Low Potential Ocular Irritation of Arginine-Based Gemini Surfactants and Their Mixtures with Nonionic and Zwitterionic Surfactants

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Purpose. The aim of this study was to find new biocompatible surfactants and mixtures with low ocular irritant action for application in pharmaceutical formulations and to establish a relationship between their structure and their potential ocular irritant activity.

Methods. An alternative method to the Draize *in vivo* test, based on the adverse effects of surfactants on the cytoplasmic membrane of red blood cell, was used to evaluate the potential ocular irritation of the surfactants.

Results. It was found that the hemolytic activity of arginine-based gemini surfactants increased with the aliphatic alkyl chain lengths of the hydrophobic tail. The addition of the surfactant with an alkyl chain length of 10 carbon atoms to cocoamidopropilbetaina (TB), decylglucoside (APG), and N^{α}-lauroyl-arginine ethyl ester (LAE) increases the hemolytic activity moderately for the mixtures with TB and LAE (1.1- and 1.5-fold, respectively) and strongly for APG (five-fold).

Conclusions. The new arginine-based gemini surfactants constitute a suitable alternative to commercial surfactants because of their natural origins, which make them biocompatible and renewable products. Based on their hemolytic activity as an alternative to the Draize test, these new arginine-based gemini surfactants and their mixtures can be classified as mild irritants. This fact constitutes an advantage, especially for pharmaceutical and cosmetic applications.

KEY WORDS: hemolysis; arginine-based surfactants; red blood cell; lipoamino acids; ocular irritation.

INTRODUCTION

Surfactants, in accordance with their surface or interface activities, are among the most versatile and frequently applied excipients in pharmaceutical technology. Among the different applications of surfactants it has been shown that they can be used to give prolonged release from gels through the partition of drugs to micelles (1). However, the application of pharmaceutical or cosmetic products containing these compounds should avoid skin or eye irritation or other side reactions. Therefore, special attention must be paid to these facts from the very first moment when preformulation trials are started, and the development of new nonirritant surfactants is of considerable interest.

Amino acid–based surfactants have attracted much interest as environmentally friendly surfactants because of their biocompatibility, low toxicity, excellent emulsifying properties, and antimicrobial activity (2,3). An obvious strategy to increase the efficiency and the efficacy of amino acid–based single chain structures is to build up dimeric structures from lipoamino acids. Among the amino acid–based gemini surfactants, our interest is focused on arginine derivatives such as the bis(N^{α}-acyl-L-arginine)-α,ω-polymethylenediamide dihydrochloride [bis(Args)] (Fig. 1a).

An important characteristic of gemini surfactants is their great potential in mixtures with other surfactants. In most practical applications, mixtures of surfactants rather than individual surfactants are used. This is because mixing surfactants with different hydrophobic and hydrophilic groups enhances the performance of the final product. This occurs when synergism exists between the surfactants present in the mixture (4).

In this work we investigated the potential ocular irritation of arginine-based gemini cationic surfactants such as $C_3(OA)_2$, $C_3(CA)_2$, $C_3(LA)_2$, $C_3OH(LA)_2$ (Fig. 1a), and their mixtures with conventional widely used decylglucoside (APG), tego-betaine (TB), and N^{α}-lauroyl-arginine ethyl ester (LAE) surfactants. The potential ocular irritation of these arginine-based gemini surfactants and mixtures was assayed by the red blood cell assay (5). Eye irritation potency has traditionally being scored using the Draize rabbit eye test (6), proposed as a routine procedure to assess irritancy in safety evaluation. The red blood cell assay constitutes one of the alternatives to the Draize test to avoid the use of animals for ethical reasons (7,8). The leaking of erythrocytes is mainly caused by a low surfactant concentration, usually below the critical micellar concentration (CMC), whereas erythrocyte rupture is initiated by rather high concentrations of amphiphilic substances or micelles (9). For this reason, we also investigate if there is any relationship between the hemolytic activity of the surfactants studied and their CMC.

MATERIAL AND METHODS

Surfactants

Bis(N^{α}-acyl-L-arginine)-α,ω-polymethylenediamide dihydrochloride (bis(Args) was synthesized in our lab following the procedure described previously (10) (Fig. 1a).

Cocoamidopropilbetaine (Tego-betaine T-50; TB) was from Goldschmidt AG (Germany); decylglucoside (Plantcare 2000; APG) was from Henkel AG (Germany); and N^{α}-lauroyl-arginine ethyl ester (LAE) was a generous gift of Laboratorios Miret S.A. (Barcelona, Spain). Sodium dodecyl sulfate (SDS) was obtained from Sigma Aldrich (Germany)

Determination of Critical Micellar Concentration

Aqueous surfactant solutions of different concentrations were prepared and allowed to equilibrate at 25°C for 24 h. A Krüss KA12 tensiometer with a roughened platinum plate attached to a precision torsion balance was used for measuring equilibrium surface tensions. The critical micellar concen-

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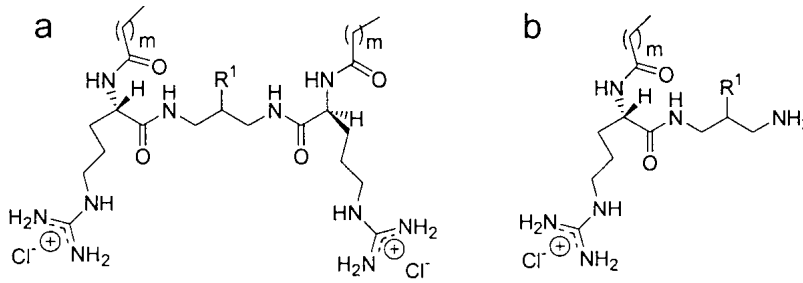

Fig. 1. Chemical structure and nomenclature of gemini surfactants (a) and their synthetic precursors (b).

tration (CMC) was determined from the break point of the surface tension/concentration curves (11).

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 isotonic phosphate buffer saline (PBS) containing 22.2 mM Na_2HPO_4, 5.6 mM KH_2PO_4, 123.3 mM NaCl, and glucose 10.0 mM in distilled water (pH 7.4). The cells were then suspended in isotonic saline solution (NaCl 0.9%) at a cell density of 8×10^9 cell/ml.

Incubation Media

Because of the lack of solubility of the arginine-based surfactants in PBS solution, different solvents have been tested to find the best one to dilute these surfactants. The hemolysis test of the SDS was performed in PBS, Tris-mannitol, and NaCl 0.9% solutions for comparative purposes.

Hemolysis Assay

Different volumes ranging from 10 to 80 μl of surfactant solution (from 1 mg/ml) were introduced in polystyrene tubes to assay various concentrations (from 5 to 1000 μg/ml) of these surfactants. Aliquots of 25 μl of erythrocyte suspension were added to the tubes and incubated for 10 min, with constant shaking, at room temperature. After incubation, the tubes were centrifuged (5 min at $1500 \times g$), and, finally, the percentage of hemolysis was determined by comparing the absorbance (540 nm) of the supernatant with that of control samples totally hemolyzed with distilled water (5). From the hemolysis results, the dose–response curve was determined, and the concentration that induces the hemolysis of 50% of the cells (HC_{50}) in the erythrocyte suspension was subsequently calculated.

Potential Ocular Irritation

The potential ocular irritation of the surfactants was studied with 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 irritation index was determined according to the lysis/denaturation ratio (L/D) obtained dividing the HC_{50} (μg/ml) by the denaturation index. The denaturation index (DI) of each surfactant was determined by comparing the hemoglobin denaturation induced by the surfactant and SDS as positive control. Hemoglobin denaturation was determined after inducing hemolysis by adding 10 mg/ml of the surfactant or SDS and measuring the absorption ratio of the supernatant at 575 nm and 540 nm. The resulting L/D ratio is used instead of the ocular irritancy score in the acute phase of *in vivo* evaluation. The surfactants can be classified according to this L/D ratio as nonirritant (>100), slight irritant (>10), moderate irritant (>1), irritant (>0.1), and very irritant (< 0.1) (5).

RESULTS AND DICUSSION

The red blood cell test is a rapid *in vitro* screening assay to assess the acute eye irritation potential that could be induced by surfactants or final products containing them and has been proposed as a good alternative to the Draize test (5).

It was found that when the arginine-based gemini surfactants were dissolved in aqueous PBS buffer solutions, a precipitate appeared in a few minutes. To avoid these solubility problems and, therefore, to obtain reliable results on the hemolytic activity, isotonic NaCl solution (0.9% w/v) and Tris-mannitol buffer (pH 7.4) were assayed as alternative incubation media. In both solutions the arginine-based gemini surfactants were soluble at the concentrations used for the assay. Both incubation media were validated with SDS, which was used as the positive control. Similar hemolytic activity was observed when the surfactant was dissolved in PBS and in NaCl solution (HC_{50} 42.5 and 44.3 μg/ml, respectively). By contrast, the hemolytic activity of SDS was lower when Tris-mannitol buffer was used (HC_{50} 122.2 μg/mL). On the basis of these results, the whole study was performed using NaCl solution as incubation medium. The reduction of the irritant effect of the surfactant can be explained by the formation of Tris dodecyl sulfate, which has less hemolytic activity, as has been demonstrated in previous works where the effect of Tris as counterion has been pointed out (12).

Results of hemolysis obtained at different concentrations for gemini surfactants and their synthetic precursors (Fig. 1b) are presented in dose–response curves (Fig. 2). When a hydroxyl group was added to the gemini $C_3(LA)_2$ and its precursor $C_3(LA)$, no effect on the hemolytic activity was induced. Thus, the addition of this group does not contribute to additional benefits or irritation effects of the surfactant. The

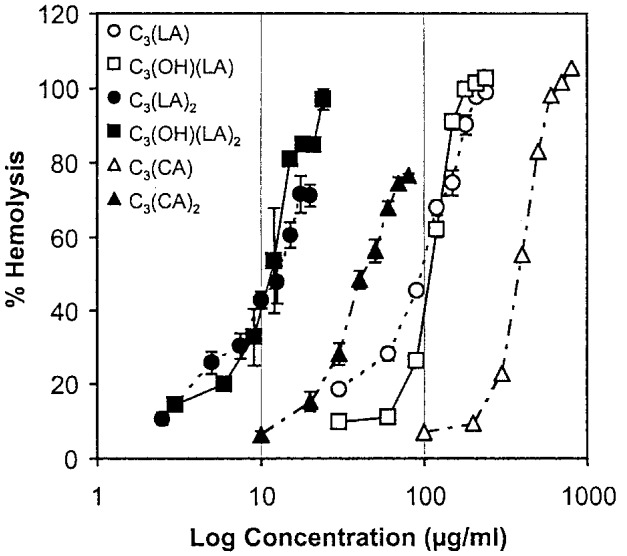

Fig. 2. Hemolysis induced by gemini surfactants and their synthetic precursors. Results are expressed as mean ± standard deviation of three experiments.

study of the hemolytic activity of the synthetic precursors of these arginine-based gemini surfactants is an important issue because the (bio)degradation products from these gemini surfactants may be structurally similar. Moreover, technical-grade preparations of these surfactants may contain variable amounts of starting materials or synthetic intermediates, which can have different toxicity profiles. It is interesting to note that the synthethic precursors of these arginine-based gemini surfactants were about 15-fold less hemolytic than the corresponding gemini surfactants. This could result, among other things, from their lower hydrophobicity because they have two polar groups and just one hydrophobic aliphatic alkyl chain. This fact is of great interest because the presence of this intermediate in the final product would not affect the potential ocular irritation of the end product.

When surfactants are added to the erythrocyte suspension in an aqueous medium, they could first distribute between the erythrocyte membrane and the solution by adsorption until equilibrium is reached. The surfactant–erythrocyte membrane interaction at sublytic concentration could be governed by the affinity of each surfactant for the aqueous medium or the membrane, a factor that is closely related to the hydrophobicity of surfactants and consequently to the critical micellar concentration (CMC) (13). Hemolysis probably begins when the erythrocyte membranes are saturated with the surfactant molecules. Table I shows the CMC and the 50% hemolytic concentration (HC_{50}) of the different surfactants studied. From these results, we found that there exists a good correlation between the CMC and HC_{50} ($r = 0.9998$) of the gemini surfactants. It is also interesting to note that the CMC decreases in the opposite direction to the number of carbon atoms present in the alkyl chain length (Fig. 1a). In agreement with these data, we may conclude that the longer the alkyl chain length, the higher is the hemolytic activity (i.e., the lower the HC_{50} values). In the same way, a similar correlation was found with lysine derivate surfactants (14), thus supporting this hypothesis. In contrast to these results, the commercial surfactants studied and the mixtures did not present this correlation in a similar way to other studies with nonionic alkylpolyglycosides (15), probably because of the difficulty of polymeric surfactants to form micelles.

Mixtures of different kinds of surfactants are particularly attractive because the performance of the final product is superior to those of the individual surfactants, thus increasing the range of applications. In the present work, we studied the hemolytic activity of the mixtures of the conventional surfactants such as decylglucoside (APG) and tego-betaine (TB) with the arginine-based gemini surfactant $C_3(LA)_2$. In addition, mixtures of $C_3(LA)_2$ with another arginine-based surfactant such as N-lauroyl-L-arginine ethyl ester were also investigated. We selected $C_3(LA)_2$ because preliminary studies in our laboratory indicated that it gave a better performance to its mixtures in terms of CMC reduction (data not shown).

Table I lists the CMC values for each surfactant mixture. The minimum α values [minimum molar fraction of $C_3(LA)_2$ that produces a significant decrease in the CMC value in the mixture] are 0.17, 0.30, and 0.20 for APG, TB, and LAE, respectively. The data revealed that a small molar fraction of $C_3(LA)_2$ in the mixture decreased its CMC about five- to

Table I. CMC, Hemolytic Effect, Denaturation Index, Lysis/Denaturation Ratio and Classification of Gemini, Commercial and Surfactant Mixtures

Surfactant	CMC (10^{-6} M)	HC^{a}_{50} (µg/ml)	DI[b]	L/D[c]	Classification
$C_3(OA)_2$	700	>1000	0.3	>3,000	Non irritant
$C_3(CA)_2$	30	48.1 ± 0.4	12.1	4.0	Moderate
$C_3(LA)_2$	4.7	12.5 ± 0.4	8.2	1.5	Moderate
$C_3(OH)(LA)_2$	7.0	12.1 ± 0.6	11.4	1.0	Moderate
LAE	100	38.4 ± 1.1	13.3	2.9	Moderate
LAE + $C_3(LA)_2$	20	34.4 ± 0.4	14.7	2.3	Moderate
TB	167	34.4 ± 2.2	14.4	2.4	Moderate
TB + $C_3(LA)_2$	32	22.4 ± 0.6	9.4	2.4	Moderate
APG	167	251.9 ± 5.8	14.2	17.8	Slight
APG + $C_3(LA)_2$	15	47.0 ± 0.8	12.3	3.8	Moderate

[a] Hemolytic concentration causing 50% hemolysis; results are expressed as mean ± standard deviation.
[b] Denaturation index
[c] Lysis/denaturation ratio

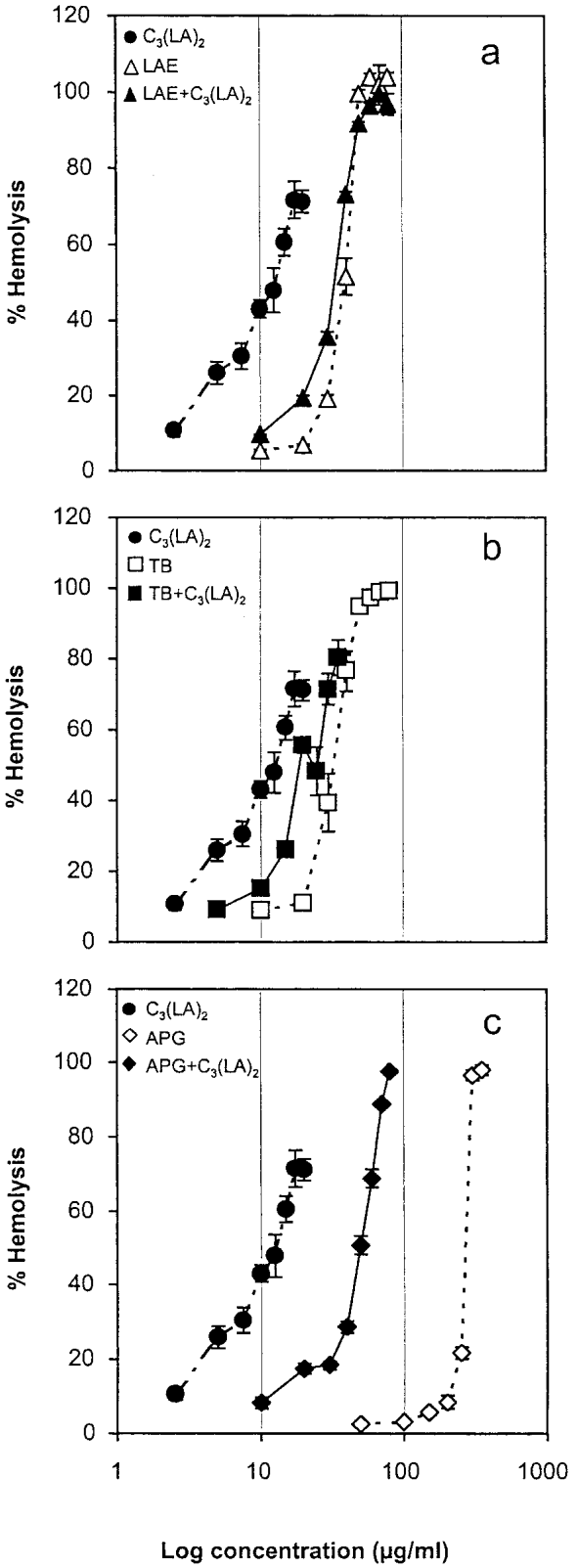

Fig. 3. Hemolysis induced by LAE (a), TB (b), APG (c), and their mixtures with $C_3(LA)_2$. Results are expressed as mean ± standard deviation of three experiments.

10-fold compared to the individual APG, LAE, and TB surfactants.

The addition of $C_3(LA)_2$ to conventional surfactants induced small changes in the hemolytic activity except for the decylglucoside (APG). In this case the hemolytic activity increased fivefold (i.e., the HC_{50} is smaller for the mixture than for the surfactant alone) (Fig. 3). As has been pointed before, when $C_3(LA)_2$ is mixed with APG, LAE, and TB, there was a reduction of the CMC of the mixture. This reduction in the CMC was parallel to the reduction observed in the HC_{50} of the mixture, which indicates that the formation of micelles may be responsible for the hemolytic action of this gemini surfactant alone or with other surfactants.

Table I also shows the denaturation index (DI), the lysis/denaturation ratio (L/D), and the potential ocular irritation of the different surfactants and mixtures studied. The gemini surfactants and their mixtures with conventional nonionic or zwitterionic surfactants tested in the present study are mild and even nonirritant in a way comparable to the commercial surfactants tested. Moreover, because of their physicochemical characteristics, the amount of these new compounds required in formulations will be lower than conventionally available surfactants, and thus, less ocular irritation is expected when these are applied in different cosmetic or pharmaceutical consumer goods. According to the results of the present work we can conclude that the new arginine-based gemini surfactants and their mixtures constitute a promising alternative to other commercial surfactants.

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