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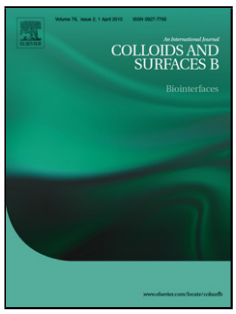

Title: VOLUME EXPANSION OF ERYTHROCYTES IS NOT THE ONLY MECHANISM RESPONSIBLE FOR THE PROTECTION BY ARGININE-BASED SURFACTANTS AGAINST HYPOTONIC HEMOLYSIS

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VOLUME EXPANSION OF ERYTHROCYTES IS NOT THE ONLY MECHANISM RESPONSIBLE FOR THE PROTECTION BY ARGININE-BASED SURFACTANTS AGAINST HYPOTONIC HEMOLYSIS

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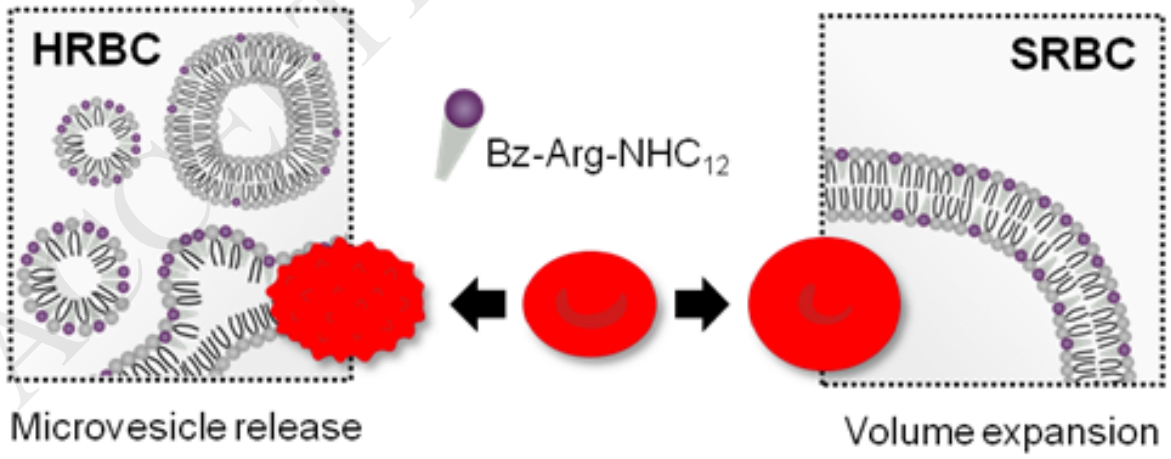
Graphical Abstract



Highlights

- Arginine-based surfactant Bz-Arg-NHC_{12} has both hemolytic and antihemolytic effects.
- Both processes depend on the lipid composition of the erythrocyte membrane.
- SRBC is more resistant than HRBC to the hemolytic effect of the surfactant.

- The degree of protection showed for SRBC was about 50% lower than for HRBC.
- HRBC could respond to osmotic stress by releasing membrane lipids to the medium.

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ABSTRACT

A novel arginine-based cationic surfactant N^{α}-benzoyl-arginine dodecylamide (Bz-Arg-NHC_{12}) was synthesized in our laboratory. In this paper we study the interaction of Bz-Arg-NHC_{12} with sheep and human red blood cells (SRBC and HRBC respectively) due to their different membrane physicochemical/biophysical properties. SRBC demonstrated to be slightly more resistant than HRBC to the hemolytic effect of the surfactant, being the micellar structure responsible for the hemolytic effect in both cases. Moreover, besides the hemolytic effect, a dual behavior was observed for the surfactant studied: Bz-Arg-NHC_{12} was also able to protect red blood cells against hypotonic lysis for HRBC in a wide range of surfactant concentrations. However, the degree of protection showed for SRBC was about 50% lower than for HBRC. In this regard, a remarkable volume expansion was evidenced only for SRBC treated with Bz-Arg-NHC_{12}, although no correlation with the antihemolytic potency (pAH) was found. On the contrary, our surfactant showed a greater pAH when human erythrocytes were submitted to hypotonic stress, with a low volume expansion, showing a higher amount of solubilized phospholipids in the supernatant when compared with SRBC behavior. Surface plasmon resonance measurements show the molecular interaction of the surfactant with lipid bilayers from HRBC and SRBC lipids, demonstrating that in the latter neither microvesicle release or lipid extraction occurred. Our results demonstrate that the volume expansion of erythrocytes is not the only mechanism responsible for the protection by surfactants against hypotonic hemolysis: volume expansion could be compensated via microvesicle release or by the extraction of membrane components upon collisions between red blood cells and surfactant aggregates depending on the membrane composition.

Keywords: Amino acid-based surfactants; hemolysis; antihemolytic potency; membrane physicochemical properties; microvesicles.

1. **INTRODUCTION**

Surfactants are ingredients included in many products used in pharmaceutical formulations and cosmetics, particularly in topical and transdermal administration of different active compounds. *Skin delivery* offers many advantages over conventional oral and invasive techniques of drug delivery such as its simplicity and convenience. However, its efficiency is restricted by the barrier function of the stratum corneum (SC), which structure is analogous to a wall: corneocytes are embedded in multiple lipid bilayers of ceramides, fatty acids, cholesterol and cholesterol esters [1]. These bilayers form regions of semicrystalline, gel and liquid crystals domains, establishing intercellular micro-routes through which many molecules can easily penetrate. Significant attention has been paid to the development of more effective methods in order to improve skin drug delivery kinetics and broaden the range of drugs that can be administrated through transdermal permeation [2]. Among them, *penetration enhancers* can increase skin permeability via different mechanisms, including enhanced drug solubility, increased partitioning into the SC, and reversible alteration of the lipid region of the SC, either through lipid extraction, fluidization of its crystalline structure or introduction of other modes of structural reorganization [3]. Surfactants (anionic, cationic and nonionic) represent one of the most commonly used penetration enhancers applied in the pharmaceutical industry [4].

In the particular case of surfactants, they can increase the solubility and mobility of hydrophobic or organic compounds through the stratum corneum [2]. However, irritation and damage to the skin barrier is mainly associated with ionic surfactants. Those surfactants resembling biological amphiphiles have demonstrated the ability to influence permeation and increase drug transport through the skin, and therefore are promising candidates to be incorporated as penetration enhancers in skin delivery formulations [5]. Among this kind of tensioactives, bio-based surfactants are synthetic compounds based on natural amphipathic structures such as glycolipids, lipopeptides, phospholipids and fatty acids. Lipo-amino acids have been

exhaustively studied, demonstrating good surface activity and aggregation properties, broad biological activity, and low toxicity profile [6]. In particular, arginine based surfactants are a remarkable group of cationic surfactants with antimicrobial activity against a broad spectrum of bacteria and fungi, enhanced biodegradability and low toxicity. Consequently, arginine-based surfactants have been proposed as an alternative to quaternary ammonium halides, which have a questionable intrinsic toxicity as well as a questioned biodegradability [7,8].

A novel arginine-based cationic surfactant was synthesized in our laboratory using papain (an endopeptidase from *Carica papaya* latex) adsorbed onto polyamide as biocatalyst. The classical substrate N^{α}-benzoyl-arginine ethyl ester hydrochloride (BAEE) was the arginine donor, whereas dodecylamine was used as nucleophile for the condensation reaction. The final product, N^{α}-benzoyl-arginine dodecylamide (Bz-Arg-NHC_{12}, Fig. 1), has shown antimicrobial activity against both Gram-positive and Gram- negative bacteria, revealing its potential use as preservative and effective disinfectant, as it was able to reduce 99% the initial bacterial population after only one hour of contact [9]. Bz-Arg-NHC_{12} demonstrated lower hemolytic activity and was less eye-irritating than the commercial cationic surfactant cetrimide. A similar trend was also observed when the *in vitro* cytotoxicity was tested against hepatocytes (HepG2) and fibroblasts (1BR.3.G) cell cultures, proving that Bz-Arg-NHC_{12} was less toxic than Cetrimide [9]. Recently, the antifungal activity of this compound was also described against phytopathogenic strains [10].

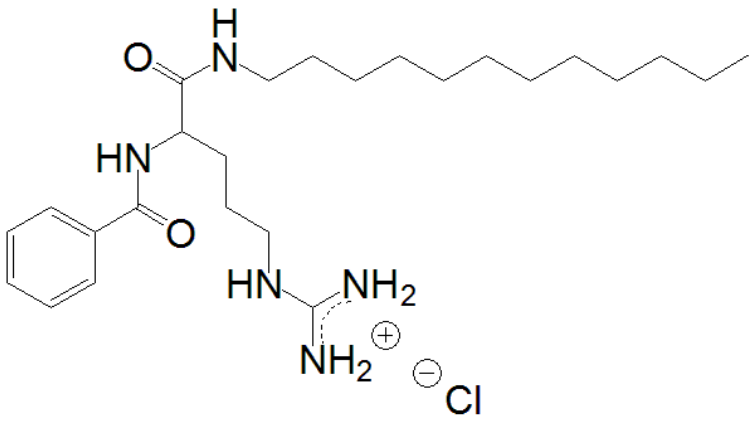

Fig 1. N^{α}-benzoyl-arginine dodecylamide (Bz-Arg-NHC_{12}) chemical formula.

Erythrocyte membranes are commonly used as a simplified model system to study the interaction of surfactants with biological membranes. In this paper we studied the lytic/protective effect of Bz-Arg-NHC_{12} on sheep and human red blood cells (SRBC and HRBC respectively) due to their differences in membrane lipid composition. It is important to consider that ruminants are mammals with a unique and striking phospholipid organization in red blood cell membranes, lacking phosphatidylcholine (PC, the most abundant phospholipid in other mammals' red blood cell membranes) and having high levels of sphingomyelin (SM) and cholesterol (CHO). All these characteristics causes the membrane to have a high degree of lipid order [11,12], which is more similar to the one described on the lipid matrix of the skin in comparison to the lipid order of HRBC membranes.

In this context, the aim of this work is to gain a deeper insight into the hemolytic and hypotonic hemolysis protection mechanisms of Bz-Arg-NHC_{12} as an estimation of surfactant-membrane interaction and toxicity assessment. To this end, we evaluate the dependence of both processes on the lipid composition of the erythrocyte membrane. Moreover, the hemolytic and antihemolytic mechanisms involved would give valuable information concerning to a general toxicological mechanism, providing a hot spot to focus considering the potential use of amino acid-based surfactants in biomedical applications.

2. MATERIALS AND METHODS

2.1. Ethics Statement

Blood from healthy sheep was obtained following the protocol approved by the Research Ethics Committee of the *Facultad de Ciencias Veterinarias* of the *Universidad Nacional de La Plata* (CICUAL 129/09) in accordance with the international guidelines for the care and use of laboratory animals. Human blood was obtained by venipuncture of healthy volunteers from our laboratory staff (CIPROVE, La Plata, Argentina) who gave the appropriate informed consent.

All this procedure was approved by the *Comité de Bioética y Ética de la Investigación de la Facultad de Ciencias Médicas de la Universidad Nacional de La Plata* (COBIMED) according to the requirements of the Declaration of Helsinki and the Argentinean legislation concerning Public Health (laws 25326 and 26529). EDTA was used to prevent blood clotting in both cases.

2.2. Surfactant synthesis

Bz-Arg-NHC_{12} was synthesized in our laboratory using papain, an endopeptidase from *Carica papaya* latex, adsorbed onto polyamide as biocatalyst according to Fait *et al.*(2015) [9].

2.3. Preparation of red blood cells suspensions

Red blood cells (RBC) were separated from sheep and human blood by centrifugation at 1300×g for 15 min at room temperature and washed three times with phosphate buffer solution (PBS) containing 123.3 mM NaCl, 22.2 mM Na_2HPO_4 and 5.6 mM KH_2PO_4 in MilliQ® nanopure water (pH 7.4; 300 mOsm/L).

2.4. Hemolysis assays

Aliquots of each RBC suspension (25 µL) were exposed to different concentrations of the surfactant dissolved in PBS (1 mL) at a final hematocrit (*Ht*) of 2%. Samples were incubated at 37°C for 10 min and then centrifuged at 1300×g for 10 min. Finally, the extent of hemolysis was quantified spectrophotometrically measuring the hemoglobin release to the supernatant at 540 nm, and contrasted to the supernatant of RBC treated with MilliQ® nanopure water (100% hemolysis) [13]. A sigmoidal (Boltzmann) fit was applied to dose-response curves using the OriginPro 8® software and the concentration inducing 50% hemolysis (HC_{50}) was calculated for each compound.

2.5. Protection against hypotonic hemolysis evaluation

Surfactant's protection against hypotonic hemolysis was examined in hypotonic PBS, diluted to an osmolarity so that untreated samples (absence of the surfactant) showed 80–90% of hemolysis (127 mOsm/L for HRBC and 150 mOsm/L for SRBC) [14]. RBC suspensions (25 µL) were incubated with different concentrations of the surfactant, dissolved in hypotonic PBS (1 mL) for 10 min at 37°C with constant shaking. Intact cells were separated by centrifugation at 1300×g for 10 min, and the extent of hemolysis in the supernatant was determined as previously described. Each experiment was performed at least three times using three replicate samples for each surfactant concentration tested. Statistical analyses were conducted using one-way analysis of variance (ANOVA) to determine the differences between the data sets, followed by Tukey's post hoc tests for multiple comparisons using the GraphPad Prism® software. $P<0.05$ was considered to denote significance. The surfactant's concentrations resulting in the maximum protection against hypotonic hemolysis (cAH_{max}) were estimated from the dose-response curves and the antihemolytic potency (pAH) was expressed as the hemolysis reduction percentage compared to untreated samples. Additionally, morphological changes in both HRBC and SRBC treated with the surfactant at the corresponding cAH_{max} were studied using an optical microscope (Nikon Eclipse TS100). Images were acquired with a Nikon 391CU 3.2M CCD digital camera and analyzed by means of the Micrometrics SE Premium® software.

2.6. Erythrocyte volume expansion calculation

Volume expansion determination of HRBC and SRBC treated with the surfactant was carried out according to Nogueira *et al.* (2012) [15].Osmolarities inducing 50% of hemolysis (C_{50}) were calculated from the *hemolysis vs. medium osmolarity* plots obtained for HRBC and SRBC incubated in the presence of Bz-Arg-NHC_{12} at the corresponding cAH_{max} (supplementary material). Theoretical calculations of the volume expansion induced by the surfactant were carried out according to Ponder (1948) [16], who proposed that the relation between the critical

hemolytic volume (V_h) and the osmotic concentration inducing 50% hemolysis (C_{50}) is described by Eq. (1):

$$V_h = V_{na} + V_a \times \frac{C_{iso}}{C_{50}} \qquad (1)$$

Where V_{na} is the osmotically non-active volume, representing 30% of the normal RBC volume (V_0); V_a is the osmotically active part of the RBC volume, representing 70% of V_0; and C_{iso} is the isotonic concentration (300 mOsm/L). Considering V_0 = 98 and 34 fL, V_{na} = 29.4 and 10.2 fL and V_a = 68.6 and 23.8 fL for HRBC and SRBC, respectively. V_0 was measured by introducing a blood sample into a Mindray BC-3000 Plus Auto Hematology Analyzer (Shenzhen, China). Percentage of volume expansion was calculated comparing the V_h of control and treated cells.

2.7. Membrane solubilization analysis

The extent of solubilized phospholipids (PL_s) was analyzed in the supernatant of HRBC and SRBC incubated with Bz-Arg-NHC_{12}. Aliquots of the erythrocyte suspensions previously prepared (25 µL) were exposed to surfactant at different concentrations, both below and above the corresponding CMC, dissolved in isotonic PBS (1 mL). After 10 min incubation at 37°C, supernatant was separated from intact cells by centrifugation (1300×g, 10 min) and hemoglobin release was spectrophotometrically determined as previously described. PL_s were extracted from the supernatant using a mixture of chloroform:methanol (2:1). Finally, organic phase was separated and evaporated in a SpeedVac and the amount of PL_s were quantified spectrophotometrically by a phosphate assay [17].

2.8. Surface plasmon resonance (SPR) measurements

SPR measurements were performed in MP-SPR Navi™ 200 (BioNavis, Finland). The instrument comprises a dual-channel detection system with simultaneous measurement in two flow cells with an angular-scan range of 40–78 degrees. The device is equipped with two

independent lasers (670 and 785 nm) in both channels. Typical flows employed were 10-500 µL/min and all solutions were prepared with MilliQ® nanopure water.

2.8.1. Preparation of the sensor surfaces

Commercial gold substrates (SPR102-AU) were purchased from Bionavis™. Prior to use, the gold substrates were washed with NH_3:H_2O_2:H_2O (1:1:2) at 90°C during 10 min, rinsed with water and ethanol and dried with N_2. DL-dithiothreitol (DTT) covered gold substrates were prepared by incubation of the bare substrates on 50 µM DTT ethanolic solution for 30 min at room temperature. The DTT-gold substrates were immediately rinsed with ethanol and dried with N_2.

2.8.2. Liposome binding

Small unilamellar vesicles (SUVs) prepared from lipids of HRBC and SRBC were employed to prepare supported bilayers. Briefly, RBC ghosts were obtained by hypotonic lysis (buffer Tris 5 mM, pH 7.4) for 30 min at 4°C from RBC stock suspensions (1 mL). After centrifugation at 10000×g for 15 min at 4°C, the supernatant was removed and the ghosts were washed several times with lysis buffer till the supernatant remained clear, free of hemoglobin. Total lipids were then extracted from the RBC ghosts by the procedure of Folch *et al*. (1957) [18]. The organic phase was separated and dried by evaporating the solvent under a stream of nitrogen and then placing the samples under high vacuum for 2 h. Multilamellar vesicles (MLVs) were prepared by hydrating the RBC lipids in 20 mM Tris, 150 mM NaCl, pH 7.4 (TBS buffer), prepared with high-purity water (Millipore SuperQ). Finally, SUVs were obtained by sonication of the MLVs for 30 min and used for the *in situ* generation of lipid bilayers on the surface of the SPR sensor chip. DTT-gold substrates were placed in the SPR device and the SPR curves (from 52 to 78 degrees) were registered each 3.5 s. The running buffer was TBS with Ca^{2+} 3 mM at a flow of 10 µL/min. After 10 min of baseline stabilization, the liposome suspension 0.2 mg/mL was injected at 10 µL/min during 20 min in both flow cells. After 10 min of stabilization, a quick

pulse of running buffer at 500 µL/min was performed in order to remove weakly adsorbed material. The amount of bound lipid was calculated from the change in signal at the angle of maximum derivative ($\Delta\Theta_{bilayer}$) from the initial baseline and the final baseline. $\Delta\Theta_{bilayer}$ were employed to normalize the results in different experiments, with typical values around $\Delta\Theta$~0.5.

2.8.3. Surfactant affinity measurements

After supported bilayer preparation, surfactant solutions of 125, 250 and 500 µM in TBS buffer with DMSO 3% were injected at 10 µL/min during 25 min through the main channel while buffer (TBS + DMSO 3%) was injected in the control channel. Measurements were made by duplicate in freshly made supported bilayers.

3. RESULTS AND DISCUSSION

3.1. Effect of Bz-Arg-NHC_{12} on red blood cells

In order to clarify the relationship between the membrane's biophysical properties and the hemolytic and antihemolytic mechanisms induced by Bz-ArgNHC_{12}, we analyzed the susceptibility of RBC isolated from two different species (sheep and human) to this arginine-based surfactant. Despite their relative simplicity (*i.e.* absence of nucleus and organelles), RBC have enough functions in common with more specialized cells, and the given results may be good indicators of the biological activity of the surfactants at physiological conditions [15]. Within this context, SRBC and HRBC were selected as model systems based on their differences in the molar proportions of the major lipid classes in the RBC's membranes. Briefly, although the cholesterol (CHO) content was roughly similar among both RBC membranes, the main difference was found in the relative amounts of PC and SM: SRBC are known to have a higher proportion of SM in their membranes (~42%) than HRBC (12%), while the highest amount of PC is found in HRBC (17%) since SRBC membranes lack of this lipid in their composition. In addition, the molar ratio of PE, PS and PI together was only slightly different between the lipid content of the RBC membranes of both species [11,12].

Characterization and quantification of the biological membrane interactions of the surfactants was conducted by hemolysis studies. It is widely accepted that the measurement of hemoglobin release from RBC is a reliable experimental approach to studying plasma membrane permeabilization by various chemical compounds. Furthermore, the evaluation of the hemolytic activity of surfactants is a process of fundamental and practical importance, and can be used as a means for their toxicological assessment which is especially significant for their medical applications [19].

In this regard, Bz-Arg-NHC_{12} showed hemolytic activity against both HRBC and SRBC in isotonic medium. The extent of hemolysis obtained at different surfactant concentrations was

presented in a dose-response plot (Fig. 2), from which HC_{50} values were obtained (Table 1). The HC_{50}/CMC ratio was also calculated, since it gives a hint about the surfactant's form responsible for the hemolytic effect. Considering the CMC value calculated for Bz-Arg-NHC_{12} was 0.085 mM [20], HC_{50}/CMC ratios for HRBC and SRBC were 16.6 and 18.7 respectively. This data suggests that SRBC are slightly more resistant than HRBC to hemolysis induced by the surfactant, and that its micellar form would be the entity responsible for the hemolytic effect in both cases. However, differences in the shape of the dose-response curves were evidenced (Fig. 2), reflecting an influence of the membrane biophysical properties on the mechanism involved.

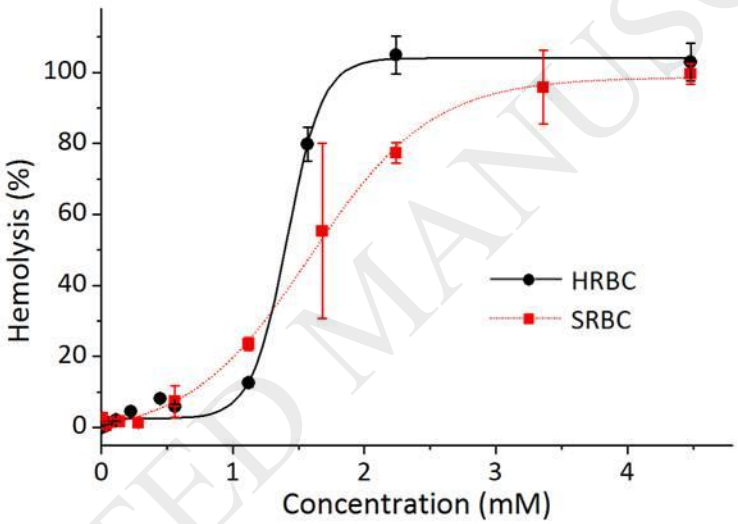

Fig. 2. Hemolysis induced by Bz-Arg-NHC_{12} in human and sheep red blood cells (HRBC and SRBC respectively). Values are plotted as the mean ± SD of three independent experiments.

3.2. Effect of Bz-Arg-NHC_{12} on the resistance of RBC towards hypotonically induced lysis

Concentration-dependent surfactant effects on erythrocyte membranes are well known. Amphiphiles induce shape alterations, vesiculation and hemolysis in RBC in isotonic media. In addition, at low surfactant/lipid molar ratios and in hypotonic media, some surfactants have the capability to protect erythrocytes against osmotic lysis [21,22]. Within this context, the

influence of surfactants on the cellular osmotic resistance can be used as an index of plasma membrane interactions.

In order to assess the ability of Bz-Arg-NHC_{12} to protect RBC against hypotonic hemolysis, experiments were conducted in hypotonic PBS buffer at a concentration at which approximately 80-90% of hemolysis was observed for untreated RBC after 10 min incubation at 37°C. Results revealed that in the presence of Bz-Arg-NHC_{12} at different concentrations, protection of HRBC and SRBC against hypotonic lysis was observed (Fig. 3). In each case, the maximum protective concentration for Bz-Arg-NHC_{12} (cAH_{max}) was determined from the dose-response curves as described in the materials and methods section. cAH_{max} values together with the antihemolytic potency (pAH) are listed in Table 1.

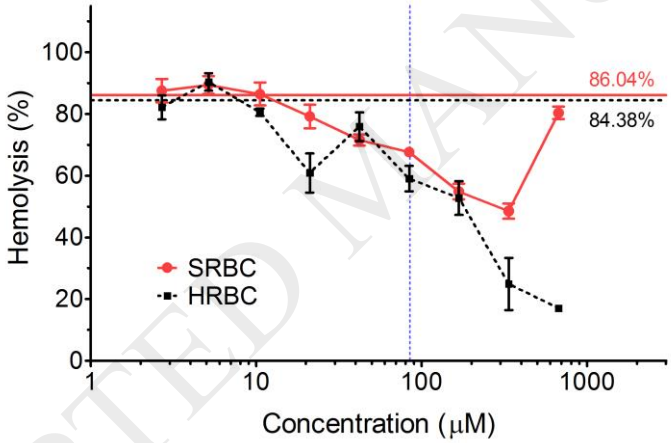

Fig. 3. Antihemolysis profiles showing hypotonically induced hemolysis dependence on Bz-Arg-NHC_{12} concentration (logarithmic scale) for HRBC and SRBC. Straight horizontal lines correspond to the percentage of hemolysis of untreated RBC in hypotonic medium (127 mOsm/L and 150 mOsm/L for HRBC and SRBC, respectively). Values are plotted as the mean ± SD of three independent experiments. The vertical dotted line indicates the surfactant concentration corresponding to CMC.

cAH_{max} values obtained corresponded to 47.5% and 21.0% of the corresponding HC_{50} for HBRC and SRBC respectively. In both cases, the interaction of the surfactant with the plasma membrane allows the cell to resist hypotonic media before solubilization/lysis begins. As the HC_{50}, cAH_{max} values for RBC of both species were higher than the CMC (Table 1). Thus, a dual behavior was observed for the surfactant studied, showing a wider range of protective concentrations when HRBC were tested. Furthermore, the degree of protection (described by the cAH_{max} and pAH values) of Bz-Arg-NHC_{12} for SRBC was about 50% lower than the one registered for HRBC, showing a rapid increase in the extent of hemolysis beyond the cAH_{max}.

The antihemolytic mechanism induced by surfactants in hypotonic media is still not fully understood but two potential mechanisms have been proposed [22]. On the one hand, the surfactant monomer insertion leads to a membrane enlargement by decreasing the lateral membrane tension, which allows RBC to swell, increasing their critical lytic volume before the leakage. On the other hand, another possible explanation would be that the increase in the membrane ionic permeability induced by surfactants leads to transmembrane flip–flop rate changes and morphological alterations. Consequently, the equilibrium of ion concentrations inside and outside the cell is modified, which reduces the hypotonic hemolysis.

In order to clarify the mechanism involved in the case of Bz-Arg-NHC_{12}, the theoretical calculation of the RBC volume expansion was performed using the relation proposed by Ponder (1948) as described by Eq. 1 in the methodology section [16]. Results are presented in Table 1. In this regard, the volume expansion of HBRC induced by Bz-Arg-NHC_{12} was around 4%. Surprisingly, for SRBC treated with the surfactant, a remarkable volume expansion was evidenced, although no correlation with the antihemolytic potency was found. Our results indicate that an increase in the critical hemolytic volume is not a general mechanism responsible for the Bz-Arg-NHC_{12} antihemolytic activity.

Nogueira *et al.*(2012) reported results about the effect of another class of amino acid-based surfactants on the resistance of erythrocytes against hypotonically induced lysis [15]. Their results support that the surfactant-membrane interactions in isotonic medium (hemolysis) were first conducted by the cationic charge density and position rather than by the hydrophobicity (given by the alkyl chain length) of the surfactant molecules. On the contrary, this was not the case for the antihemolytic effect, in which instance the hydrophobicity of the compounds had a greater influence. In their study, both hemolytic and antihemolytic activities were tested against human and rat RBC. It is worth noticing their findings corroborate previous studies, in which erythrocytes from different species —having specific phospholipid composition and mechanical properties of the cytoskeleton [23,24]— also had distinctive hemolytic behavior in the presence of surfactants [25,26]. However, no connection between the hemolytic and antihemolytic behavior of the surfactants studied and the physicochemical properties of membrane have been proposed. In regard to the protection against hypotonic lysis induced by the surfactants, Nogueira *et al*, found no direct correlation between the antihemolytic profile —given by the shape of the dose-response curves in hypotonic medium— and antihemolytic potency (pAH). Moreover, they found no significant correspondence between the antihemolytic potency and cell volume expansion for all surfactants tested. According to their conclusions, the increased membrane fluidity observed in hypotonic conditions, together with the cell volume expansion, might support the mechanism involving the enlargement of the stretching capacity of the membrane together with changes in its ionic permeability [15].

Moreover they reported a significant correlation between the antihemolytic potency and the CMC of the lysine based surfactants for rat RBCs, but not for HRBC. On the other hand the CMC correlated with cell volume expansion in human RBC, but not in rat RBC. However, the different behavior between rat and human erythrocytes is not exhaustively explained. Ultimately, they conclude that the ability of this group of lysine-based surfactants to protect against hypotonic hemolysis correlates, in general, directly with the CMC and inversely with

the alkyl chain length of the product, depending the surfactant-mediated hemolysis protection on the monomer form and not on the micellar structures. Our findings are contradictory to these results, since the antihemolytic activity of Bz-Arg-NHC_{12} was evidenced mainly at surfactant concentrations above the CMC (Fig. 3).

3.3. Solubilization of phospholipids by Bz-Arg-NHC_{12}

In order to correlate the lack of volume expansion results and the antihemolytic potency of Bz-Arg-NHC_{12} observed for HRBC with an alternative mechanism involving lipid extraction — such as the shedding of microvesicles or lipid extraction upon micellar collisions due to the presence of surfactant micelles—, solubilized phospholipids (PL_s) were quantified in the supernatant of RBC from both species incubated in the presence of the surfactant.

Fig. 4 shows percentage of hemolysis and solubilization of HRBC and SRBC membranes induced by Bz-Arg-NHC_{12} at concentrations both below and above the CMC. Results evidenced that the amount of PL_s in the supernatant of HRBC treated with our surfactant was significantly higher than that attained for SRBC, giving an insight on the reason why there is no volume expansion in this case despite the higher pAH observed.

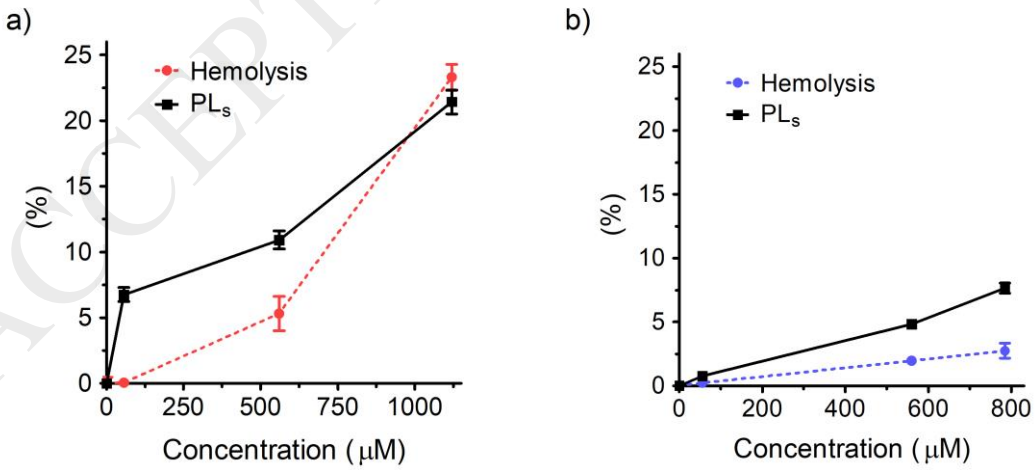

Fig. 4. Hemolysis and membrane solubilization —assayed as phospholipid solubilization— of human (Panel a) and sheep (Panel b) red blood cells treated with Bz-Arg-NHC_{12}. Values are plotted as the mean ± SD of three independent experiments.

This finding supports the idea that volume expansion would not be the main mechanism of protection against hypotonic hemolysis in the case of HRBC treated with Bz-Arg-NHC_{12}. Instead, HRBC could respond to osmotic stress by releasing membrane lipids to the medium, either by induced exovesiculation or by lipid extraction upon collisions with surfactant micelles present in the surrounding medium. This process could lead to some hemoglobin leakage without complete cell lysis.

As we previously demonstrated, HRBC irreversibly lose surface area and volume due to exovesiculation induced byBz-Arg-NHC_{12} [20]. When incorporated into the membrane, this kind of arginine-based compound—having a bulky head with a benzoyl group attached to it—are unable to equilibrate rapidly between the outer- and the inner-membrane monolayers. Consequently, and in order to compensate the expansion of the outer monolayer without compromising the bilayer structure, microvesicles are released and a shape transition from discocyte to echinocyte takes place (Fig. 5). Our hypothesis is that exovesiculation can be also observed in the case of protection against hypotonically induced hemolysis by Bz-Arg-NHC_{12}, explaining the ability of HRBC to resist hypotonic lysis despite the absence of cell volume expansion. Furthermore, no microvesicle release was observed when SRBC were incubated in the presence of Bz-Arg-NHC_{12} (data not shown). As a matter of fact, it is important to point out that when SRBC were treated with Ca^{2+} and ionophore A23187 no detectable microvesicles were found in the supernatant, indicating that SRBC's membrane lipid composition is a restraining factor for microvesicle release [27]. That is, different mechanisms can be involved in the protection against hypotonic hemolysis induced by a given surfactant, depending not only on the surfactant's hydrophobicity, and cationic charge density and position, but also on the membrane's composition.

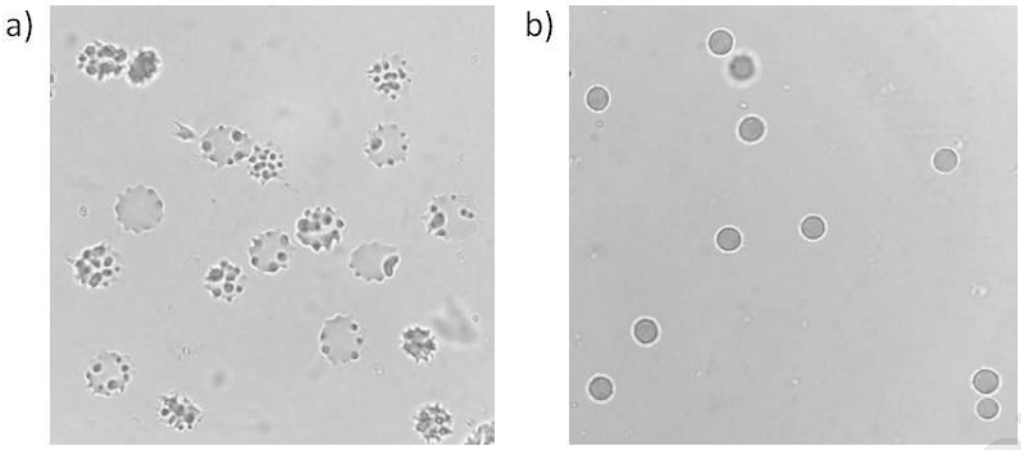

Fig 5. Representative optical-microscopy images (1000×) showing morphological changes in human (Panel a) and sheep (Panel b) erythrocytes induced by Bz-Arg-NHC_{12} at the corresponding cAH_{max} (672.1 and 336.1 μM, respectively).

3.4. Interaction between surfactant and membrane lipids measured by SPR

SPR constitutes a powerful biosensing technique for monitoring biomolecular interactions in real-time without labeling requirements. Biosensor experiments involve immobilizing one reactant on a surface and monitoring its interaction with a second component in solution. SPR biosensors measure the change in refractive index of the solvent near the surface that occurs during complex formation or dissociation [28].

Using supported lipid bilayers and SPR spectroscopy, we tested the binding and lipid mobilization capacity of Bz-Arg-NHC_{12} on HRBC and SRBC membranes. For that purpose, lipids were extracted from RBC and immobilized forming a bilayer onto the surface of a sensor chip. Following injection of 125 μM of Bz-Arg-NHC_{12} on chips covered with lipids extracted from HRBC (Fig. 6, Panel a) a drop of the signal below the baseline was observed, indicating the removal of membrane lipids from the chip. When the concentration of Bz-Arg-NHC_{12} was increased, a net increment in the SPR signal was evidenced, suggesting an increase in the binding of the surfactant molecules to HRBC lipids, which exceeded the decreasing response caused by the lipid extraction. As a result, SPR responses in these cases must include both the

binding of the surfactant to the surface of the lipid bilayer along with the extraction of the lipids. In contrast, a different profile was observed when Bz-Arg-NHC_{12} was injected on chips covered with lipids extracted from SRBC. In this case, after surfactant injection an increase of the SPR signal was observed, indicating the binding of Bz-Arg-NHC_{12} molecules to the immobilized lipids (Fig. 6, Panel b). An increment in the binding was detected as the concentration of the surfactant increased, evidenced as an increment in the SPR signal.

When 250 and 500 µM of surfactant were injected, the lower signals obtained in the sensorgrams for HRBC lipids compared to those of SRBC lipids suggest the combination of both processes —surfactant binding and removal of lipids from the bilayer— occurring simultaneously in HRBC membranes. Based on these results, we attribute the decrease of the response in the SPR sensorgrams to lipid extraction from the immobilized HRBC lipids. The reduction of the signal observed in the case of bilayers consisting of lipids extracted from HRBC supports our hypothesis about the alternative mechanism for HRBC resistance against hypotonically induced hemolysis in the presence of Bz-Arg-NHC_{12}. Neither microvesicle release or lipid extraction by action of the surfactant aggregates were evidenced when the surfactant interacted with the bilayers consisting of lipids extracted from SRBC. In this case only the association of surfactant molecules with the lipid bilayer seemed to occur.

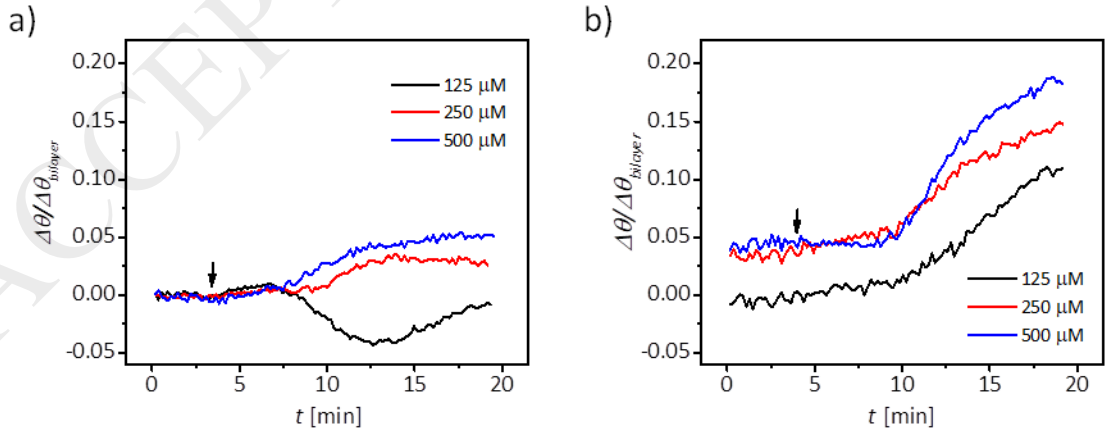

Fig. 6. SPR sensorgrams obtained with injections of surfactant solutions (125, 250 and 500 µM) on supported lipid bilayers made of lipid extracted from HRBC (Panel a) and SRBC (Panel b).

Time of the injection at 10 μL/min is pointed with and arrow. SPR signals are normalized to the amount of immobilized bilayer.

By means of a computer-executed multivariate analysis, a comparison has been made between the osmotic fragility of mammalian erythrocytes and twenty-three species-specific variables [24]. Statistically significant correlations were found between cell fragility and mean cell volume, and PC and SM membrane content: while mean cell volume has a direct effect on the osmotic response, PC and SM content determines the membrane behavior. Cell volume (the only dimensional variable) showed a strong negative correlation (-0.84) with fragility —*i.e.* fragility decreases as cell volume increases. This is not an unexpected phenomenon, as at a given hypotonic concentration, the larger the cell, the smaller the percentage increase in volume and the later the onset of hemolysis occurs. It is important to note that the mean volume of SRBC is lower than that of their human counterparts. Thus, more membrane surface is exposed in the first than in the latter. This emphasizes that the differences in susceptibility towards the surfactant studied in this paper are due to their composition rather than to differences in surface exposure.

In terms of the membrane lipid composition of the phospholipid fractions, PC appears to have a stabilizing influence on the cell membrane, whereas SM correlates significantly with cell fragility. These three parameters —cell volume and membrane content of SM and PC— are in favor of a greater osmotic fragility of the SRBC in comparison with HRBC.

SRBC can incorporate a much lower amount of surfactant molecules into the bilayer before the protective effect is lost and hemolysis takes place, as seen in Table 1. Previous suggestions regarding the effect of surfactants on the resistance of RBC towards hypotonically induced lysis are based mainly on the idea that the intercalated molecules increase 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 hypotonic pressure. This would permit the cell to swell to a greater volume before it lyses in the hypotonic medium.

However, no volume expansion was found when the assay was performed with HRBC, although these cells were able to resist a wide range of surfactant concentrations before lysis take place. The physicochemical properties of the SRBC membrane, determined by its lipid composition (enriched in cholesterol and SM), does not allow the release of microvesicles and consequently volume expansion takes place before lysis begins.

4. CONCLUSIONS

Human and sheep red blood cells are both susceptible to Bz-Arg-NHC_{12}. The surfactant studied in this paper has shown a biphasic behavior protecting RBC against hypotonic hemolysis at low concentrations but inducing hemolysis at higher ones. All results suggest that the mechanism by which Bz-Arg-NHC_{12} produces either hemolysis or protects RBC from osmotic lysis seems to depend crucially on the lipid composition of the membrane. Briefly, results proved that in RBC with membranes with high content of SM and absence of PC —*e.g.* SRBC—, the antihemolytic mechanism involves surfactant monomer insertion into the membrane, leading to its enlargement, which allows RBC to swell, increasing its critical lytic volume before lysis occurs. However, in RBC with membranes with relatively similar content of PC and SM —*e.g.* HRBC— the volume expansion (cell swelling) induced by monomer insertion is compensated via shedding of mixed microvesicles from the membrane or by the extraction of membrane components caused by collisions between the cells and the surfactant aggregates present in the surrounding medium. Fig. 7 presents a schematic representation of both phenomena. These two mechanisms are supported by SPR studies, which have corroborated the interaction of the surfactants with the lipid bilayer, and the lipid released from bilayer composed by lipid extracted from HRBC.

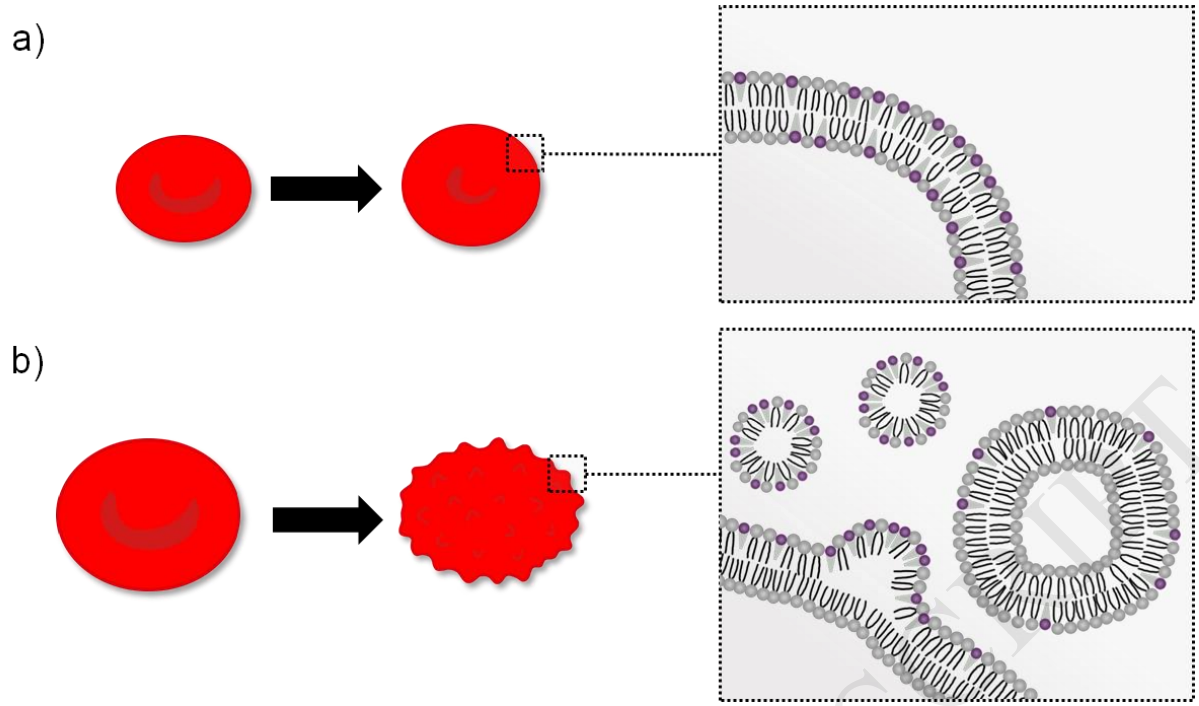

Fig. 7. Simplified scheme of the main antihemolytic mechanisms observed for Bz-Arg-NHC_{12} depending on the RBC membrane composition. While in SRBC (Panel a) —which membrane has high content of SM and absence of PC— the surfactant monomer insertion into the membrane, leads to its enlargement, allowing SRBC to increase its critical lytic volume, in HRBC (Panel b) —which membrane has relatively similar content of PC and SM— the volume expansion is compensated via microvesicle release or by the extraction of membrane components upon collisions between red blood cells and surfactant aggregates. N.b.: microvesicles and mixed micelles are represented in transverse cross section.

On the basis of our overall results, we conclude that cell volume expansion is not a general mechanism for surfactant protection against hypotonic hemolysis, since it depends not only on the chemical structure of the surfactant, but —for a given surfactant— on the composition and biophysical properties of the cell membrane.

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REFERENCES

[1] A.C. Williams, H.G.M. Edwards, B.W. Barry, The "Iceman": molecular structure of 5200-year-old skin characterised by Raman spectroscopy and electron microscopy., Biochim. Biophys. Acta. 1246 (1995) 98–105. doi:10.1016/0167-4838(94)00189-N.

[2] H. Zhang, Y. Zhai, X. Yang, G. Zhai, Breaking the Skin Barrier: Achievements and Future Directions, Curr. Pharm. Des. 21 (2015) 2713–2724. doi:10.2174/1381612821666150428124406.

[3] S. Kumar, M. Zakrewsky, M. Chen, S. Menegatti, J.A. Muraski, S. Mitragotri, Peptides as skin penetration enhancers: Mechanisms of action, J. Control. Release. 199 (2015) 168–178. doi:10.1016/j.jconrel.2014.12.006.

[4] M.E. Lane, Skin penetration enhancers, Int. J. Pharm. 447 (2013) 12–21. doi:10.1016/j.ijpharm.2013.02.040.

[5] R.S. Teixeira, T.F.G.G. Cova, S.M.C. Silva, R. Oliveira, M.J. Araújo, E.F. Marques, A.A.C.C. Pais, F.J.B. Veiga, Lysine-based surfactants as chemical permeation enhancers for dermal delivery of local anesthetics, Int. J. Pharm. 474 (2014) 212–222. doi:10.1016/j.ijpharm.2014.08.002.

[6] L. Pérez, M.T. Garcia, I. Ribosa, M.P. Vinardell, A. Manresa, M.R. Infante, Biological properties of arginine-based gemini cationic surfactants, Environ. Toxicol. Chem. 21 (2002) 1279–1285. doi:10.1002/etc.5620210624.

[7] A. Pinazo, M.A. Manresa, A.M. Marques, M. Bustelo, M.J. Espuny, L. Pérez, Amino acid–based surfactants: New antimicrobial agents, Adv. Colloid Interface Sci. 228 (2016) 17–39. doi:10.1016/j.cis.2015.11.007.

[8] J.A. Castillo, A. Pinazo, J. Carilla, M.R. Infante, M.A. Alsina, I. Haro, P. Clapés,

Interaction of Antimicrobial Arginine-Based Cationic Surfactants with Liposomes and Lipid Monolayers, Langmuir. 20 (2004) 3379–3387. doi:10.1021/la036452h.

[9] M.E. Fait, G.L. Garrote, P. Clapés, S. Tanco, J. Lorenzo, S.R. Morcelle, Biocatalytic synthesis, antimicrobial properties and toxicity studies of arginine derivative surfactants, Amino Acids. 47 (2015) 1465–1477. doi:10.1007/s00726-015-1979-0.

[10] M.E. Fait, H. Costa, L. Bakas, C.D.T. Freitas, S.R. Morcelle, Antifungal Activity of Arginine-Based Surfactants, Curr. Bioact. Compd. 14 (2018) 1–9. doi:10.2174/1573407214666180131161302.

[11] S. Maté, J. V. Busto, A.B. García-Arribas, J. Sot, R. Vazquez, V. Herlax, C. Wolf, L. Bakás, F.M. Goñi, N-nervonoylsphingomyelin (C24:1) prevents lateral heterogeneity in cholesterol-containing membranes., Biophys. J. 106 (2014) 2606–16. doi:10.1016/j.bpj.2014.04.054.

[12] K.S. Koumanov, C. Tessier, A.B. Momchilova, D. Rainteau, C. Wolf, P.J. Quinn, Comparative lipid analysis and structure of detergent-resistant membrane raft fractions isolated from human and ruminant erythrocytes, Arch. Biochem. Biophys. 434 (2005) 150–158. doi:10.1016/j.abb.2004.10.025.

[13] W.J.W. Pape, U. Pfannenbecker, U. Hoppe, Validation of the red blood cell test system as in vitro assay for the rapid screening of irritation potential of surfactants., Mol. Toxicol. (1987) 525–536. http://europepmc.org/abstract/MED/3509700 (accessed February 27, 2014).

[14] L. Sánchez, V. Martínez, M. Rosa Infante, M. Mitjans, M. Pilar Vinardell, Hemolysis and antihemolysis induced by amino acid-based surfactants, Toxicol. Lett. 169 (2007) 177–184. doi:10.1016/j.toxlet.2007.01.003.

[15] D.R. Nogueira, M. Mitjans, M.A. Busquets, L. Pérez, M.P. Vinardell, Phospholipid Bilayer-Perturbing Properties Underlying Lysis Induced by pH-Sensitive Cationic Lysine-Based Surfactants in Biomembranes, Langmuir. 28 (2012) 11687–11698. doi:10.1021/la300626y.

[16] E. Ponder, Hemolysis and related phenomena, in: Grunner and Strattor, New York, 1948.

[17] P.S. Chen, T.Y. Toribara, H. Warner, Microdetermination of Phosphorus, Anal. Chem. 28 (1956) 1756–1758. doi:10.1021/ac60119a033.

[18] J. Folch, M. Lees, G.H.S. Stanley, A simple method for the isolation and purification of total lipids from animal tissues, J Biol Chem. 226 (1957) 497–509. http://www.jbc.org/content/226/1/497.short.

[19] N. Joondan, S. Jhaumeer Laulloo, P. Caumul, D.E.P. Marie, P. Roy, E. Hosten, Synthesis, physicochemical properties and membrane interaction of novel quaternary ammonium surfactants derived from l-Tyrosine and l-DOPA in relation to their antimicrobial, hemolytic activities and in vitro cytotoxicity, Colloids Surfaces A Physicochem. Eng. Asp. 511 (2016) 120–134. doi:10.1016/j.colsurfa.2016.09.050.

[20] M.E. Fait, M. Hermet, F. Comelles, P. Clapés, H.A. Alvarez, E. Prieto, V. Herlax, S.R. Morcelle, L. Bakás, Microvesicle release and micellar attack as the alternative mechanisms involved in the red-blood-cell-membrane solubilization induced by arginine-based surfactants, RSC Adv. 7 (2017) 37549–37558. doi:10.1039/C7RA03640J.

[21] N. Fatma, M. Panda, W.H. Ansari, Kabir-ud-Din, Solubility enhancement of anthracene and pyrene in the mixtures of a cleavable cationic gemini surfactant with conventional surfactants of different polarities, Colloids Surfaces A Physicochem. Eng. Asp. 467 (2015) 9–17. doi:10.1016/j.colsurfa.2014.11.010.

[22] M. Manaargadoo-Catin, A. Ali-Cherif, J.-L. Pougnas, C. Perrin, Hemolysis by surfactants — A review, Adv. Colloid Interface Sci. 228 (2016) 1–16. doi:10.1016/j.cis.2015.10.011.

[23] M. Arias, J.C. Quijano, V. Haridas, J.U. Gutterman, V. V. Lemeshko, Red blood cell permeabilization by hypotonic treatments, saponin, and anticancer avicins., Biochim. Biophys. Acta. 1798 (2010) 1189–96. doi:10.1016/j.bbamem.2010.03.018.

[24] M.F. Coldman, M. Gent, W. Good, Relationships between osmotic fragility and other species-specific variables of mammalian erythrocytes, Comp. Biochem. Physiol. 34 (1970) 759–772. doi:10.1016/0010-406X(70)90997-7.

[25] M.A. Vives, M.R. Infante, M.P. Vinardell, Comparative Study of the Resistance of Different Erythrocytes to Haemolysis by the Surfactant Lysine Laurate*, Pharm. Sci. 3 (1997) 601–604. doi:10.1111/j.2042-7158.1997.tb00505.x.

[26] H. Hägerstrand, M.X. Danieluk, M.X. Bobrowska-Hägerstrand, A. Iglič, A. Wróbel, B. Isomaa, M. Nikinmaa, Influence of band 3 protein absence and skeletal structures on amphiphile- and Ca2+-induced shape alterations in erythrocytes: A study with lamprey (Lampetra fluviatilis), trout (Onchorhynchus mykiss) and human erythrocytes, Biochim. Biophys. Acta - Biomembr. 1466 (2000) 125–138. doi:10.1016/S0005-2736(00)00184-X.

[27] D. Allan, R.H. Michell, Calcium ion-dependent diacylglycerol accumulation in erythrocytes is associated with microvesiculation but not with efflux of potassium ions., Biochem. J. 166 (1977) 495–9. http://www.ncbi.nlm.nih.gov/pubmed/339908.

[28] R. Rich, Advances in surface plasmon resonance biosensor analysis, Curr. Opin. Biotechnol. 11 (2000) 54–61. doi:10.1016/S0958-1669(99)00054-3.

SUPPLEMENTARY MATERIAL

C_{50} determination

The assays consisted on the incubation of 25 µL of the erythrocyte suspensions (HRBC and SRBC) in the presence of Bz-Arg-NHC_{12} at the corresponding cAH_{max} dissolved in PBS of different osmolarities (1 mL), ranging from isotonic (300 mOsm/L) to hypotonic (85 mOsm/L) solutions, for 10 minutes at 37°C. Control experiments were carried out in the presence of PBS at the same osmolarities but in the absence of the surfactant. Hemolysis degrees were determined following the procedure previously described and dose-response curves were plotted. A sigmoidal (Boltzmann) fit was applied to each plot using the Origin Pro 8® software and osmolarities inducing 50% of hemolysis (C_{50}) were obtained (Fig. S1)[15].

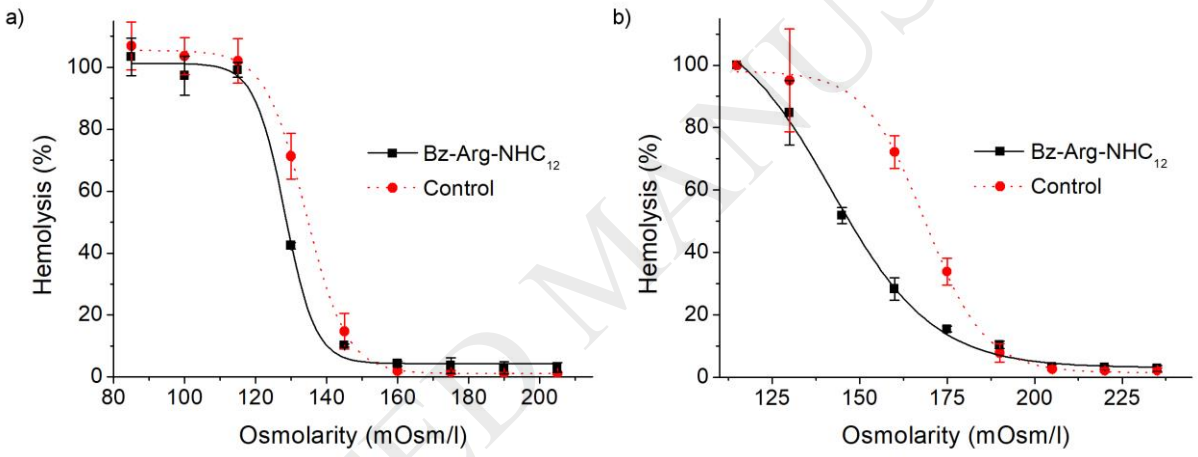

Fig. S1. Osmotic lysis of human (Panel a) and sheep (Panel b) red blood cells in serial dilutions of PBS (control) and PBS added with Bz-Arg-NHC_{12} at the cAH_{max} (672.1 µM and 336 µM respectively). Values are plotted as the mean ± SD of three independent experiments.

Table 1. Concentration inducing 50% hemolysis (HC_{50}), maximum protective concentration against hemolysis (cAH_{max}), antihemolytic potency (pAH), and theoretical calculation of the volume expansion (Vol Exp) induced by Bz-Arg-NHC_{12}

	HC_{50} (µM)	cAH_{max} (µM)	pAH (%)	Vol Exp (%)
HRBC	1412.8 ± 15.8	672.1	67.32	3.8 ± 0.6
SRBC	1585.3 ± 26.9	336.1	37.54	15.15 ± 0.7