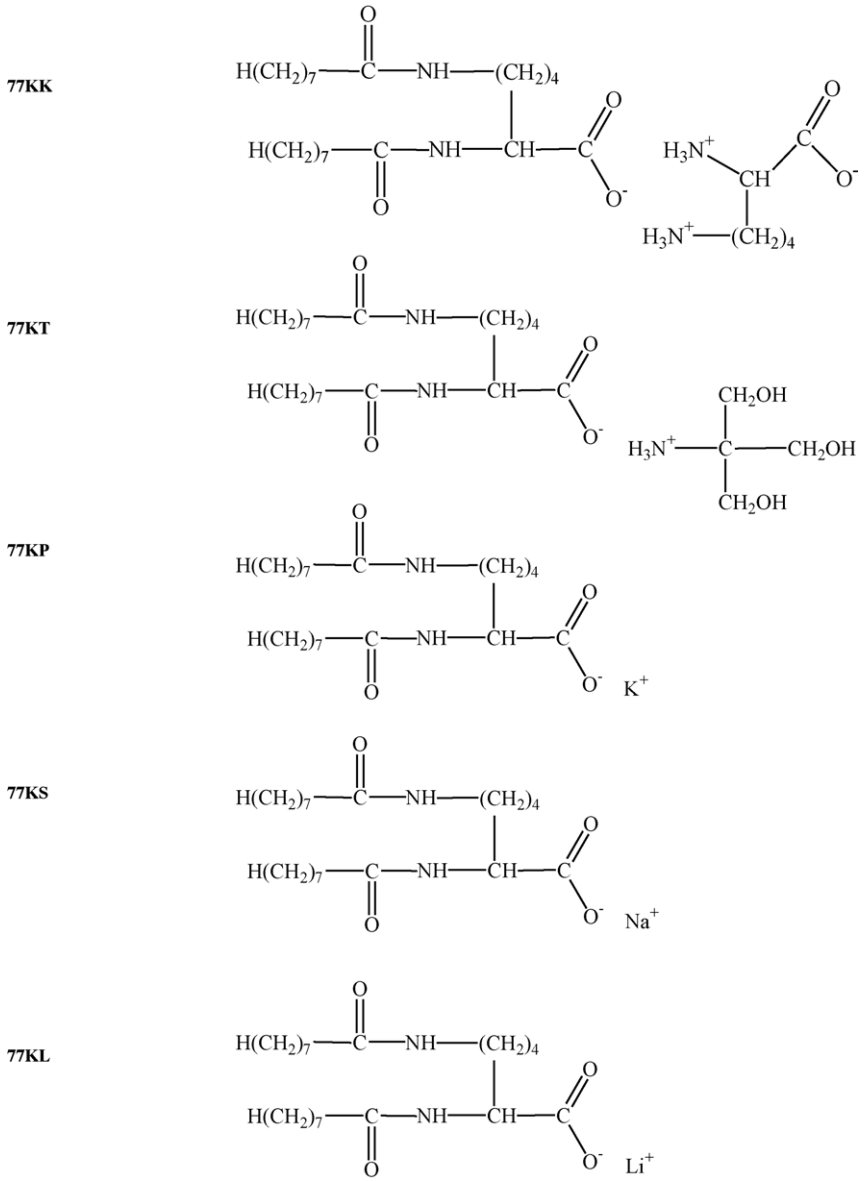


Fig. 1. Molecular structure of the lysine derivative anionic surfactants and codes. K, T, P, S, L represent lysine, Tris, potassium, sodium and lithium, respectively.

Anionic surfactants consist of two hydrophobic tails of octanoic acid bound to the amino acid lysine, and were obtained by condensation of fatty acyl radicals with the α-amino and ε-amino groups of lysine. The α-carboxylate group of lysine confers the anionic charge to the amphiphile. All compounds have a common hydrophobic moiety bound to the lysine residue, whereas counterions of various sizes and natures (heavy organic ones: Tris and lysine; light inorganic ones: sodium, lithium and potassium) were associated with the free carboxylic group of the lysine.

### 2.3. Cytotoxicity evaluation on HaCaT cells

#### 2.3.1. Culture of HaCaT cell line

The spontaneously immortalized human keratinocyte cell line HaCaT was grown in DMEM medium (4.5 g/l glucose) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM Hepes buffer and 1% penicillin (10,000 U/ml)–streptomycin (10,000 μg/ml) mixture at 37 °C, 5% $CO_2$. The HaCaT keratinocyte cell line was routinely cultured into 75 $cm^2$ culture flasks.

When the cells were approximately 80% confluent, they were harvested with trypsin/EDTA and seeded at a density of $5 \times 10^4$ cells/ml into the central 60 wells of 96-well plates and then incubated for 24 h at 37 °C, 5% $CO_2$. The triplicate runs were undertaken with different passage cells.

#### 2.3.2. Experimental treatments

Surfactants were dissolved in DMEM medium supplemented with 5% FBS, 2 mM L-glutamine, 10 mM Hepes buffer and 1% of the antibiotic mixture. They were prepared in a range of concentrations from 7 μg/ml to 500 μg/ml. After removal of the medium culture, HaCaT cells cultured in the 96-well plates were exposed to the serial dilutions of the surfactants previously sterilized by filtration. Controls, containing culture medium only, were included in each plate and they were independent for each of the different surfactants tested. Plates were incubated at 37 °C, 5% $CO_2$ for 24 h.

#### 2.3.3. NRU assay

The NRU assay was performed as described by Borenfreund and Puerner (1985).

Following treatment with the surfactant dilutions, the medium was aspirated and neutral red solution (50 μg/ml in RPMI medium without phenol red and serum) was added. After 3 h of incubation at 37 °C, 5% $CO_2$ medium was aspirated, cell cultures in the wells were washed twice with PBS and a solution containing 50% ethanol absolute–1% acetic acid in distilled water was added to extract the dye.

After 10 min on a microtitre-plate shaker, the absorbance of neutral red was measured at a wavelength of 550 nm in a Bio-Rad 550 microplate reader.

#### 2.3.4. MTT assay

A tetrazolium-based assay was used to determine the cytotoxicity of the surfactants following Mosmann (1983).

MTT was dissolved in PBS (5 mg/ml) and added to the cells in a 1:10 dilution in medium without phenol red and serum. After the treatment with surfactants, medium was removed and replaced by 100 μl of the MTT solution per well and the plates were incubated for 3 h. Thereafter, cultures were washed once with PBS and 100 μl of dimethylsulphoxide per well was added to dissolve the purple formazan product while shaking for 10 min at room temperature. The absorbance of the resulting solutions was read at 550 nm wavelength in a Bio-Rad 550 microplate reader.

### 2.4. Preparation of erythrocyte suspension

Erythrocytes were obtained from the blood of healthy volunteers from Hospital Clinic (Barcelona, Spain). They were washed three times in a phosphate buffer solution containing 123.3 mM NaCl, 22.2 mM $Na_2HPO_4$ and 5.6 mM $KH_2PO_4$ in distilled water (pH 7.4; 300 mos mol/l). The cells were then suspended at a density of $8 \times 10^9$ cells/ml.

### 2.5. Hemolysis assay

We followed the method described by Pape et al. (1987) as an alternative to the classical Draize test (Draize et al., 1944). The 25 μl aliquots of erythrocyte suspension were exposed to various concentrations of surfactants dissolved in PBS solution (from 100 μg/ml to 800 μg/ml) in a total volume of 1 ml. Following incubation at 37 °C for 10 min and centrifugation for 10 min at 3000 rpm, the absorbance of the supernatant was measured at 540 nm in a spectrophotometer. The percentage of hemolysis was determined by comparing the absorbance of the samples with that of the control totally hemolysed with distilled water. Dose–response curves were determined from hemolysis results and the concentrations inducing 50% hemolysis ($HC_{50}$) were calculated.

### 2.6. Evaluation of potential ocular irritation

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 of the surfactant or SDS by measuring the ratio of absorbance at 575 nm and 540 nm. The surfactants can be classified according to this L/D ratio as non-irritant (>100), slightly irritant (>10), moderately irritant (>1), irritant (>0.1) and very irritant (<0.1) (Pape et al., 1987).

### 2.7. Photohemolysis assay

The method applied is a modification of that described by Pape et al. (2001).

Various concentrations of surfactants (from 100 μg/ml to 800 μg/ml) were added to 25 μl aliquots of erythrocyte suspension in 24-well plates and were exposed to an UV lamp (Ultra-vitalux®, Osram,

Germany) (UVA 960 μW/cm$^2$ (6.9 J/cm$^2$) and UVB 220 μW/cm$^2$ (1.58 J/cm$^2$) for 2 h. The energy output of the lamp was checked periodically before each experiment with a UVX Digital Radiometer (Ultra-violet Products Inc., San Gabriel, CA, USA).

$HC_{50}$ was determined in irradiated and non-irradiated plates. A compound can be considered photoirritant if HC50 decreases significantly after UV radiation.

### 2.8. Statistical analysis

The cytotoxicity of each surfactant was expressed as a percentage of viability compared with control wells (the mean optical density of untreated cells was set to 100% viability) in terms of its $IC_{50}$ (concentration of surfactant causing 50% death of the cell population), calculated from the concentration–response curves by linear regression analysis. NRU assay results are expressed as the percentage of uptake of neutral red dye by the lysosomes and MTT assay results as the percentage of reduction of tetrazolium salt by the viable cells.

Both NRU and MTT experiments were performed at least three times using three wells for each surfactant concentration tested. For the hemolysis assay, three replicate samples were tested and for the photohemolysis assay, three wells. Both hemolysis and photohemolysis assays were carried out in triplicate. All data were compared by one-way analysis of variance (ANOVA) and Student's *t*-test using the SPSS® software (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to denote significance.

## 3. Results and discussion

### 3.1. Cytotoxicity of surfactants in HaCaT cells as determined by MTT and NRU assays

The cytotoxic effects of lysine derivative and commercial surfactants were evaluated via the cell membrane integrity of HaCaT cells. A colorimetric assay measuring the ability of live cells to take up the neutral red dye and the capacity for viable cells to metabolize a tetrazolium colourless salt to a blue formazan (MTT assay) were used as an indirect measurement of cell viability to predict skin irritancy. Cell cytotoxicity assays are amongst the most common in vitro bioassay methods employed to predict toxicity and corrosive effects. Therefore, it is important to study thoroughly the response of cell mechanisms upon exposure to chemical compounds (Lawrence, 1997; Wilhelm et al., 2001).

Previous studies have suggested that cultured human keratinocytes allow us to predict dermal irritancy caused by several surfactants in human subjects (Korting et al., 1994; Ward et al., 1998; Wilhelm et al., 2001). Keratinocytes are a biologically relevant target for skin irritants because they are the first living cells that contact externally applied compounds. The material required for primary keratinocyte cultures is limited and variable, and susceptibility to irritants varies with the number of passages. Therefore, we used HaCaT cells as a model, because this non-tumorigenic, spontaneously immortalized keratinocyte cell line provides an almost unlimited supply of identical cells, ensuring high intra- and interlaboratory reproducibility (Spielmann et al., 1998), and in vitro cytotoxicity in a human keratinocyte line (HaCaT) and human in vivo data for a homologous series of anionic surfactants are strongly correlated (Wilhelm et al., 1994).

However, the keratinocyte monolayer culture technique cannot directly replace in vivo methods because cell culture lacks some of the physiological and morphological properties of intact skin, such as a selective barrier and interaction between cell types. Thus, although the irritation potential may be overestimated, it can function as a useful, rapid pre-screening tool (Lawrence, 1997), but the resulting data should be interpreted cautiously.

To compare the cytotoxicity of the compounds, three commercial surfactants were employed as reference: an irritant anionic surfactant, sodium dodecyl sulphate, a slightly irritant amphoteric surfactant, tegobetaine and a highly irritant cationic surfactant, hexadecyl trimethyl ammonium bromide.

All tested surfactants had cytotoxic effects, as shown by the decrease in neutral red uptake and reduction of MTT salt. Typical concentration–response curves from NRU and MTT assays were recorded for all surfactants with $IC_{50}$ ranging from 500 μg/ml to 7 μg/ml. The rates of cytotoxicity ($IC_{50}$s) and the curve profiles obtained by both in vitro tests were similar. However, $IC_{50}$ values listed in Table 1 reveals that surfactants derived from lysine were less cytotoxic and thus predicted to be less irritant than the three commercial compounds taken as model irritants as demonstrated by statistical analysis. The $IC_{50}$ values obtained by NRU analysis were higher than those with MTT (Table 1) and the differences found were statistically significant ($P < 0.05$).

The cytotoxicity values of the five anionic surfactants differed significantly and the less cytotoxic and thus less irritant were the surfactants with heavy counterions, as revealed by both NRU and MTT in vitro methods. Nevertheless, we did not found differences between surfactants with counterions of the same size.

Table 1
Cytotoxicity of surfactants in HaCaT cells in terms of $IC_{50}$ (μg/ml)

| Surfactant | NRU assay | MTT assay |
|---|---|---|
| 77KK | 299.95 ± 20.82* | 194.73 ± 14.85* |
| 77KT | 246.40 ± 13.25* | 163.23 ± 9.27* |
| 77KP | 211.55 ± 10.62* | 145.80 ± 10.80* |
| 77KS | 199.63 ± 21.58* | 129.50 ± 7.33* |
| 77KL | 222.67 ± 26.16* | 129.55 ± 13.34* |
| SDS | 47.60 ± 5.60 | 31.40 ± 2.90 |
| HTAB | 4.53 ± 0.06 | 2.31 ± 0.60 |
| TGB | 76.73 ± 4.51 | 28.53 ± 5.17 |

Mean ± S.E.M. of three experiments.
* Significantly different from commercial surfactants ($P < 0.05$), Student's *t*-test.

In general, the physicochemical properties of surfactants are a crucial factor in eliciting skin irritation. Anionic surfactants are broadly accepted as potent irritants of human and animal skin; cationic surfactants are reputedly at least equally irritant, but more cytotoxic than anionic ones, while the irritation potential of non-ionic ones is considered the lowest (Effendy and Maibach, 1995).

The anionic surfactants tested in this study were less cytotoxic than SDS, which is the most widely used surfactant in gels and shampoos, and has been studied as a model irritant for studying mechanisms of acute irritation (Barany et al., 1999). They were also less cytotoxic than tegobetaine, which is considered as non-toxic and non-allergenic, alleviating the irritating effects of SDS (Rigano et al., 2000), and as example of detergent coming into use in shampoos and toothpaste given the rare cases of allergic reactions (Barany et al., 1999; Fowler, 1993). Both NRU and MTT assay identified the cationic surfactant HTAB as the most cytotoxic and thus the most irritant, as expected from previous results.

The cytotoxicity index of each surfactant obtained from both methods was correlated with the $IC_{50}$ of the surfactant SDS, taken as a control given its anionic behaviour. The *N* index was obtained by dividing the $IC_{50}$ of the surfactant by the $IC_{50}$ of SDS. Thus, we compared the cytotoxicity and irritancy potential of SDS and the surfactants tested. The anionic surfactants derived from lysine were four to six times less cytotoxic than SDS (Fig. 2). These results suggest that the lysine derivative surfactants are much less irritant than SDS and thus, could be employed as additives or constituents in many topical formulations.

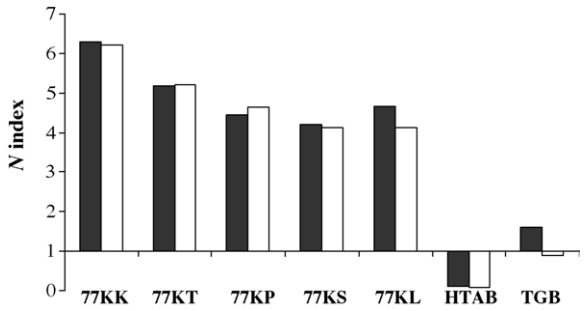


Fig. 2. Cytotoxicity on HaCaT cells measured by NRU (dark bars) and MTT assay (white bars) of surfactant referred to SDS. *N* index means how many times a surfactant is less cytotoxic compared to SDS.

### 3.2. Red blood cell assay to predict potential ocular irritation

The potential ocular irritation of the surfactants was studied using a method based on the use of red blood cells 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 classical in vivo methods for the determination of ocular irritancy have been replaced by in vitro methods in the last years. The red blood cell lysis assay is a rapid in vitro screening tool for evaluating the acute irritation potential induced by surfactants or surfactant-containing preparations and has been proposed as a reliable alternative to the Draize test. It distinguishes damage to the membrane and damage to proteins as endpoints that correlate with lesions on the conjunctiva, iris and cornea in the rabbit eye and the acute inflammatory responses during cutaneous irritancy testing (Pape et al., 1987). Moreover, erythrocytes are useful targets for ocular irritation assessment, as demonstrated elsewhere (Pape and Hoppe, 1991; Sugai et al., 1991).

The hemolysis, denaturation index and the corresponding classification of the surfactants according to their irritation potential showed that lysine derivative surfactants were less irritant than the reference surfactants tested (Table 2). According to the hemolytic activity of the five anionic salts, we found differences related to the counterion size. Therefore, those with heavy counterions presented the highest $HC_{50}$ ($P < 0.05$), being 77KT the less hemolytic.

Given the complexity of the human eye and skin, we should interpret these results with caution as no single in vitro assay can mimic at all the events occurring in the real eye or skin. Thus, a battery of methods needs to be employed to evaluate the irritation caused by cosmetic and pharmaceutical ingredients.

Table 2
$HC_{50}$ values, denaturation index (DI), lysis/denaturation ratio (L/D) and classification of the surfactants by their potential ocular irritation according to their L/D ratio

| Surfactant (MW) | $HC_{50}$ (μg/ml) (mean ± S.E.M.) | DI | L/D | Classification |
|---|---|---|---|---|
| 77KK (545.7) | 321.30 ± 22.37[a] | 4.83 | 66.52 | Slight irritant |
| 77KT (519.74) | 560.96 ± 16.03[a,b] | 6.46 | 86.84 | Slight irritant |
| 77KP (437.6) | 289.17 ± 2.41[a] | 5.01 | 57.72 | Slight irritant |
| 77KS (421.5) | 260.67 ± 11.17[a] | 5.56 | 46.88 | Slight irritant |
| 77KL (405.6) | 473.07 ± 9.72[a,*] | 6.23 | 75.93 | Slight irritant |
| SDS (288.4) | 43.6 ± 1.5 | 100 | 0.43 | High irritant |
| HTAB (598.4) | 43 ± 1.5 | 46.5 | 0.92 | High irritant |
| TGB (532.4) | 34.4 ± 2.2 | 14.4 | 2.39 | Middle irritant |

Mean ± S.E.M. of three experiments.
[a] Significantly different from commercial surfactants.
[b] Significantly different among surfactants with heavy counterions.
[*] Significantly different among surfactants with light counterions ($P < 0.05$), Student's *t*-test.

### 3.3. Photohemolysis assay

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 (Spielmann et al., 1994a). Thus, a photohemolysis assay has been developed as an alternative to the use of animals. This test revealed an overall good correlation to human in vivo data (Spielmann et al., 1994b).

The aim of this assay was to study the phototoxic potential of various compounds by their ability to disturb the erythrocyte membrane, oxidize hemoglobin or both under exposure to UV light. This method is useful and inexpensive and allows rapid screening of tested compounds. The combined photo–RBC test can be considered as an in vitro method that can provide useful data, in particular about photodynamic effects on cellular proteins and biomembranes given their accuracy, sensitivity and positive predictability (Pape et al., 2001).

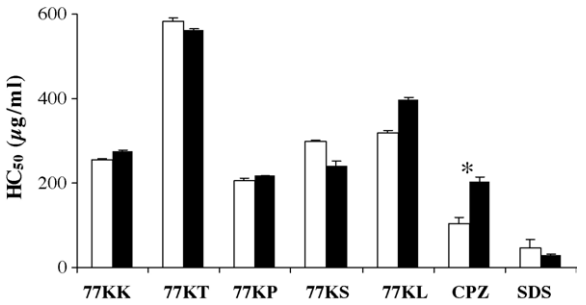


Fig. 3. Photohemolysis ($HC_{50}$) induced by the surfactants in human red blood cells. White bars represent irradiated cells and dark bars, non-irradiated cells. Chlorpromazine (CPZ) as positive control and SDS as negative control. [*]Significantly different from non-irradiated cells ($P < 0.05$), Student's *t*-test, mean ± S.E.M. of three experiments.

In this assay, two compounds were included as non-photoirritant (SDS) and photoirritant controls, e.g. the neuroleptic drug chlorpromazine (Eberlein-Konig et al., 1997).

The $HC_{50}$ of irradiated and non-irradiated red blood cells was similar for each surfactant, indicating no phototoxic action (Fig. 3) despite the high levels of UVB upon exposure to the erythrocytes. The $HC_{50}$ of chlorpromazine decreased significantly after UV radiation, confirming its photoirritant activity.

## 4. Conclusions

The dermal irritation predicted from the cytotoxic effects on HaCaT cells demonstrated that all lysine derivative surfactants were less cytotoxic than SDS. Likewise, our surfactants were less eye-irritating than SDS and none showed phototoxic effects. Surfactants with heavy counterions were the less skin and eye-irritating.

In conclusion, these surfactants are a promising alternative to commercial anionic surfactants given their low ocular and dermal irritancy. These properties offer great potential for topical preparations.

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# ARTÍCULO 3

## Ecotoxicidad en *Daphnia magna* y actividad antimicrobiana de los tensioactivos

## The biological properties of lysine-derived surfactants

Artículo publicado en *Amino Acids*

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## Resumen

Como consecuencia de las múltiples y variadas aplicaciones de los tensioactivos, éstos pueden aparecer en el medioambiente una vez desechados. Uno de los ambientes donde los factores ambientales adversos inciden de manera más directa es el acuático. Por tanto, es importante asegurar su inocuidad frente al medio ambiente. Por otro lado, los tensioactivos desempeñan un papel crucial en numerosos procesos biológicos y además pueden ser utilizados como agentes antimicrobianos.

En este trabajo, se ha evaluado la toxicidad acuática mediante el ensayo de inmovilización en el crustáceo *Daphnia magna* y las propiedades antimicrobianas en una serie de bacterias y levaduras de una familia de tensioactivos aniónicos derivados de lisina con diferente contraión. Los resultados obtenidos en *Daphnia magna* muestran que los tensioactivos 77KT y 77KL fueron los menos tóxicos de los tensioactivos estudiados mientras que los valores de MIC de los ensayos de susceptibilidad antimicrobiana muestran que los tensioactivos sólo son activos frente a las levaduras y la bacteria gram-negativa *Bordetella bronchiseptica*. No se encontró ninguna relación entre el tipo de contraión asociado a los tensioactivos y las propiedades estudiadas. Las concentraciones aplicadas estuvieron por debajo de la CMC, indicando que los monómeros y no las micelas fueron los responsables de la acción sobre el crustáceo y los microorganismos.

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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 $N^{\alpha}$,$N^{\varepsilon}$-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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# ARTÍCULO 4

## Determinación de IL-1α en queratinocitos humanos tratados con los tensioactivos

## Determination of interleukin-1α in human keratinocyte cells as a predictor of skin irritation from lysine-based surfactants

Artículo aceptado en *Toxicology Letters*

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### Resumen

Los tensioactivos derivados del aminoácido lisina han demostrado ser poco tóxicos en estudios anteriores y por ese motivo, han sido propuestos como ingredientes en preparados de aplicación tópica sin que representen un riesgo medioambiental.

Con el objetivo de completar el perfil toxicológico de estos tensioactivos, se ha valorado la producción de IL-1α (intracelular y liberada al medio de cultivo) en una línea celular de queratinocitos humanos (NCTC 2544) para predecir el potencial irritante dérmico. Paralelamente, se ha determinado la viabilidad celular de las células tratadas con los tensioactivos mediante el ensayo MTT. Los queratinocitos, debido a su localización en la epidermis, desempeñan un papel muy importante en el inicio, modulación y mantenimiento de las respuestas inflamatoria e inmunológica a través de la producción de citocinas, especialmente de IL-1α, que puede ser liberada en respuesta a un gran abanico de estímulos.

Los resultados muestran que los tensioactivos son menos potentes en estimular la síntesis y la liberación de la citocina al medio de cultivo que el tensioactivo aniónico SDS, observándose una ligera tendencia de los tensioactivos con contraión orgánico a ser menos irritantes. La liberación de IL-1α y la viabilidad celular constituyen parámetros efectivos para predecir el potencial efecto irritante dérmico y se confirma el bajo efecto irritante de los derivados de lisina, que los hace aptos para formulaciones de aplicación tópica.

# Determination of interleukin-1α in human NCTC 2544 keratinocyte cells as a predictor of skin irritation from lysine-based surfactants

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## Abstract

Lysine-derivative surfactants are a class of amino acid-based surfactants synthesized as lecithin analogues that deserve particular attention because of their low toxicity and high biocompatibility. To complete the toxicological profile of these surfactants, IL-1α production (cell-associated and release to the culture medium) was determined as an *in vitro* method for predicting skin irritation. In addition, an MTT assay was used as a viability marker in keratinocytes NCTC 2544. Keratinocytes are a biologically relevant target for developing *in vitro* techniques to assess skin irritants: Moreover, they are the principal source of the proinflammatory cytokine IL-1α in the epidermis. Lysine-derivatives proved to be less potent in stimulating IL-1α synthesis and induced a lower release of this cytokine into the culture medium when compared to the anionic surfactant sodium dodecyl sulfate. Due to their low irritancy potencial, lysine-based surfactants may offer promising applications in pharmaceutical and cosmetic preparations.

*Keywords*: Lysine-based surfactant; Skin irritation; Interleukin-1α

## 1. Introduction

Surfactants, due to their surface and interface activities, are among the most common and versatile components of pharmaceutical and cosmetic preparations, cleansers, soaps and shampoos. Thus, the concentrations used in commercial formulations should avoid adverse reactions such as skin irritation and damage. For this reason, the search for new non-irritative surfactants has generated considerable interest, although pre-formulation developmental trials are needed (Benassi et al., 2003). Furthermore, there is a pressing need to develop biodegradable and biocompatible surfactants with low toxicity, and excellent emulsifying properties, particularly in light of increasing concerns about energy consumption and environmental and toxicological dangers (Richter and Kanaut, 1998). Therefore, it is necessary not only to use renewable, low-cost raw materials but also to design compounds with an inert structure. An interesting and useful strategy is the synthesis of surfactants with structures analogous to natural compounds. These surfactants are characterized by a non-conventional structure derived from amino acids, oligosaccharides, or glycerols. Among amino acid-based surfactants, we focused on the diacyl lysine derivative surfactants prepared as lecithin analogues (Seguer et al., 1994; Vives et al., 1999).

$N^{\alpha}$, $N^{\varepsilon}$-dioctanoyl lysine derivatives were selected for the present study as homologues with eight carbon atom chains proved the least hemolytic and irritant, and thus the most suitable for practical applications (Macian et al., 1996). We have also recently shown that these surfactants not only present potentially low eye and skin irritation compared with widely used commercial surfactants (Sanchez et al., 2004, 2006a), but also low acute aquatic toxicity when tested on freshwater crustaceans (*Daphnia magna*). Thus all of these surfactants appeared environmentally friendly despite of their high effective concentrations (Sanchez et al., 2006b).

Before humans can be exposed to such substances, the tendency of new chemicals to cause skin irritation must be determined. The method published by Draize et al. (1944) which assessed skin irritation and corrosion in rabbits has been used for decades to classify and evaluate the risks inherent to new chemicals. However, this method has been widely criticized for several reasons: the subjectivity involved in obtaining irritancy scores; the irreproducibility of results; the inability to obtain relevant information concerning the potential toxicity mechanisms of chemicals; and increasing efforts to limit animal testing (Eun and Suh, 2000). Therefore, considerable effort has been made towards developing and evaluating *in vitro* as replacements for traditional *in vivo* assays (Garthoff, 2005). *In vitro* methods offer some advantages over *in vivo* systems owing to their reproducibility, availability (Osborne and Perkins, 1994) and their capacity to provide relevant mechanistic information (Pappinen et al., 2005).

Therefore, conventional cell cultures are used to test direct interactions of test agents in living cells (Ward et al., 1998). A great variety of protocols have been published in the

literature, encompassing a wide range of responses believed relevant to *in vivo* skin irritation (Medina et al., 2000: Sugibayashi et al., 2002).

Human keratinocytes represent the major cell type in the epidermis and play a key role in skin inflammatory and immunological reactions due to their anatomical location (Barker et al., 1991; Berardesca and Distante, 1995). Because of their ability to produce a large range of inflammatory mediators, immersed keratinocyte cultures are usually chosen to develop *in vitro* methods for evaluating irritants (Corsini et al., 1996).

In response to physical and chemical stresses like skin irritants, keratinocyte cells produce and release inflammatory cytokines, chemokines, growth-promoting factor, and other signaling factors that rapidly generate cutaneous inflammation. This suggests, that the measurement of such keratinocyte responses may be useful for the evaluation of chemical toxicological properties related to irritants. Cytokine production and response are the mayor mechanism used by epidermal cells to participate in immune and inflammatory responses (Williams and Kupper, 1996). Of the cytokines produced by keratinocytes, IL-1 is among of the most interesting, since it is produced constitutively by keratinocytes and retained under normal conditions into the cells. IL-1$\alpha$ is a very important inflammatory mediator and is believed to be the main switch initiating inflammation (Coquette et al, 2000) and the body's response to microbial invasion, and tissue injury (Dinarello, 1996). In keratinocyte cells, biologically active IL-1$\alpha$ predominates, large amounts of which can be released in response to a range of stimuli. Here, the hypothesis is that every time that keratinocytes are damaged by chemicals, UVB light or by microorganism infections, IL-1$\alpha$ is released as a primary event in skin defense (Kupper and Groves, 1995). In fact, it is accepted as an early marker of irritation both in *in vivo* and *in vitro* systems (Corsini et al., 1996; Corsini and Galli, 1998).

The aim of this study was to investigate the toxicological effects of five anionic lysine derivative surfactants containing heavy and light counterions within their structure and to predict their dermal irritation potential using *in vitro* methods. One human keratinocyte cell line (NCTC 2544) was used as *in vitro* model to evaluate IL-1$\alpha$ production as an inflammatory indicator while the MTT reduction assay was used as a viability marker.

## 2. Materials and Methods

### *2.1. Materials*

L-lysine monohydrochloride, L-Lysine, sodium dodecyl sulfate (SDS), Tris(hydroxymethyl)aminomethane, NaCl, and the bases NaOH, LiOH and KOH were purchased from Merck (Darmstadt, Germany). RPMI 1640 medium without glutamine, RPMI 1640 without phenol red and glutamine, L-Glutamine, trypsin/EDTA, phosphate buffer solution (PBS) without calcium and magnesium, penicillin (10,000 U/ml)/streptomycin (10,000 $\mu$g/ml) mixture and fetal bovine serum (FBS) were

supplied by Cambrex Bio Science (Verviers, Belgium). MTT salt was acquired from Sigma-Aldrich (St Louis, MO, USA). 75 $cm^2$ flasks and 24-well plates were obtained from TPP (Trasadingen, Switzerland).

*2.2. Synthesis of surfactants*

Five anionic surfactants of the derived from $N^{\alpha}$,$N^{\varepsilon}$-dioctanoyl lysine containing counterions of different chemical compositions were tested (Fig. 1): lysine salt (77KK), tris(hydroxymethyl)aminomethane salt (77KT), sodium salt (77KS), lithium salt (77KL) and potassium salt (77KP). They were synthesized as previously described (Vives et al., 1999; Sanchez et al., 2004).

*2.3. Culture of NCTC 2544 keratinocytes*

The human keratinocyte cell line NCTC 2544 was cultured in RPMI 1640 medium supplemented with 10 % FBS, 2 mM L-glutamine and 1 % penicillin (10,000 U/ml)/streptomycin (10,000 µg/ml) mixture and maintained in 75 $cm^2$ culture flasks at 37 ºC, 5 % $CO_2$.
When the cells reached approximately 80 % confluency, they were harvested with trypsin/EDTA and seeded at a density of 5 x $10^4$ cells/ml into 24-well plates and then incubated until attaining confluency at 37 ºC, 5 % $CO_2$.

*2.4. Surfactant treatment*

Surfactants were dissolved in RPMI medium supplemented with 1 % FBS, 2 mM L-glutamine and 1 % of the antibiotic mixture. Lysine derivative surfactants were prepared in a range of concentrations from 20 µg/ml to 180 µg/ml and SDS, from 5 µg/ml to 50 µg/ml, respectively. After removing the medium culture, the cells grown in 24-well plates were exposed to serial surfactant dilutions, previously sterilized by filtration. Controls, containing culture medium only, were included in each plate, and were maintained independently for each of the different surfactants tested. The chemicals and the controls were added in a volume of 300 µl per well. Plates were incubated at 37 ºC, 5 % $CO_2$ for 24 h.

*2.5. IL-1alpha determination*

Following surfactant exposure, conditioned medium was recovered, centrifuged and used to determine extracellular IL-1$\alpha$ (IL-1$\alpha$ release). Keratinocyte monolayers were washed once with PBS, then lysed in 300 µl of PBS containing 0.5 % of Triton X-100 and the resulting solution was centrifuged and used for intracellular IL-1$\alpha$ (cell-associated IL-1$\alpha$) determination. Both IL-1$\alpha$ release and cell-associated IL-1$\alpha$ were determined using an

IL-1alpha ELISA kit according to the manufacturer's instructions (Diaclone Research, France).

The cell-associated IL-1$\alpha$ amount were plotted against surfactant concentrations on exponential graphs and the corresponding $EC_{50}$ values (the concentration that induces a 50 % increase in cell-associated IL-1$\alpha$) were calculated from the dose-response curves equation.

*2.6. MTT assay prodedure*

A tetrazolium-based assay was used to determine the cell viability (Mosmann, 1983) of the surfactants in parallel with IL-1$\alpha$ determinations. MTT salt was dissolved in PBS (5 mg/ml) and added to the cells in a 1:10 dilution in RPMI medium without phenol red and serum. Following surfactant treatment in 24-well plates, the medium was removed and replaced by 300 µl/well of MTT solution and the plates were incubated for 3 h. Thereafter, cultures were washed once with PBS and 300 µl/well of DMSO was added to dissolve the purple formazan product, shaking for 10 min at room temperature. 100 µl aliquots of MTT extraction solution were transferred from each well to corresponding wells of a 96-well plate and the absorbance was read a 550 nm wavelength using a Bio-Rad 550 microplate reader.

The absorbance values were plotted against surfactant concentrations on exponential graphs and the corresponding $IC_{50}$ values (the concentration of surfactant causing 50 % inhibiton of cell growth) were calculated from the dose-response curves equation.

*2.7. Statistical analysis*

Results are expressed as the mean ± S.E. of at least three independent experiments. Statistical significance was determined by one-way analysis of variance (ANOVA) and the Student's *t*-test using SPSS® software (SPSS INC., Chicago, IL, USA). $P<0.05$ was considered significant.

## 3. Results and Discussion

The use of surfactants in everyday life is almost unavoidable. Thus the development of less irritative, less toxic, consumer-friendly surfactants is, of great interest. Lysine-derivative surfactants constitute a novel class of amino acid-based surfactants that can be regarded as an alternative to conventional synthetic surfactants based their multi-functionality, the renewable source of raw materials used during their synthesis and the low irritation potential revealed in previous studies (Vives et al., 1999; Sanchez et al., 2004, 2006a). These characteristics make them excellent candidates as additives in pharmaceutical and cosmetic formulations.

Keratinocytes are a biologically relevant target for the development of *in vitro* techniques for assessing skin irritants (Osborne and Perkins, 1994; Van de Sandt et al., 1999; Eun and Suh, 2000). Moreover, it has become clear that keratinocytes participate actively in skin inflammatory and immunological reactions (Mckenzie and Sauder, 1990; Barker et al., 1991).

Skin irritants induce or upregulate the cutaneous expression of cytokines, including those that are required for immune responses and that participate in inflammatory reactions. Chemical irritants such as surfactants can trigger various inflammatory processes induced by proinflammatory cytokines. In fact, the release of inflammatory mediators from keratinocyte cultures has been proposed as an *in vitro* approach for assessing surfactant-induced irritation (Corsini et al., 1996; Ward et al., 1998).

Due to their epidermal location and their central role in irritative contact dermatitis, keratinocyte and cytokine production offers a simplified *in vitro* model to evaluate the potential toxicity of chemicals with cutaneous applications (Sauder and Pastore, 1993).

In this study, IL-1$\alpha$ production (both cell-associated and that released into culture medium) was investigated in human keratinocytes following their exposure to lysine derivative surfactants. The anionic surfactant SDS was selected as a reference surfactant, while untreated cells were included as a control in each assay. The intracellular content and release of IL-1$\alpha$ into the culture medium were determined by ELISA while an MTT assay was used as an indicator of cell viability. In some cases, the amount of cytokine was not detected by the IL-1$\alpha$ ELISA kit due to the limit of detection (31.25 pg/ml).

Our results regarding the cell-associated IL-1$\alpha$ and IL-1$\alpha$ release for lysine derivative surfactants are presented in Fig. 2. IL-1$\alpha$ production yielded clear discrimination among the new surfactants (Fig. 2) and SDS (Fig. 3). SDS provoked cell-associated IL-1$\alpha$ as well as release of this cytokine at lower concentrations than our surfactants. The higher IL-1$\alpha$ release measured approximately 150 pg/ml at the higher concentration of lysine derivatives (180 µg/ml) while for SDS, the IL-1$\alpha$ release measured 300 pg/ml at the lower concentration (50 µg/ml). In some cases, the IL-1$\alpha$ release was only detected at the surfactant highest concentrations. For lysine derivative surfactants, IL-1$\alpha$ release was significantly after exposure to concentrations higher than 150 µg/ml ($P<0.05$).

In the same way, lysine derivatives at 120 or 150 µg/ml induced the highest cell-associated IL-1$\alpha$ (500 to 1000 pg/ml) whereas SDS at 30 µg/ml induced almost 800 pg/ml of cell-associated IL-1$\alpha$. Cell associated IL-1$\alpha$ for lysine derivatives was found to be significantly different from 100 µg/ml to 150 µg/ml ($P<0.05$). These results demonstrate that SDS is the surfactant with the greatest capacity for increasing IL-1$\alpha$ production ($P<0.05$).

All lysine-based surfactants induced lower levels of IL-1$\alpha$ synthesis and release to the culture medium when compared with SDS, suggesting they are less irritative than the reference surfactant. As clearly shown in both figures 2 and 3 all surfactants induced a

dose-related increase in both released and cell-associated IL-1α, indicating a neosynthesis of IL-1α. At the highest concentration it was observed a decrease in cell-associated IL-1α and an increase in IL-1α release, due to the loss of cell membrane integrity. $EC_{50}$ (the concentration that induces a 50 % increase in cell-associated IL-1α) was calculated for comparative purposes from concentration-IL-1α synthesis curves (Table 1). The $EC_{50}$ values showed that lysine derivative surfactants were less potent than SDS in stimulating the synthesis of IL-1α ($P<0.05$). In addition, 77KT was the surfactant with the lowest capacity for increasing this proinflammatory cytokine, as demonstrated by its higher $EC_{50}$ values. SDS showed the lowest $EC_{50}$, which was 2,5 to 4,5 folds below that of our surfactants.

The MTT dye-metabolism assay was useful to rank human skin irritancy levels of diluted surfactants, to include anionic, cationic, and nonionic classes. In addition to indicating the value of the approach for *in vitro* screening of surfactants, it also will provide a useful model for understanding differences in mechanisms of surfactant irritancy (Perkins et al., 1999). IL-1α production from the cell cultures appeared to be a more sensitive endpoint for distinguishing and rank-ordering products that range in irritancy.

We observed an inversed correlation between MTT values and the maximum amount of IL-1α released (a low $IC_{50}$ value implies a high release of IL-1α) suggesting that *in vitro* IL-1α release and cell viability are effective parameters for predicting skin irritation in a manner similar to other skin equivalents (Corsini and Galli, 1997; Coquette et al., 2003). When comparing both *in vitro* methods, we found high rank correlation ($r= 0.9196$) between $EC_{50}$ and MTT values.

In conclusion, our results show that IL-1α synthesis and release is a valuable in vitro parameter for identifying and classifying irritants, for confirming the low skin irritation potential of the lysine based surfactants compared to SDS, for determinating their potential use in topical formulations and for confirming the capacity of IL-1α production as a predictor of skin irritation.

**Acknowledgements**

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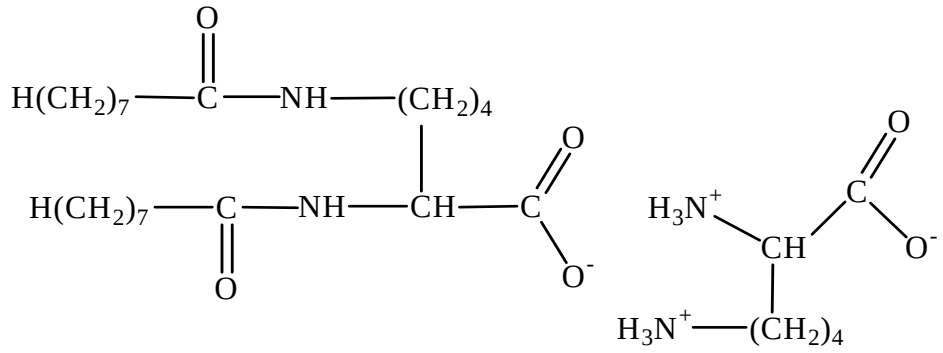
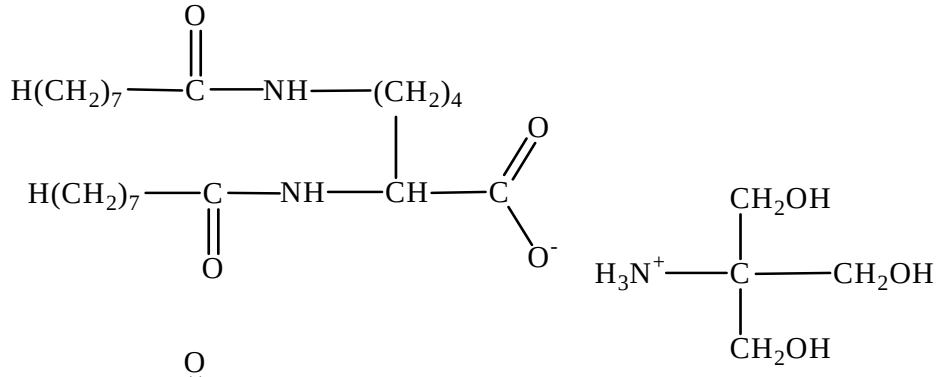
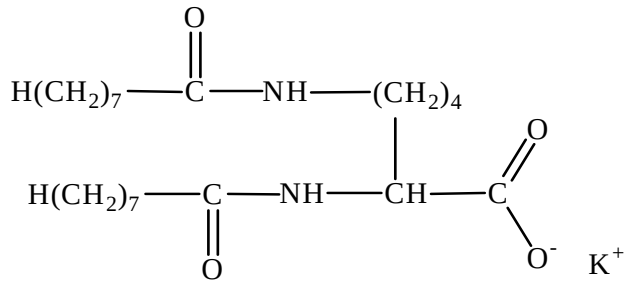
77KK



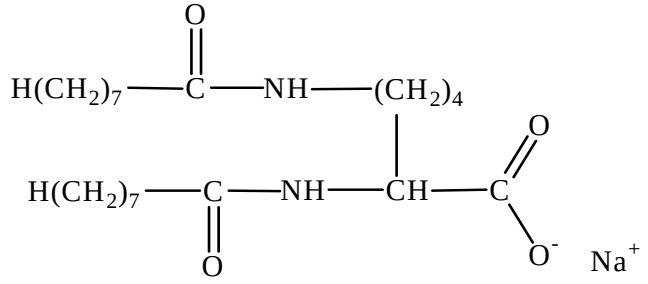
77KT



77KP



77KS



77KL

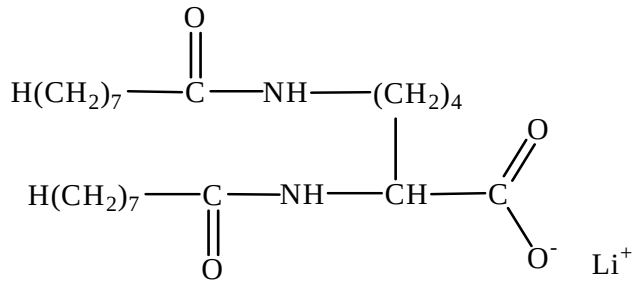


Fig. 1. Molecular structure of lysine derivative surfactants and codes. K, T, P, S, L represent lysine,tris, potassium, sodium and lithium, respectively.

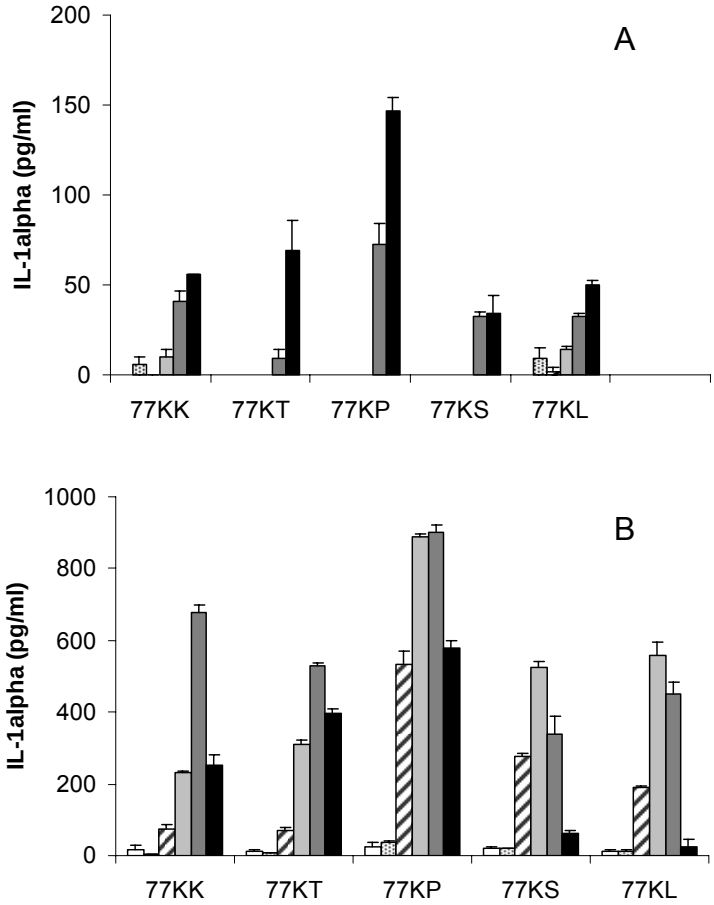



Fig. 2. Treatment with lysine based surfactants increased IL-1alpha release to the culture medium (A) and cell-associated IL-1alpha (B). The concentrations tested were 20 (white bar), 50, 100, 120, 150 and 180 µg/ml (black bar).

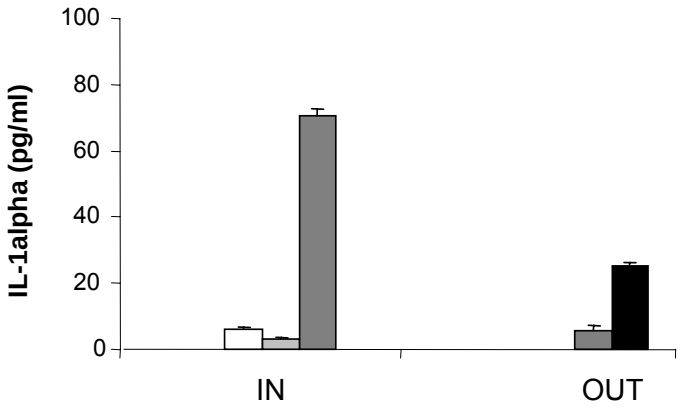



Fig. 3. Treatment with SDS increased cell-associated IL-1alpha and release to the culture medium. The concentrations tested were 15 (white bar), 25, 30 and 50 µg/ml (black bar).

Table 1

Cell-associated IL-1alpha increase ($EC_{50}$) and MTT assay ($IC_{50}$) results in keratinocyte cells following treatment with surfactants

| Surfactant (MW) | MTT assay, $IC_{50}$ µg/ml | IL-1$\alpha$, $EC_{50}$ pg/ml |
|---|---|---|
| 77KK (545.7) | 152.1 $\pm$ 3.3* | 77.25 ± 0.4* |
| 77KT (519.7) | 146.8 $\pm$ 3.1* | 80.44 ± 1.8* |
| 77KP (437.6) | 123.4 $\pm$ 5.3* | 44.01 ± 3.7* |
| 77KS (421.5) | 114 $\pm$ 11.6* | 62.68 ± 5.3* |
| 77KL (405.6) | 130.4 $\pm$ 2.6* | 73.58 ± 1.1* |
| SDS (288.4) | 30.2 $\pm$ 8.5 | 17.12 ± 0.2 |

Mean ± SE of three independent experiments.

* Significantly different from SDS ($P<0.05$), Student's *t-test*.

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# ARTÍCULO 5 (MANUSCRITO)

## Interacción de los tensioactivos con la bicapa lipídica de los eritrocitos

## Disturbance of erythrocyte lipid bilayer fluidity by amino acid--based surfactants

Artículo pendiente de publicación en *Toxicology Letters*

## Resumen

Los tensioactivos tienen la capacidad de permanecer en los medios de organismos vivos e interaccionar con las bicapas lipídicas de las membranas celulares. Esto es lo que ocurre cuando entran en contacto con la piel o los ojos y provocan irritación.

Con el objetivo de profundizar en el estudio del mecanismo de interacción de los tensioactivos con las membranas celulares, se han escogido dos familias de tensioactivos derivados de aminoácidos, los derivados de lisina (estudiados en esta Tesis) y otros derivados de arginina. Se ha estudiado la protección frente a la hemólisis hipotónica (antihemólisis) y las alteraciones de la fluidez de membrana mediante anisotropía de fluorescencia, utilizando como modelo el eritrocito debido a su fácil obtención, manipulación y la simplicidad de su estructura.

Todos los tensioactivos derivados de aminoácidos mostraron un comportamiento bifásico, si bien los derivados de arginina mostraron potencias antihemolíticas superiores a los derivados de lisina. Los experimentos de anisotropía de fluorescencia con las sondas DPH y TMA-DPH mostraron una tendencia de los tensioactivos a perturbar la región externa de la bicapa lipídica, sin afectar la interna.

# Disturbance of erythrocyte lipid bilayer fluidity by amino acid-based surfactants

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**Abstract**

Surfactants have the special ability to interact with the lipid bilayer of cell membranes. The red blood cell is one of the most popular cellular membrane models used to study the mechanisms underlying surfactant-induced osmotic cell resistance. To increase our knowledge regarding the mechanisms of surfactant membrane interaction, we studied the action of several anionic and cationic amino acid-based surfactants on hypotonic hemolysis as well as the alterations on membrane fluidity using fluorescence anisotropy. Results showed two different antihemolytic behaviors among amino acid-based surfactants, both related to the maximal protective concentration. Anisotropy measurements demonstrated that almost all of the surfactants studied disturbed the external region of the erythrocyte membrane without affecting the core of the bilayer. How the physico-chemical properties and structure of these compounds determine the protection against hypotonic hemolysis and affect dynamics of the lipid bilayer is discussed in detail.

*Keywords*: hypotonic hemolysis; plasma membrane; fluorescence anisotropy; volume expansion.

## Introduction

Surfactants, due to their surface and interface properties, are among the most versatile and frequently applied excipients in pharmaceutical, cosmetics, and technology-based industries. They are employed in large quantities every day on a worldwide scale as constituents of many different products [1].

Since it is well known that surface-active compounds can adversely affect the environment, the biodegradability and biocompatibility of surfactants have become almost as important as their functional performance to the consumer. One interesting strategy to minimize their environmental effects is to synthesize new molecules with analogues structures to such natural compounds as lipo-amino acids.

Amino acid-based surfactants have attracted much interest as environmentally friendly surfactants because of their biodegradability, low aquatic toxicity, low hemolytic activity and their use of renewable sources of raw materials for their synthesis [2-4]. Our group has considerable experience in the synthesis of surfactants derived from amino acids. Indeed, we have recently developed new families of lysine and arginine-based surfactants [5,6]. Both families have been widely studied in recent years in attempts to evaluate their potential risks for eye and skin irritation. Previous reports from our laboratory using *in vitro* methods revealed low toxicity when compared to conventional surfactants [7,8]. Among these methods the red blood cell lysis assay, which quantifies adverse effects of surfactants on the cytoplasmatic membrane, is a specific *in vitro* tool for evaluating the acute irritant potential induced by surfactants or surfactant-containing preparations [9]. However, the way surfactants interact with biological membranes is not clearly understood and different research groups have made great efforts to clarify the molecular processes involved in surfactant-induced cell membrane lysis [10-12], which is very closely related to surfactant toxicity. Because the human erythrocyte has no internal organelles and since it is the simplest cellular model obtainable, it is the most popular cell membrane system to study the surfactant-membrane interaction [13].

The influence of surfactants on osmotic cell resistance can be used as an index of plasma membrane interactions. Surfactants interact with erythrocyte membranes in a biphasic way by protecting against hypotonic hemolysis at low concentrations and inducing hemolysis at higher concentrations [11,12,14-18]. The mechanism underlying the antihemolytic effect is not fully understood. To increase our knowledge regarding possible mechanisms of surfactant interactions with erythrocyte membranes, we investigated the actions of several amino acid-based surfactants on hypotonic hemolysis and the alterations on membrane fluidity.

A better understanding of surfactant cell lysis may assist in developing surfactants with enhanced selectivity, and in widening their range of applications.

## Material and methods

### *Materials*

L-lysine monohydrochloride, L-Arginine monohydrochloride, L-Lysine, caprylic acid, Tris(hydroxymethyl)aminomethane, Sodium dodecyl sulfate (SDS), methanol, NaCl, $Na_2HPO_4$, $KH_2PO_4$, and the bases NaOH, LiOH and KOH were purchased from Merck (Darmstadt, Germany). Fatty acids mixture was kindly supplied by Calià and Parés (Barcelona, Spain). Fluorescent probes DPH (1,6-diphenyl-1,3,5-hexatriene) and TMA-DPH (1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate) were purchased from Molecular Probes (Eugene, OR). Hexadecyltrimethylammonium bromide (HTAB) was obtained from Sigma-Aldrich (St Louis, MO, USA).

### *Surfactants tested*

Two new classes of amino acid based surfactants were investigated in this study:

a) Three different cationic $N^{\alpha}$-acyl arginine derivatives were tested: $N^{\alpha}$–lauroyl-L-arginine methyl ester (LAM), $N^{\alpha}$–myristoyl-L-arginine methyl ester (MAM) and a mixture of different $N^{\alpha}$–acyl-L-arginine methyl derivatives (Figure 1). LAM and MAM were synthesized in our laboratory as previously described [19,20]. The surfactant mixture was synthesized for the first time in our laboratory using a fatty acids mixture from coconut extract (caprylic acid, 5.84 %, capric acid, 4.62 %, lauric acid, 53.04 %, myristic acid, 18.12 %, palmitic acid, 8.68 %, stearic acid, 9.47 %). Our procedure involved the introduction of fatty acid residues as acid chlorides [21].

b) Five anionic surfactants, with counterions of different chemical natures from the type Nα,Nε-dioctanoyl lysine were tested (Figure 2): lysine salt (77KK), tris(hydroxymethyl)aminomethane salt (77KT), sodium salt (77KS), lithium salt (77KL) and potassium salt (77KP). They were synthesized in our laboratory as previously described [7,12].

The molecular weight (MW), the critical micellar concentration (CMC), the charge, the number and length of alkyl chains of each compound are shown in Table 1.

### *CMC determination*

The CMC values were calculated as previously described for Nα,Nε-dioctanoyl lysine surfactants [22]. Critical micelle concentrations (CMCs) were determined by measuring 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.

Conductometry was measured with a Thermo Orion® connected to a water-flow thermostat maintained at 25 ºC. 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.

*Incubation media*

Lysine-based surfactants and SDS were dissolved in a PBS buffer. As the arginine-based surfactants in PBS solution are not soluble, different solvents were tested to find the best for dilution. Arginine-based surfactants and HTAB were dissolved in NaCl 0.9 % solution.

*Preparation of erythrocyte suspensions*

Human blood was obtained from the Blood Bank of the Hospital Clinic (Barcelona, Spain). The erythrocytes were washed three times in a phosphate buffer solution (PBS) containing 123.3 mM NaCl, 22.2 mM $Na_2HPO_4$ and 5.6 mM $KH_2PO_4$ in distilled water (pH 7.4; 300 mOsmol/L). The cells were then suspended at a cell density of $8 \times 10^9$ cell/mL.

*Protection against hypotonic hemolysis*

A volume of erythrocyte suspension (25 µL) was incubated with different concentrations of each surfactant dissolved in hypotonic solutions of PBS or NaCl. The final volume present in the vial was 1 mL. After incubation at room temperature, with constant shaking for 10 minutes and centrifugation at 3000 rpm for 10 minutes, the percentage of hemolysis was determined by comparing the absorbance at 540 nm of the samples with that of the control totally hemolysed with distilled water. The concentrations resulting in maximum protection ($cAH_{max}$) against hypotonic hemolysis were estimated from dose-response curves.

Surfactant antihemolysis was examined in buffer diluted to an osmolarity of about 80-90 % hemolysis of untreated samples. The antihemolytic potency of the surfactants, measured after 10 min incubation at room temperature, was expressed as the hemolysis reduction percentage compared with samples not treated with surfactants.

*Volume expansion calculation*

To determine the percentage of volume expansion induced by surfactants, a 25 µL volume of the erythrocyte suspension was incubated with an appropriate concentration of each compound dissolved in PBS or NaCl solutions of different osmolarities, ranging from isotonic (300 mOsmol/L) to hypotonic (110 mOsmol/L). Using the same procedure described above, the degree of hemolysis was determined. Dose-response curves were determined from hemolysis results and those osmolarities inducing 50 % hemolysis ($C_{50\%}$) were then calculated. To avoid differences in the hemolysis ratios resulting from the different blood donors, a hemolysis control curve was performed without adding surfactant. The osmolarity of the solutions was estimated by the freezing point method using a cryoscopic osmometer (Osmomat 030).

Theoretical calculations of the volume expansion induced by surfactants were carried out according to Ponder [23], who proposed that the association between the critical hemolytic volume ($V_h$) and the osmotic concentration inducing 50 % hemolysis ($C_{50\%}$) is described by the equation:

$$V_h = V_{na} + V_a \times (C_{iso} / C_{50\%}),$$

where $V_{na}$ is the osmotically nonactive volume representing 30 % of the normal erythrocyte volume ($V_o$ = 98 fL), $V_a$ is the osmotically active part of the erythrocyte volume representing 70 % of $V_o$, and $C_{iso}$ is the isosmotic concentration. Taken together:

$V_{na}$ = 29.4 fL

$V_a$ = 68.6 fL

$C_{iso}$ = 0.300 osmol/L

$C_{50\%}$ = value in osmol/L determined for the control or for the surfactant

The relationship between $V_h$ of the control and $V_h$ of treated cells was calculated and expressed as a percentage.

*Fluorescence emission anisotropy measurements*

To determine cell membrane fluidity, DPH and TMA-DPH fluorescent probes were selected. To carry out the steady-state fluorescence anisotropy measurements of the probes in treated and untreated red blood cells, the erythrocyte suspensions (hematocrit of 0.01 %) in PBS or NaCl were labeled with the fluorescent dyes (final concentration in samples $10^{-6}$ M) at room temperature for 1 hour. Steady-state anisotropy measurements were carried out with an AB-2 spectrofluorometer SLM-Aminco using polarizers in the L configuration in a quartz cuvette under constant stirring at room temperature. Samples were illuminated with the linearly (vertically or horizontally) polarized monochromatic light ($\lambda_{ex}$ = 365 nm) and the emitted fluorescence intensities ($\lambda_{em}$ = 425 nm) parallel or perpendicular to the direction of the excitation beam (slit-widths: 8 nm) were recorded. Fluorescence anisotropy (*r*) was calculated automatically by software provided with the instrument, according to:

$$r = ( I_{vv} – I_{vh}G ) / ( I_{vv} + 2I_{vh}G ),$$

where $I_{vv}$ and $I_{vh}$ represented the components of the light intensity emitted, respectively, in parallel and in perpendicular to the direction of the vertically polarized excitation light. A factor $G = I_{hv} / I_{hh}$ was used to correct the inequality of the detection beam to horizontally and vertically polarized emission [24].

*Statistical analysis*

Hypotonic hemolysis assays were carried out in triplicate. All anisotropy fluorescence values were expressed as the means ± standard error (SE) of at least 3 independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) and Student's *t*-test using the SPSS® software (SPSS Inc. Chicago, IL, USA).

## Results

*Protection against hypotonic hemolysis*

These experiments were carried out in a hypotonic buffer yielding approximately 80-90 % hemolysis of untreated erythrocytes after 10 min incubation at room temperature. The erythrocytes were added to the hypotonic buffer containing various concentrations of the surfactants and $cAH_{max}$ values were then determined (Figure 3).

The effect of lysine derivative surfactants on hypotonic hemolysis was compared to the anionic surfactant SDS, while arginine derivatives were compared to the cationic HTAB. Both tensides (SDS and HTAB) are widely used as reference compounds [25,26].

All compounds under investigation protected erythrocytes against hypotonic hemolysis. The maximum protective concentration for each compound ($cAH_{max}$) is listed in Table 2. $HC_{50}$ values (concentration of surfactant that induces 50 % hemolysis in isotonic medium) shown in Table 2 were determined as previously described [8,27]. In the case of lysine derivatives, $cAH_{max}$ values were close to their $HC_{50}$ values while reductions in hemolysis measured about 35 %, except for 77KL, which presented an antihemolytic potency of 76 %. For arginine derivatives, the $cAH_{max}$ values were approximately half their $HC_{50}$ values, while their antihemolytic potencies varied from 64 % to 79 %. The protection induced by SDS and HTAB was 64 % and 45 %, respectively, and their $cAH_{max}$ values were half their $HC_{50}$ values. In all cases $cAH_{max}$ values were below CMC values (Table 1).

The dose-response curves representing the degree of protection induced by surfactants are reported in Figure 3. Biphasic behavior was observed for all surfactants studied. The anionic surfactants 77KK, 77KT, 77KS and 77KP and the cationic HTAB showed similar antihemolytic potency. In the same way, the cationic surfactants LAM, MAM and CCR and the anionic SDS presented similar degrees of protection. Therefore, no significant differences in antihemolytic potency between the groups of differently charged compounds could be found.

It is interesting to note that two different profiles of how the surfactants protect against hypotonic hemolysis exist. The compounds 77KL, LAM and CCR had a wide range of protective concentrations, whereas the other surfactants rapidly increased the hemolysis rate beyond their $cAH_{max}$ values.

The results shown in Figure 4 indicate that the addition of surfactants caused a shift of the lysis curve to the left, revealing an increase in osmotic resistance. In the case of 77KL, the hemolysis rate did not reach 100 % at the lowest osmolarity tested compared with the other anionic surfactants, which is due to their high protective effects against hypotonic hemolysis (Figure 3A). Although some hemolysis occurred in mildly hypotonic mediums with MAM, it protected the erythrocytes against osmotic lysis in way similar to LAM and CCR, which is related to their similar maximum antihemolytic capacities (Figure 3B). Among the cationic surfactants, HTAB was the less effective protector against hypotonic hemolysis.

Dose-response curves (Figure 4) allowed the theoretical calculation of the erythrocyte volume expansion (Table 2). The highest volume expansion induced by arginine-based surfactants is related with their great antihemolytic potency. Interestingly, the anionic surfactant 77KL showed similar antihemolytic potency without perceptible changes in erythrocyte volume.

*Changes in erythrocyte membrane fluidity by surfactant treatment*

The fluorescent probes DPH and TMA-DPH were incorporated into the membrane of erythrocytes treated with surfactants in order to dissect their effects on lipid dynamics at different bilayer regions. DPH is a hydrophobic molecule incorporated in the region near the center of the bilayer [28]. TMA-DPH molecules, on the other hand, accumulate almost exclusively in the outer leaflet of cell membranes because of their polar head (trimethylammonium groups) [29]. Given that fluorescence measurements are very sensitive to medium turbidity, which can result in dispersion, the experiments were carried out in isosmotic mediums to avoid hemolysis. The surfactant concentrations chosen for assessing the membrane fluidity were selected according to their $cAH_{max}$ and $HC_{50}$ values. For all cationic surfactants, 77KL, and SDS, anisotropy measurements were performed at their $cAH_{max}$ values. However, the concentrations chosen for 77KK, 77KT, 77KP and 77KS were below their $cAH_{max}$ values because these were very close to their respective $HC_{50}$ values and would hemolyse the samples.

The effects exerted by the surfactants on membrane fluidity, as measured by the fluorescent probes, are shown in Table 3. Low anisotropy values (*r*) correspond to increased fluidity of cell membrane. None of the surfactants tested altered the core of the membrane as demonstrated by the DPH anisotropy values. However, the arginine derivatives (LAM, MAM and CCR), some lysine derivatives (77KK, 77KT and 77KP) and SDS modified the erythrocyte membrane fluidity on the external region of the membrane as demonstrated by the reductions in anisotropy TMA-DPH values.

## Discussion

The action of surfactants on plasma membranes is surprisingly complex. We have studied a group of surfactants with a wide range of physico-chemical properties in order to clarify the possible relationship between surfactants and their effect on cell membrane. The most accepted hypothesis is that surfactants are intercalated into the lipid bilayer of the membrane, such that the hydrophilic region is located at the hydrophilic/hydrophobic interface of the membrane and the hydrophobic region at the core of the bilayer [30,31]. Concentration-dependent surfactant effects on erythrocyte membranes are well known and widely described in the literature. High concentrations are hemolytic and membrane solubilization is often observed, while at low concentrations detergents are often protective in hypotonic solution [12,17,32]. A great number of compounds with amphiphilic character [33-35] display this biphasic (protective/lytic) behaviour upon interaction with the

erythrocyte membrane. However, the mechanism underlying the antihemolytic effect is not fully understood. Previous suggestions regarding this mechanism are based mainly on the idea that the intercalated compound increases either the membrane-area/cell-volume ratio or the stretching capacity of the bilayer, thereby allowing the cell to attain a critical hemolytic volume, which would aid in restraining the hypo-osmotic pressure [14,36]. This would permit the cell to swell to a greater volume before it lyses in the hypotonic medium [17]. However, this explanation has been contradicted by experiments that have shown how varying decreases in antihemolysis do not affect the critical hemolytic volume [37,38].

When we studied antihemolysis and the volume changes induced by amino acid-based surfactants, the antihemolytic potency appeared to correlate with the volume expansion. However, the anionic surfactant 77KL contradicted this trend due to its high antihemolytic potency (76 %) and low volume expansion (2.7 %). This report indicates that an increase in critical hemolytic volume solely based on the incorporation of amphiphiles into the bilayer is not a general mechanism that might explain surfactant-induced antihemolysis. Several authors [10,39] have proposed that the decrease in hemolysis observed under hypotonic conditions could be explained by a phospholipid rearrangement. In this way, an increase in permeability would result, and thus, a fast ion efflux, which would decrease the existing osmotic difference between the interior and exterior of the cell. It has also been suggested that band 3, an anion exchange protein in human erythrocytes, participates in hypotonic hemolysis [40]. Therefore, a possible interaction of surfactant-band 3 could be implicated in the antihemolysis activity of these compounds.

On the other hand, as the cationic arginine-based surfactants and the anionic surfactant 77KL exhibited a clear trend towards higher antihemolytic potencies, no significant differences in antihemolytic potency between the groups of differently charged compounds could be found. No explanation for the varying degrees of protection could be deduced from the molecular structure of the surfactants. It could not be attributed either to the length of the alkyl chain or to the nature of the molecular polar head.

One of the important parameters relating to the structure and functional state of the cell membrane is membrane fluidity [24]. To determine whether membrane fluidity was modified by surfactant treatment, the fluorescent probes DPH and TMA-DPH were incorporated into the membranes of erythrocytes. Knowledge of the probe's location is essential for a consistent interpretation of the observed fluorescence polarization. DPH is a hydrophobic molecule that is incorporated in the region near the center of the bilayer. Differences in the fluorescence polarization of this probe may reflect a direct effect on the motion of the lipid molecules in the core region of the bilayer [28]. The TMA-DPH molecules are believed to accumulate and remain almost exclusively in the outer leaflet of the cell membrane, since their polar head (trimethylammonium groups) are anchored at the lipid-water interface while hydrocarbon moieties enter the lipid part of the membrane. Therefore, fluidity assessed by steady-state fluorescence with different probes reveals the arrangement and mobility of membrane components at different regions of the bilayer [29].

From our findings, it is obvious that the fluidity of the erythrocyte membrane was modified by treatments with all of the arginine derivative surfactants, 77KK, 77KT, 77KP ($p$<0.01) and SDS ($p$<0.05). Our fluorometric experiments clearly showed that the perturbation caused in membranes by the amino-acid based surfactants incorporated therein was higher in the polar region of erythrocytes membranes and decreased with depth of incorporation, as demonstrated by TMA-DPH and DPH anisotropy values, respectively. A possible explanation for this fact is that the most common phospholipids in the bilayer are 16 to 18 carbons in length while the alkyl chain length of the tested surfactants was between 8 to 14 carbons. Therefore, these surfactants could not be incorporated more deeply into the membrane bilayer.

It is known that compounds containing counterions interact with biological and model membranes with different efficiencies [41]. This hypothesis is also supported by our results, specifically in the case of lysine derivative surfactants, which only differ in their counterions. The anisotropy data revealed that 77KK, 77KT and 77KP increased membrane fluidity whereas 77KS and 77KL had no effect. The counterion is also implicated in the differences in the antihemolytic potency and the hemolytic activities of this class of surfactants. However, no relationship between the antihemolytic potency and changes in membrane fluidity could be deduced from the chemical nature of the counterion.

Although the mechanisms of action of various surfactants was evaluated in light of their physicochemical properties, the fact that minor changes in surfactant properties may cause dramatic alterations of membrane fluidity or antihemolytic potency supports the idea that specific surfactant-lipid and surfactant-protein interactions should also be considered [42-44].

## Conclusions

We did not find a relationship between antihemolytic potency and changes in membrane fluidity in the series of surfactants studied. We believe that the antihemolytic effect is a complex process in which the structure of the amphiphile (including the counterion) and the specific composition of the membrane play a crucial role. Further investigations are needed to clarify the mechanisms involved in antihemolysis. These studies might focus on the permeability changes and the possible role of the anion exchanger Band 3 protein which would allow further growth in the understanding of the mechanism of surfactant-membrane interaction.

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Table 1

Physico-chemical properties of the surfactants studied.

| Surfactant | MW[a] | CMC[b] ($10^3$µg/mL) | Charge | Number of alkyl chains | Length of alkyl chain |
|---|---|---|---|---|---|
| 77KK | 545.7 | 1.8 | Anionic | 2 | C8 |
| 77KT | 519.7 | 2.3 | Anionic | 2 | C8 |
| 77KP | 437.6 | 1.9 | Anionic | 2 | C8 |
| 77KS | 421.5 | 3.0 | Anionic | 2 | C8 |
| 77KL | 405.6 | 2.9 | Anionic | 2 | C8 |
| LAM | 406.6 | 2.2 | Cationic | 1 | C12 |
| MAM | 434.7 | 0.7 | Cationic | 1 | C14 |
| CCR | 418.4 | 1.7 | Cationic | 1 | Variable (C6-C16) |
| SDS | 288.4 | 2.3 | Anionic | 1 | C12 |
| HTAB | 598.4 | 0.4 | Cationic | 1 | C16 |

[a]Molecular weight, [b]critical micellar concentration.

Table 2

Hemolysis, antihemolysis and theoretical calculation of the volume expansion induced by the surfactants.

| Surfactant | $HC_{50}$[a] (μg/mL) | $cAH_{max}$[b] (μg/mL) | Antihemolytic potency (%) | Volume expansion (%) |
|---|---|---|---|---|
| 77KK | 321.3 ± 22.4 | 390 | 41 | 1.6 |
| 77KT | 560.9 ± 16.0 | 750 | 35 | 4.6 |
| 77KP | 289.2 ± 2.4 | 300 | 34 | 3.3 |
| 77KS | 260.7 ± 11.2 | 270 | 38 | 3.6 |
| 77KL | 473.1 ± 9.7 | 430 | 76 | 2.7 |
| LAM | 58.8 ± 3.6 | 30 | 79 | 12.3 |
| MAM | 52.4 ± 3.4 | 10 | 64 | 9.1 |
| CCR | 63.9 ± 8.6 | 25 | 77 | 12.7 |
| SDS | 43.6 ± 1.5 | 20 | 64 | 6.7 |
| HTAB | 11.6 ± 0.1 | 5 | 45 | 5.5 |

[a]Concentration of surfactant inducing 50 % hemolysis (mean±SE) [8,27], [b]concentration resulting in maximum protection against hypotonic hemolysis

Table 3

Steady-state fluorescence anisotropy of fluorescence probes DPH and TMA-DPH incorporated into erythrocyte membranes.

| | Concentration (μg/mL) | (*r*) DPH (mean ± SE) | (*r*) TMA-DPH (mean ± SE) | (*r*) TMA-DPH reduction (%) |
|---|---|---|---|---|
| Samples in PBS | | | | |
| Untreated cells | - | 0.2393 ± 0.0076 | 0.2305 ± 0.0062 | - |
| 77KK | 300 | 0.2439 ± 0.0099 | 0.1778 ± 0.0084 ** | 23 |
| 77KT | 600 | 0.2190 ± 0.0017 | 0.1304 ± 0.0069 ** | 43 |
| 77KP | 270 | 0.2368 ± 0.0107 | 0.1717 ± 0.0094 ** | 26 |
| 77KS | 180 | 0.2485 ± 0.0139 | 0.2215 ± 0.0119 | 4 |
| 77KL | 430 | 0.2444 ± 0.0165 | 0.2266 ± 0.0113 | 2 |
| SDS | 20 | 0.2372 ± 0.0077 | 0.2060 ± 0.0068 * | 11 |
| Samples in NaCl | | | | |
| Untreated cells | - | 0.2152 ± 0.0096 | 0.2186 ± 0.0030 | - |
| LAM | 30 | 0.1988 ± 0.0060 | 0.1747 ± 0.0020 ** | 20 |
| MAM | 10 | 0.1938 ± 0.0073 | 0.1801 ± 0.0057 ** | 18 |
| CCR | 25 | 0.1972 ± 0.0040 | 0.1872 ± 0.0074 ** | 14 |
| HTAB | 5 | 0.2363 ± 0.0082 | 0.2173 ± 0.0061 | 3 |

Anisotropy measurements are represented by *r* values.

* Significantly different when compared to values obtained for untreated cells (Student's *t*-test, $p<0.05$).

** Significantly different when compared to values obtained for untreated cells (Student's *t*-test, $p<0.01$).

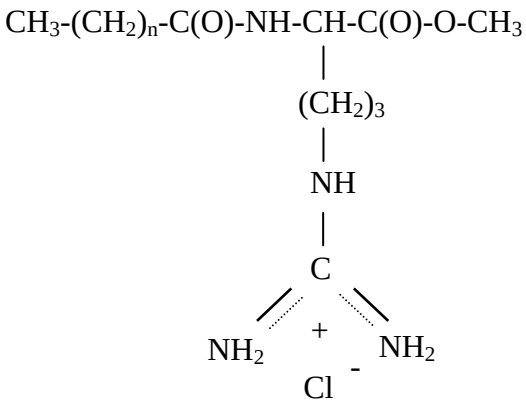


**Figure 1.** Molecular structure of arginine-derivative cationic surfactants. *n* represents the fatty acyl chain length [LAM: *n* = 10; MAM: *n* = 12; CCR: *n* = 6 (5.8 %), 10 (53.03 %), 12 (18.1 %), 14 (8.7 %), 16 (9.5 %)].

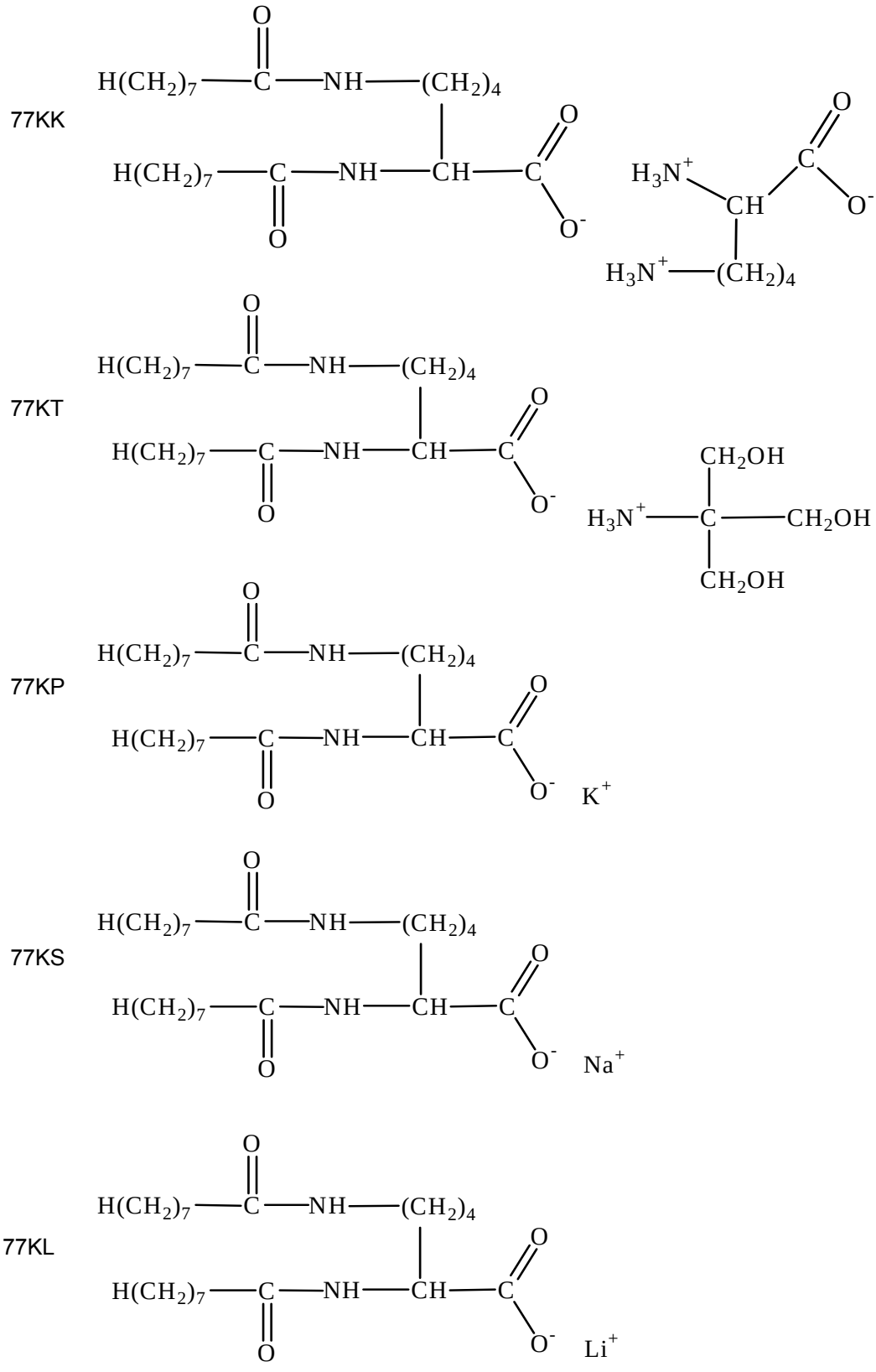


**Figure 2.** Molecular structure of lysine-derivative anionic surfactants. K, T, P, S, L represent lysine, tris, potassium, sodium and lithium respectively.

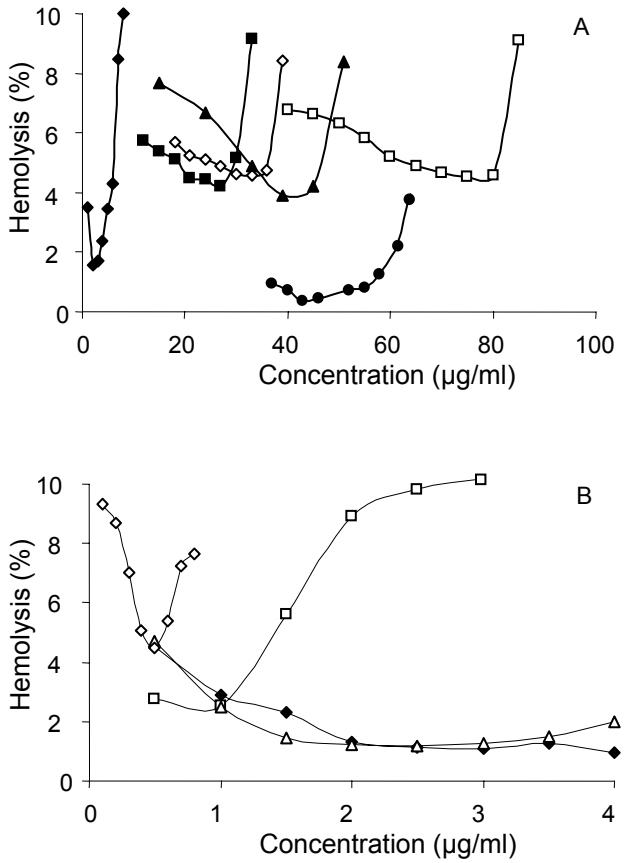


**Figure 3.** The antihemolytic and hemolytic activity of (A) 77KK (▲), 77KT (□), 77KS (■), 77KL (●), 77KP (◊) and SDS (♦); and (B) LAM (♦), MAM (□), CCR (Δ) and HTAB (◊). The determination of the antihemolytic potency was carried out in a medium of such an osmolarity of 80-90 % hemolysis of untreated erythrocytes. Each point represents the mean of at least three separate experiments.

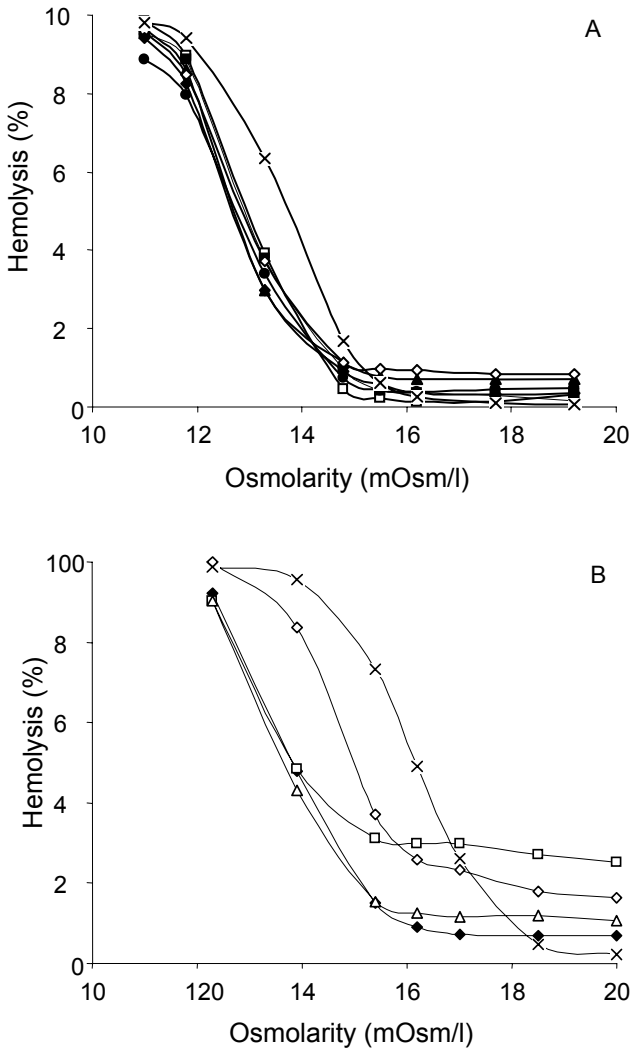


**Figure 4.** Osmotic lysis of human erythrocytes in serial dilutions of PBS (A) and NaCl (B) solutions at room temperature. Symbols in graph (A) represents: Control PBS (X), 77KK (▲), 77KT (□), 77KS (■), 77KL (●), 77KP (◊) and SDS (♦); and in graph (B): Control NaCl (X), LAM (♦), MAM (□), CCR (Δ) and HTAB (◊). Each point represents the mean of at least three separate experiments.

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