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Phospholipid bilayer perturbing-properties underlying lysis induced by pH-sensitive cationic lysine-based surfactants in biomembranes

Daniele Rubert Nogueira[1], Montserrat Mitjans[1,4], M. Antonia Busquets[2], Lourdes Pérez[3], M. Pilar Vinardell[1,4*]

[1]*Departament de Fisiologia, Facultat de Farmàcia, Universitat de Barcelona, Av. Joan XXIII s/n, E-08028, Barcelona, Spain*

[2]*Departament de Fisicoquímica, Facultat de Farmàcia, Universitat de Barcelona, Av. Joan XXIII s/n, E-08028, Barcelona, Spain*

[3]*Departamento de Tecnología Química y de Tensioactivos, IQAC, CSIC, C/Jordi Girona 18-26, E-08034, Barcelona, Spain*

[4]*Unidad Asociada al CSIC*

Autor information

[*] Corresponding author. Tel.: +34 934024505; fax: +34 934035901; e-mail address: mpvinardellmh@ub.edu (M.Pilar Vinardell).

Running headline: “Membrane perturbing-properties of lysine-based surfactants”

ABSTRACT

Amino acid-based surfactants constitute an important class of natural surface-active biomolecules with unpredictable number of industrial applications. To gain better mechanistic understanding of surfactant-induced membrane destabilization, we assessed the phospholipid bilayer-perturbing properties of new cationic lysine-based surfactants. We used erythrocytes as biomembrane models to study the hemolytic activity of surfactants and their effects on cells' osmotic resistance and morphology, as well as on membrane fluidity and membrane protein profile with varying pH. The antihemolytic capacity of amphiphiles correlated negatively with the length of the alkyl chain. Anisotropy measurements showed that the pH-sensitive surfactants, with the positive charge on the α-amino group of lysine, significantly increased membrane fluidity at acidic conditions. SDS-PAGE analysis revealed that surfactants induced significant degradation of membrane proteins in hypo-osmotic medium and at pH 5.4. By scanning electron microscopy examinations, we corroborated the interaction of surfactants with lipid bilayer. We found that varying the surfactant chemical structure is a way to modulate the positioning of the molecule inside bilayer and, thus, the overall effect on the membrane. Our work showed that pH-sensitive lysine-based surfactants significantly disturb the lipid bilayer of biomembranes especially at acidic conditions, which suggests that these compounds are promissing as a new class of multifunctional bioactive excipients for active intracellular drug delivery.

Keywords: lysine-based surfactants; hypotonic hemolysis; fluorescence anisotropy; SDS-PAGE; drug delivery

1. INTRODUCTION

Surfactants are one of the most widely used excipients in the pharmaceutical industry[1]. Their surface and interface activities in systems involving interaction with membranes have been the subject of intense study.[2-6] Surfactants derived from amino acids constitute an important class of natural surface-active biomolecules that usually have biocompatible properties and multifunctional capabilities, which make them extremely relevant for pharmaceutical applications, especially in the field of novel non-viral drug delivery devices.[4,7,8] Our group has considerable experience in the synthesis of surfactants derived from amino acids. Indeed, we recently developed new families of lysine-based surfactants,[8,9] and some of them were deeply studied here in order to find out some important characteristics of surfactant-membrane interaction. Cationic lysine-based surfactants have the advantage of offering a wide range of possibilities for structure modulation.[8,9] This feature allows the design of surfactants with low toxicity, high biodegradability and pH-sensitive activity, which make them highly suitable for practical applications in comparison to the current commercial surfactant systems.

The field of surfactant-membrane interactions is becoming a rate-limiting step of biomedical and pharmacological progress.[10] As the way surfactants interact with biological membranes is not clearly understood, various research groups have made efforts to clarify the molecular processes involved in surfactant-induced cell membrane lysis.[2,3,11,12] The extent of interaction of a surfactant with a membrane is a complex phenomenon and the properties of both the membrane and the surfactant are involved. The physico-chemical properties of a biomembrane are rather constant and defined for a physiological condition. In contrast, the factors relating to the surfactant include its affinity for the membrane, its free monomer concentration in the aqueous environment adjacent to the membrane, and its structure, which allows it to accumulate at a given membrane surface area.[13]

Cationic compounds, such as lipids, peptides, polymers and surfactants, have great potential as carriers of drugs or DNA across cell membranes.[7,14,15] This underlines the great

importance of the discovery of new bioactive excipients for the development of efficient drug delivery devices: biocompatible amino acid-based surfactants could be a promising choice for application in this field.[4,7] Previous studies by our group showed that one novel class of cationic surfactants derived from the amino acid lysine (hydrochloride salts of N^{ε}-acyl lysine methyl ester) has pH-sensitive membrane-lytic activity, which is dependent on the fine-tuning of the cationic charge position, and thus is promising as a bioactive excipient in intracellular drug delivery systems.[16] This knowledge, together with a deeper study of the interaction properties of these compounds with the phospholipid bilayer, is key to the design of efficient intracellular drug carriers with specific endosome-destabilizing activity.

Therefore, to gain better mechanistic understanding of surfactant-induced biomembrane destabilization and to complete our previous study,[16] here we assessed the phospholipid bilayer-perturbing properties of three novel cationic lysine-based surfactants derived from the salts, N^{ε} and N^{α}-acyl lysine methyl ester. We chose rat and human erythrocytes and their membranes as *in vitro* models to study the hemolytic activity of surfactants and their effects on osmotic resistance and morphology of cells, as well as on membrane fluidity and the membrane protein profile with varying pH. Because erythrocytes have no internal organelles and since they are the simplest cell models obtainable, they are the most popular cell membrane systems for studying surfactant-membrane interaction.[17] Moreover, to gain insight into the specific interaction of these compounds with the membrane, we also examined their structure-activity relationship. Knowledge of the membrane-destabilizing properties of the bioactive excipients is of great importance in attempting to develop efficient drug carriers with specific physico-chemical and biological properties, in line with the pharmaceutical industry's needs.

2. MATERIALS AND METHODS

2.1. Reagents

All solvents were reagent grade and were used without further purification. NaCl, Na_2HPO_4 and KH_2PO_4 were supplied by Merck (Darmstadt, Germany). Hexadecyl trimethyl ammonium bromide (HTAB), sodium dodecyl sulfate (SDS), methanol and glycine were from Sigma-Aldrich (St. Louis, MO, USA). Fluorescent probes DPH (1,6-diphenyl-1,3,5-hexatriene) and TMA-DPH (1-(4-trimethylammonium phenyl)-6-phenyl-1,3,4-hexatriene p-toluenesulfonate) were purchased from Molecular Probes (Eugene, OR, USA). Acrylamide PAGE 40%, methylenebisacrylamide 2%, TEMED, mercaptoethanol, ammonium persulfate and bromophenol blue used for SDS-PAGE were obtained from GE Healthcare Amersham Biosciences (Uppsala, Sweden). Finally, Precision Plus Unstained Standard was purchased at Bio-Rad (Hercules, CA, USA).

2.2. Surfactants

Three novel biocompatible amino acid-based surfactants derived from N^{ε} or N^{α}-acyl lysine methyl ester salts with one lysine as the cationic polar head (one cationic charge) and one alkyl chain were evaluated: N^{ε}-myristoyl lysine methyl ester (MKM) with one alkyl chain of 14 carbon atoms and one positive charge on the α-amino group of the lysine, N^{ε}-palmitoyl lysine methyl ester (PKM) with one alkyl chain of 16 carbon atoms and one positive charge on the α-amino group of the lysine and N^{α}-myristoyl lysine methyl ester (MLM) with one alkyl chain of 14 carbon atoms and one positive charge on the ε-amino group of the lysine. MKM and PKM have a hydrophobic chain attached to the ε-amino group of the lysine, while MLM has the hydrophobic chain attached to the α-amino group of the lysine (Figure 1). The commercial cationic surfactant HTAB was used as the reference compound. These lysine-based surfactants were synthesized in our laboratory, as described elsewhere,[8,9] and made from natural fatty acid and amino acid organic building blocks. In all cases, all building blocks were linked by amide bonds to form biodegradable molecules.

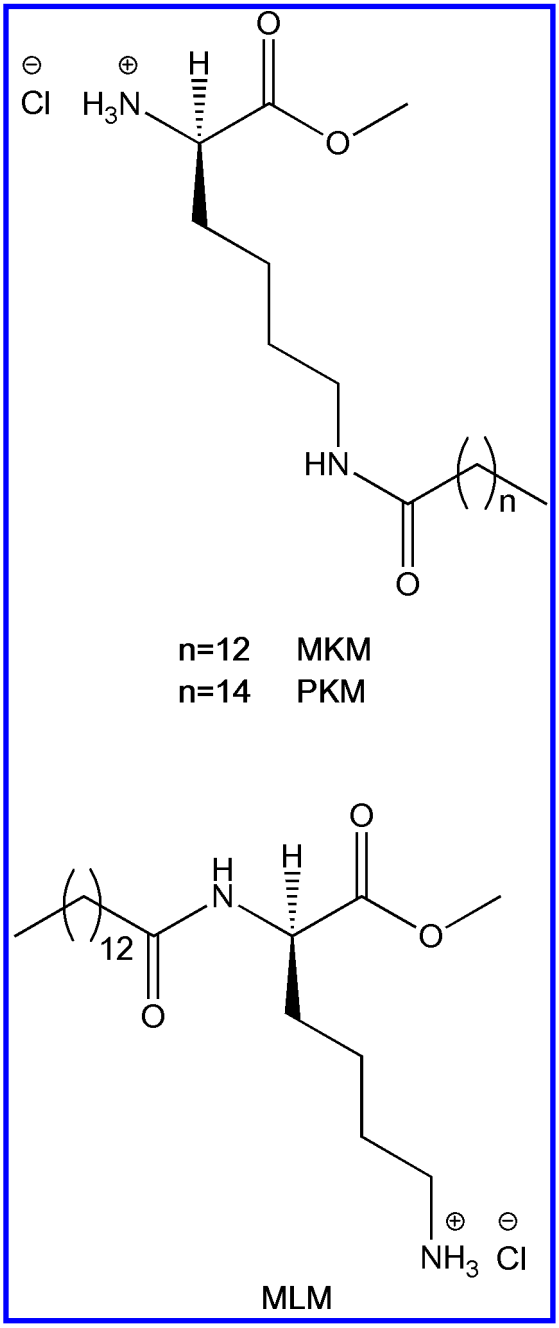


Figure 1. Molecular structures of the cationic lysine-based surfactants.

2.3. Preparation of erythrocyte suspensions

Rat blood was obtained from anesthetized animals by cardiac puncture and drawn into tubes containing EDTA. The procedure was approved by the institutional ethics committee on animal experiments. Human blood was obtained from adult healthy donors after informed consent, according to the approved institutional protocol for blood sample acquisition by venipuncture. Erythrocytes were isolated by centrifugation at 3,000 rpm at 4 ºC for 10 min and washed three

times in an isotonic phosphate buffered saline (PBS) 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 mOsmol/l). The cell pellets were then suspended in PBS solution at a cell density of 8 x 10^9 cells/ml.

2.4. Hemolysis assay

The membrane-lytic activity of the surfactants was examined by hemolysis assay. 25-µl aliquots of rat or human erythrocyte suspension were exposed to different surfactant concentrations based on the hemolytic potency of each compound (from 50 to 500 µg/ml for MKM and PKM, 10 to 60 µg/ml for MLM and 2.5 to 20 µg/ml for HTAB) and dissolved in PBS buffer in a total volume of 1 ml. The samples were incubated at room temperature for 10 minutes and then centrifuged at 10,000 rpm for 5 minutes. Two controls were prepared by resuspending erythrocyte suspension either in buffer alone (negative control) or in distilled water (positive control). Absorbance of the hemoglobin release in supernatants was measured at 540 nm by a Shimadzu UV-160A spectrophotometer (Shimadzu, Kyoto, Japan) and the hemolysis percentages were determined by comparison with the positive control samples completely hemolyzed with distilled water. Concentration-response curves were obtained from the hemolysis results and the concentration inducing 50% hemolysis (HC_{50}) was calculated.

2.5. Protection against hypotonic hemolysis

The antihemolysis activity of the surfactants was evaluated with both rat and human erythrocytes. A volume of erythrocyte suspension (25 µl) was incubated with different concentrations of each surfactant (from 25 to 125 µg/ml for MKM, 50 to 210 µg/ml for PKM and 10 to 50 µg/ml for MLM), dissolved in hypotonic solution of PBS in a total volume of 1 ml. The PBS buffer was diluted to the osmolarity provoking about 80-90% hemolysis of untreated samples. The osmotic pressure of the solutions was calculated by the freezing point method, using a cryoscopic osmometer (Osmomat 030). Antihemolysis was determined after incubating the cells

for 10 minutes at room temperature, following the procedure described above for the hemolysis assay. The concentrations resulting in maximum protection against hypotonic hemolysis (cAH_{max}) were estimated from concentration-response curves. The antihemolytic potency of the surfactants was expressed as the percentage of hemolysis reduction from the level in samples not treated with surfactants.

2.6. Volume expansion calculation

To determine the percentage of cell volume expansion induced by surfactants, a 25-µl aliquot of rat or human erythrocyte suspension was incubated with the cAH_{max} of each compound dissolved in PBS solutions of different osmolarities. The degree of hemolysis was determined by the same procedure described above. Concentration-response curves were constructed from hemolysis results and the osmolarities inducing 50% hemolysis ($C_{50\%}$) were then calculated. To avoid differences in the hemolysis ratios resulting from the different erythrocyte suspensions, a hemolysis control curve was performed without adding surfactant.

Theoretical calculations of the volume expansion induced by surfactants were carried out in line with Ponder,[18] 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:

$$V_h = V_{na} + V_a (C_{iso} / C_{50\%}) \quad (1)$$

where V_{na} is the osmotically non-active volume representing 30% of the normal erythrocyte volume (V_o = 98 fL), V_a the osmotically active part of the erythrocyte volume representing 70% of V_o, and C_{iso} is the iso-osmotic concentration. The results were: 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 the V_h of the control and V_h of treated cells was calculated and expressed as a percentage.[19]

2.7. Fluorescence anisotropy measurements

To determine cell membrane fluidity by fluorescence anisotropy, DPH and TMA-DPH fluorescent probes were used. Treated and non-treated rat cell suspensions (hematocrit of 0.01%) in PBS were labeled with the fluorescent probes (final concentration in samples 10^{-6} M) during incubation in the dark at room temperature for 1 h. The changes in membrane fluidity were evaluated in samples assayed in hypotonic condition, as well as in isotonic medium with increasing concentration range and varying pH (5.4, 6.5 and 7.4). DPH is located within the hydrophobic region of the bilayer membrane, while TMA-DPH is incorporated near the surface of the cell membrane.[20] The fluorescence anisotropy values are inversely proportional to cell membrane fluidity. A high degree of fluorescence anisotropy represents a high structural order and/or low cell membrane fluidity.[21] Steady-state anisotropy was measured with a SLM-Aminco AB-2 spectrofluorometer, using polarizers in the L configuration in a quartz cuvette at room temperature. Samples were illuminated with linearly (vertically or horizontally) polarized monochromatic light (λ_{ex} = 365 nm); and the fluorescence intensities emitted (λ_{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 the software provided with the instrument, according to the following equation:

$$r = (I_{vv} - I_{vh} \times G) / (I_{vv} + 2I_{vh} \times G) \quad (2)$$

where I_{vv} and I_{vh} represent the components of the light intensity emitted, respectively, parallel and perpendicular to the direction of the vertically polarized excitation light. The factor $G = I_{hv} / I_{hh}$ was used to correct the inequality of the detection beam to horizontally and vertically polarized emission.[21]

2.8. Erythrocyte ghost preparation

After surfactant treatment at room temperature for 10 min, human erythrocyte ghost membranes were prepared following the procedure of Fairbanks et al.[22] The packed erythrocytes were hemolyzed by hypotonic lysis in 5 mM phosphate buffer at pH 8.0. The pellet obtained by

centrifugation subsequent to hemolysis was resuspended and washed several times (by suspension in the buffer and centrifugation at 14,800 rpm for 15 min) until white ghost membranes were obtained. The protein content of erythrocyte ghosts re-suspended in PBS was measured by the Bio-Rad assay (Bio-Rad, Hercules, CA, USA), which is based on the dye-binding procedure of Bradford,[23] using bovine serum albumin (BSA) as a protein standard.

2.9. Electrophoretic analysis of membrane proteins

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the membrane proteins was performed under reducing conditions, according to the procedure of Laemmli,[24] using a Mini-PROTEAN Tetra Cell unit (Bio-Rad, Hercules, CA, USA). The slab gel consisted of a 7.5% polyacrylamide resolving gel and a 5% polyacrylamide stacking gel. The prepared human erythrocyte ghost membranes were mixed with 2x SDS sample buffer, heated at 95ºC for 5 minutes and then loaded on the gel in a concentration of 15 μg protein. Electrophoresis was carried out for 10 min at 60 V followed by 35 min at 200 V. Protein bands were viewed by staining with Coomassie brilliant blue R-250 for an hour under gentle shaking and destained with a mixture of 7.5% methanol and 10% acetic acid. The molecular weight of the membrane proteins was estimated from the molecular size marker (Bio-Rad Precision Plus Unstained Standard), ranging from 10 to 250 kDa. Densitometry analysis of Coomassie stained gel was performed using IMAT software (developed by the Scientific-Technical Services of the University of Barcelona). Actin (band 5) was chosen as the "internal standard" for quantitative calculations.[25]

2.10. Studies of erythrocyte morphology by scanning electron microscopy (SEM)

Cell morphology changes derived from the interaction of the surfactants with the erythrocyte membrane were determined by incubating intact cells with a sub-lytic concentration (10 μg/ml) of each surfactant. Samples were prepared as described elsewhere,[4] and the resulting specimens were examined under a Zeiss DSM 940A scanning electron microscope (Carl Zeiss SMT AG, Jena, Germany).

2.11. Statistical analyses

Each isotonic and hypotonic hemolysis experiment was performed at least three times using three replicate samples for each surfactant concentration tested. All anisotropy fluorescence values were also the means of three independent experiments. Results are expressed as mean ± standard error of the mean (S.E.M.). Statistical analyses were performed using Student's *t*-test or one-way analysis of variance (ANOVA) to determine the differences between the datasets, followed by Dunnett's or Tukey's *post hoc* tests for multiple comparisons using the SPSS® software (SPSS Inc., Chicago, IL, USA). $p < 0.05$ and $p < 0.005$ were considered significant. Pearson's correlation coefficients were also calculated, as indicated, by linear regression analysis using the SPSS® software.

3. RESULTS AND DISCUSSION

Surfactant-biomembrane interaction studies were performed with the aim to gain insights into the mechanisms of membrane lysis. Low concentrations of the amphiphiles (below the CMC) were chosen to determine their interaction properties, as at nonsolubilizing concentrations, surfactants serve useful purposes in biological experimentation as agents that permeabilize or perturb membrane structure in various ways.[6] Here erythrocytes were chosen as biomembrane model, whereas artificial membranes such as solid-supported lipid bilayers [26] and phosphatidylcholine vesicles [27,28] are also widely used to perform such kind of studies. Each membrane model presents its advantages and disadvantages, which makes it difficult the election of the most appropriate model. Studies using solid-supported lipid bilayers can give accurate results about corrosion of the membrane caused by surfactants at determined concentrations,[26] while phosphatidylcholine vesicles are used as model systems in search of specific biophysical mechanisms of surfactant-membrane interaction.[27,28] Erythrocytes are also largely used as biomembrane model [2-4,11,29] and, thus, might be a suitable approach to complete, correlate and/or corroborate the results obtained with the former models. Erythrocytes 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.[29,30] Therefore, these characteristics justify our choice of this model as a natural cell membrane system.

3.1. Surfactants induced hemolysis and antihemolysis

The measurement of hemoglobin released from erythrocytes is a reliable experimental approach to studying plasma membrane permeabilization by various chemical compounds. Characterization of the biological interactions of the surfactants was conducted firstly by hemolysis studies. The lysine-based surfactants had hemolytic activity in isotonic medium against rat erythrocytes, with HC_{50} values [16] dependent mainly on the position of the cationic charge and, to a lesser extent, on the length of the alkyl chain (Table 1). The surfactants MKM and PKM have the lowest p*K*a values (5.3 and 4.5, respectively) and consequently have the minor densitiy of cationic charge at physiological pH, whereas MLM, with a p*K*a = 8.1, is highly protonated. We found that MKM and PKM were considerably less hemolytic than MLM,[16] which means that the surfactant-membrane interactions in isotonic conditions were firstly affected by the charge density rather than by the hydrophobicity of the compounds. These results are in line with a previous report of our group,[31] which showed that other monocatenary lysine-based surfactants that differ in the alkyl chain length increased the hemolysis when the cationic charge density increases. Furthermore, we previously demonstrated that the charge density was directly related to the pH-sensitive activity of MKM and PKM: more protonable amino groups at acidic conditions add more charges to the polar head and consequently rises the membrane-lytic activity.[16] For these surfactants, the variation of pH promotes a variation in the protonation state that would lead to changes in their amphiphilicity and aggregation shape,[31] which, in turn, changes their interaction properties with the cell membrane. On comparing results with rat erythrocytes and with human erythrocytes, significantly higher resistance ($p < 0.05$) of the human cells to MKM and MLM was found. Our findings corroborate previous studies, in which erythrocytes from different species

(having specific phospholipid composition and mechanical properties of the cytoskeleton[32,33]) also had varied hemolytic behavior in the presence of surfactants [34,35] and of other different substances.[36,37]

Table 1. Hemolysis, antihemolysis and theoretical calculation of the volume expansion induced by the surfactants.

Surfactant (MW[a] g/mol)	CMC[b] (μg/ml)	HC_{50} [c] (μg/ml)	cAH_{max} [d] (μg/ml)	Antihemolytic potency (%)	Volume expansion (%)
Rat erythrocytes					
MKM (406.66)	650[e]	340.86 ± 14.03 [f]	37.5	41.06	10.19 ± 2.22
PKM (434.66)	260[e]	356.27 ± 13.68 [f]	50	26.55	0.79 ± 0.34
MLM (406.66)	765[f]	38.88 ± 3.51 [f]	15	44.63	5.03 ± 0.17
HTAB (598.40)	400	11.61 ± 0.88 [f]	5	57.09	4.06 ± 0.13
Human erythrocytes					
MKM (406.66)	650[e]	411.74 ± 8.78 [g]	100	63.20	11.86 ± 3.21
PKM (434.66)	260[e]	348.26 ± 4.89	150	37.94	0.79 ± 0.45
MLM (406.66)	765[f]	71.89 ± 2.56 [g]	20	58.27	13.90 ± 2.12
HTAB (598.40)	400	11.60 ± 0.1	5	45.28	5.46 ± 1.26

[a] Molecular weight
[b] Critical micelle concentration; determined in water
[c] Concentration of surfactant inducing 50% hemolysis (mean ± S.E.M.)
[d] Concentration resulting in maximum protection against hypotonic hemolysis
[e] Colomer el at.[9]
[f] Nogueira et al.[16]
[g] Significantly different from rat erythrocytes (Student's *t* test, $p < 0.05$)

The effect of surfactants on the resistance of erythrocytes on hypotonically induced lysis was also assessed with the aim to gain insight into the mechanisms of interaction. At low concentrations, well below those inducing lysis of erythrocytes, the lysine-based surfactants protected cells against their lysis in hypo-osmotic medium. The surfactants MKM and MLM showed similar antihemolytic potency, whereas PKM was the least active (Table 1). In rat erythrocytes, MLM reduced the extent of osmotically induced cell lysis by ~ 45%, whereas the least active compound PKM reduced it by ~ 25%. In human erythrocytes, MKM was the most

active compound, inhibiting more than 60% of hypotonic lysis, whereas PKM was the least active as also observed with rat erythrocytes, reducing the hemolysis by less than 40% (Table 1). This means that, differently from the hemolytic activity, the antihemolytic potency was firstly affected by the hydrophobicity rather than by the charge density of the compounds.

The dose-response curves representing the antihemolytic profile of the surfactants in rat and human erythrocytes are reported in Figure 2a and 2b, respectively. It is worth to note that, both with human and rat erythrocytes, MLM and HTAB rapidly increased the hemolysis rate beyond their cAH_{max} values, whereas the compounds MKM and PKM had a wide range of protective concentrations under hypotonic conditions. This behavior can be supported by the higher increase in the membrane fluidity at hypotonic medium induced by MKM and PKM in comparison to MLM and HTAB (see section 3.2). This might indicates that the former compounds incorporated in a higher extent into the bilayer, which in turn may support the maintenance of their antihemolytic activity over a wide concentration range. Surprisingly, we found no direct correlation between the antihemolytic profile (Figure 2) and antihemolytic potency (Table 1): PKM showed protective activity over various concentrations and the lowest antihemolytic potency, whereas MLM displayed higher antihemolytic potency and promptly lost its protective activity beyond the cAH_{max}. We can stated that the antihemolytic potency depended especially on the hydrophobicity of the compound, while the antihemolytic profile is directly affected by the position of the cationic charge. Small differences in the structure of molecules can affect their capacity to adsorb to erythrocyte membrane and, thus, their protective effects.[38] Finally, we found one meaningful correlation: the surfactants MKM and PKM that protect against hypotonic hemolysis over a wide range of surfactant concentration are the ones with the higher HC_{50} values, while the surfactants with the lower values of HC_{50} (MLM and HTAB) are those that displayed rapid loss of antihemolytic protection with increasing concentration.

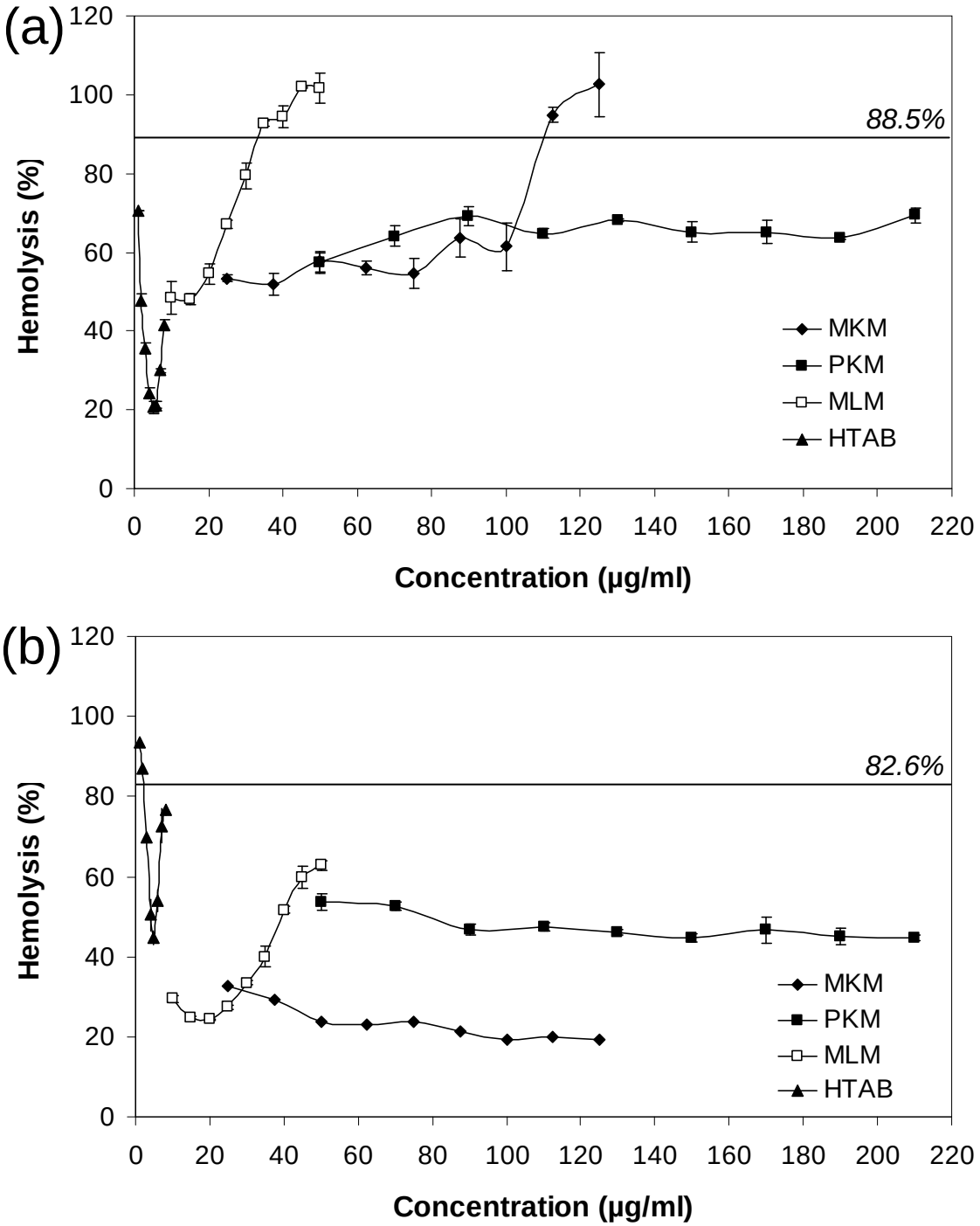


Figure 2. Dependence of the extent of hypo-osmotically induced hemolysis on surfactant concentration: (a) rat erythrocytes and (b) human erythrocytes. The straight solid line in each graph correspond to the percentage of hemolysis reached by untreated erythrocytes in hypotonic medium (88.5% and 82.6% for rat and human erythrocytes, respectively). Each point represents the mean of three independent experiments ± S.E.M. (error bars).

The effects of surfactants on the osmotic fragility of both rat and human erythrocytes, evaluated from the curves of hemolysis as a function of PBS solution osmolarities, are shown in Figure 3. The overall results indicated that the addition of the surfactants MKM, MLM and HTAB

caused a shift in lysis curve to the left, revealing that these surfactants increased the resistance of erythrocytes to hypotonic osmotic lysis. In contrast, the compound PKM barely shifted the osmotic fragility curve and so had little effect on cells' osmotic resistance. These results are consistent with the low antihemolytic potency and cell volume expansion shown by PKM (Table 1). Noteworthy is the bifunctional behavior of HTAB with human erythrocytes (Figure 3b) that was not observed with the other surfactants: it induced lower hemolysis than the untreated control cells at high osmolarities, while at low osmolarities the % of hemolysis was similar to the control cells. This indicated that HTAB has the ability to improve the cell osmotic resistance up to a critic osmolarity value, beyond that it becomes able to reach a molecular ratio in the membrane sufficient to enhance hemolysis.

In an attempt to find a correlation between antihemolytic potency and the volume changes induced by the surfactants to the cell,[2] we calculated the cell volume expansion (Table 1) using the equation (1) and the data from the concentration-response curves at different osmolarities (Figure 3). We found no significant correlations between antihemolytic potency and cell volume expansion ($r = 0.9459$ and $r = 0.7196$, $p > 0.05$, for human and rat erythrocytes, respectively). However, the trend that antihemolytic potency is related to cell volume expansion is in general followed by the surfactants even when the correlation obtained was not significant. For example, the compound PKM showed the lowest antihemolytic potency (Table 1), which can be related to negligible cell volume expansion. In contrast, MKM and MLM showed higher potency to protect against hypotonic lysis as well as enhanced cell volume expansion.

To explain the antihemolytic activity of surfactants, some mechanisms have been suggested: (i) It could be attributed to a phospholipid rearrangement,[39] which can lead to an increase in the ionic permeability of membrane and, thus, decrease the osmotic difference existing between the interior and exterior of the cell.[40] (ii) The insertion of surfactant molecules into the lipid bilayer increases either the cell surface area/volume ratio or the stretching capacity of the membrane, thereby allowing the cell to swell more and to attain a critical hemolytic volume before

the leakage.[11,41,42] (iii) It could be related to the improved elastic, mechanical and cohesive properties of the membrane resulting from the insertion of amphiphiles.[11] In our case, one individual or simultaneous mechanisms may be involved in the antihemolytic activity of the surfactants. The increased membrane fluidity observed in hypotonic condition (see section 3.2), together with the cell volume expansion might support the mechanism that the surfactant molecules can increase the stretching capacity of the membrane, while the loss of band 3 protein (see section 3.3) might be related to changes in its ionic permeability.

Several studies have attempted to demonstrate a correlation between hemolytic activity and the CMC of amphiphiles, but there are no clear conclusions in the literature.[8,11,43] In our previous study, we found no significant correlation between the HC_{50} and the CMC of the surfactants.[16] Here, we have studied the potential correlation between antihemolytic activity and the CMC. We found that the cAH_{max} values were lower than the CMC (Table 1), but no significant correlations were obtained. This indicates that the surfactant-mediated hemolysis protection depends on its monomer form and not on its micellar structures. In contrast, significant correlation was found between antihemolytic potency and the CMC of the lysine-based surfactants in rat erythrocytes ($r = 0.9995$, $p < 0.05$), but not in human cells ($r = 0.9195$, $p > 0.05$). Moreover, the CMC also correlated with the cell volume expansion in human erythrocytes ($r = 0.9973$, $p < 0.05$), but not in rat erythrocytes ($r = 0.6974$, $p > 0.05$). We can conclude from these results 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. We found that the less lipophilic amphiphiles (with shorter alkyl chain and higher CMC values) were the most protective, which was in contrast to the expected behavior: the more hydrophobic is the surfactant, the greater is its tendency to interact with the membrane and, thus, to have a strong antihemolytic potency. This contradictory behavior might be explained by the cut-off effect,[44] which could be caused by a decrease in the achievable concentration of the long-chained compound at the site of action due to its limit solubility, or by a complication in the intercalation of such amphiphile into

the lipid bilayer due to its critical chain length, resulting, thus, in a decrease in the partition coefficient. In line with our findings, this latter model has been proposed to explain the cut-off in protection against hypotonic hemolysis of erythrocytes exposed to single chain amphiphiles at concentrations well below the CMC.[45]

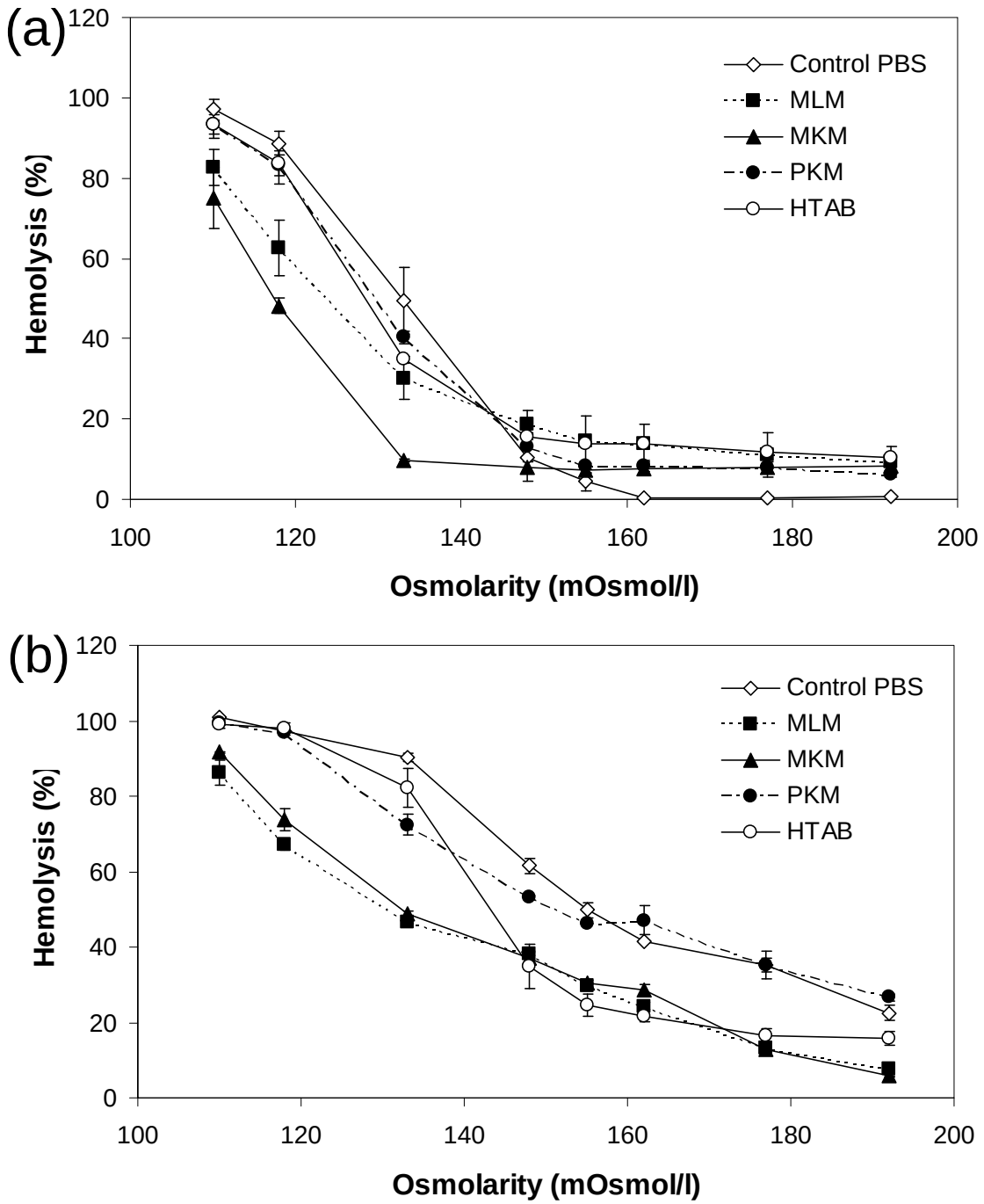
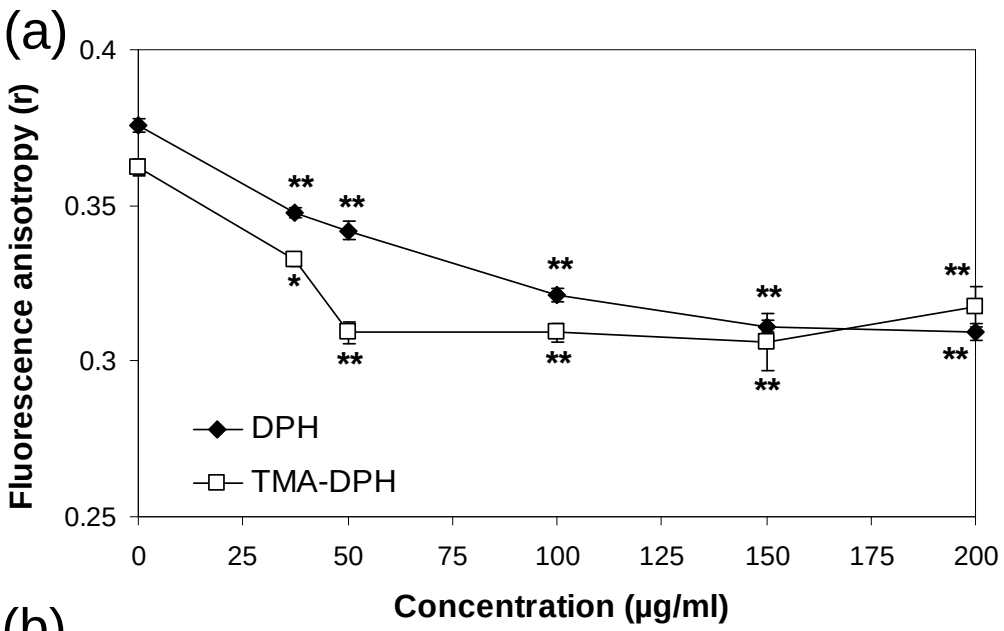


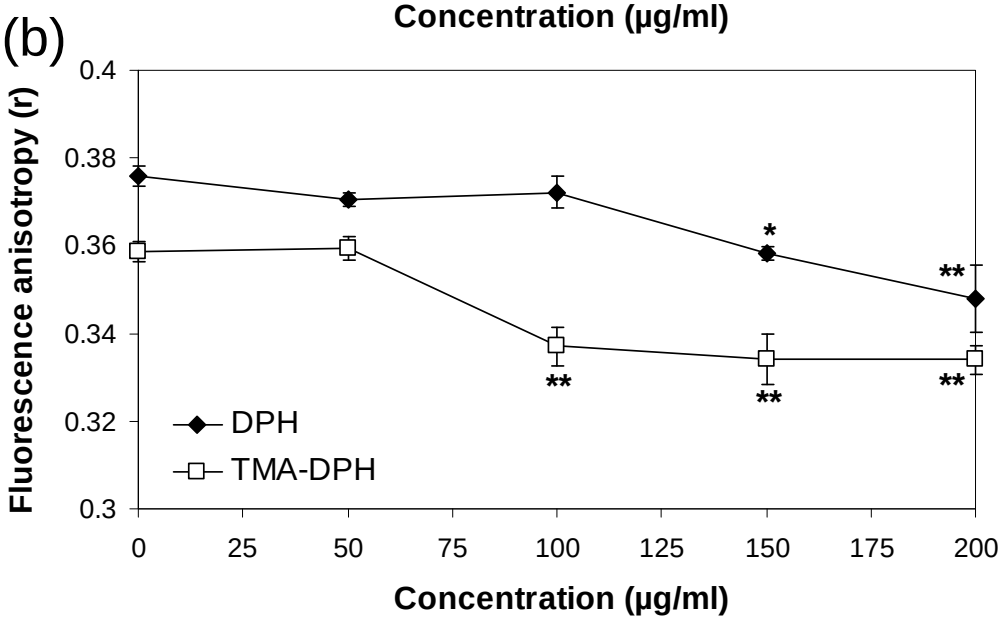
Figure 3. Osmotic lysis of (a) rat erythrocytes and (b) human erythrocytes in serial dilutions of PBS solution that reach different osmolarities at room temperature. Each point represents the mean of three independent experiments ± S.E.M. (error bars).

3.2. Effect of surfactant treatment on membrane fluidity

To determine whether surfactants disturb the phospholipid bilayer across its thickness, steady-state fluorescence anisotropy (r) was measured to test membrane fluidity, because the latter is a sensitive indicator for monitoring fluorophore binding to regions of biomembranes whose movement is constrained.[46] Membrane fluidity is an important parameter relating to the structure and functional state of the cell membrane.[21] The fluorescent probes, DPH and TMA-DPH, were incorporated into the rat erythrocyte membranes to detect the changes in membrane fluidity in the region near the center of the bilayer and in the outer leaflet of the membrane, respectively. It is known that the accumulation of surfactants on the membrane may, in consequence, cause a change in its function. Depending on the magnitude of interaction, they may cause a change in membrane permeability by alteration of lipid order, orientation and fluidity.[13]

Fluorescence anisotropy showed a significant decrease, along with the increase in surfactant concentration in isotonic conditions (Figure 4). This suggests that the lysine-based surfactants directly increase the fluidity of the phospholipid bilayer in a concentration-dependent manner. In general, the most marked decrease in anisotropy was found with the TMA-DPH probe, indicating that the external region of the bilayer is more affected by the compounds. We can conclude that, for MKM and PKM, the increase in membrane fluidity at increasing concentration might correlates directly with the increased hemolytic activity observed at the same concentration range.[16] PKM showed lower effects on packing constraints respect to MKM, which indicates inverse correlation between the alkyl chain length and the membrane fluidity. This finding is in line with the cut-off effect [44] and also with a previous report, which showed that longer tail amphiphiles lead to the formation of more ordered bilayer.[5] Surprisingly, although MLM was the most hemolytic surfactant, it displayed the minor effects on membrane fluidity even with increasing concentrations. This behavior suggests that this compound might induce bilayer solubilization due to its elevated hemolytic potency and, therefore, the increase in the bilayer fluidity might not be the main mechanism underlying its membrane-lytic activity.


(a)
Fluorescence anisotropy (r)
0.4
0.35
0.3
0.25
DPH
TMA-DPH
0
25
50
75
100
125
150
175
200
Concentration (μg/ml)


(b)
Fluorescence anisotropy (r)
0.4
0.38
0.36
0.34
0.32
0.3
DPH
TMA-DPH
0
25
50
75
100
125
150
175
200
Concentration (μg/ml)

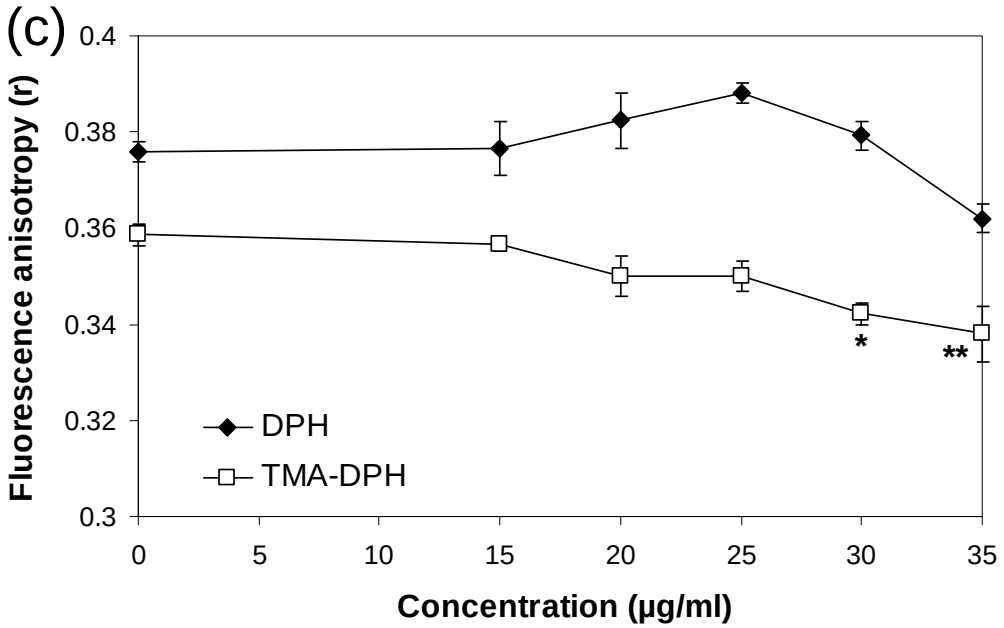

(c)
Fluorescence anisotropy (r)
0.4
0.38
0.36
0.34
0.32
0.3
DPH
TMA-DPH
0
5
10
15
20
25
30
35
Concentration (μg/ml)

Figure 4. The effect of surfactants on the membrane fluidity of rat erythrocytes as a function of concentration. (a) MKM, (b) PKM and (c) MLM. The fluorescence anisotropy (r) values determine the fluidity in both the external region (TMA-DPH) and core (DPH) of the lipid bilayer. The (r) values were compared to the control (untreated cells) by ANOVA followed by Dunnett's *post hoc* test. * $p < 0.05$ and ** $p < 0.005$ denote significant differences. Each point represents the mean of three independent experiments ± S.E.M. (error bars).

The effect of the surfactants on membrane fluidity was also assessed in isotonic conditions when the pH of the medium was varied. It was found that the penetration of the surfactants in bilayers depends on the pH of the medium (Figure 5). The core and the external region of the membrane were significantly altered by MKM ($p < 0.005$) at pH 6.5 and 5.4 and by PKM ($p < 0.05$) at pH 5.4, as demonstrated by the DPH and TMA-DPH anisotropy values, respectively. In contrast, MLM showed no significant effect with varying pH in the structural order of lipids across the entire thickness of the bilayer. Although MKM and PKM were less lipophilic (more protonated) at acidic conditions, their disturbing-properties into the lipid bilayer might be attributed to the increased electrostatic attraction between the positive polar head group (higher charge density at low pH) and the negatively charged cell membrane (see section 3.1). In turn, the lack of effects of MLM could be attributable to its tendency to solubilize the bilayer (see discussion above), which might also be due to its high charge density across the entire pH range. These results support our findings regarding the pH-responsive membrane-disruptive activity of the surfactants.[16] We found that MKM was much more potent in disrupting the plasma membrane at pH 6.5 and 5.4 while PKM also showed significantly higher lytic activity at pH 5.4. In contrast, MLM did not show pH-responsive activity. Therefore, we can conclude that the pH-sensitive activity of these compounds could be directly related to the order of the lipids in the membrane: at low pH, the incorporation of MKM and PKM into the membrane might increases and, in consequence, the membrane becomes more fluid, which lead to lysis. The higher incorporation of MKM and PKM into the membrane can be also substantiated by the SEM studies of erythrocyte morphology at acidic conditions.[16] These compounds interacted intensively with the lipid bilayer and induced a spherocyte-type deformation at pH 5.4, which can be assumed to be the last stage of the morphological change with maximum accumulation of the compound into both the outer and

inner layers of the erythrocyte membrane before cell lysis.[47] In summary, the dependence of surfactant penetration into the membrane on the position of the cationic charge is clear: compounds with the positive charge on the α-amino group of the lysine are deeply embedded in the lipid bilayer in acidic conditions.

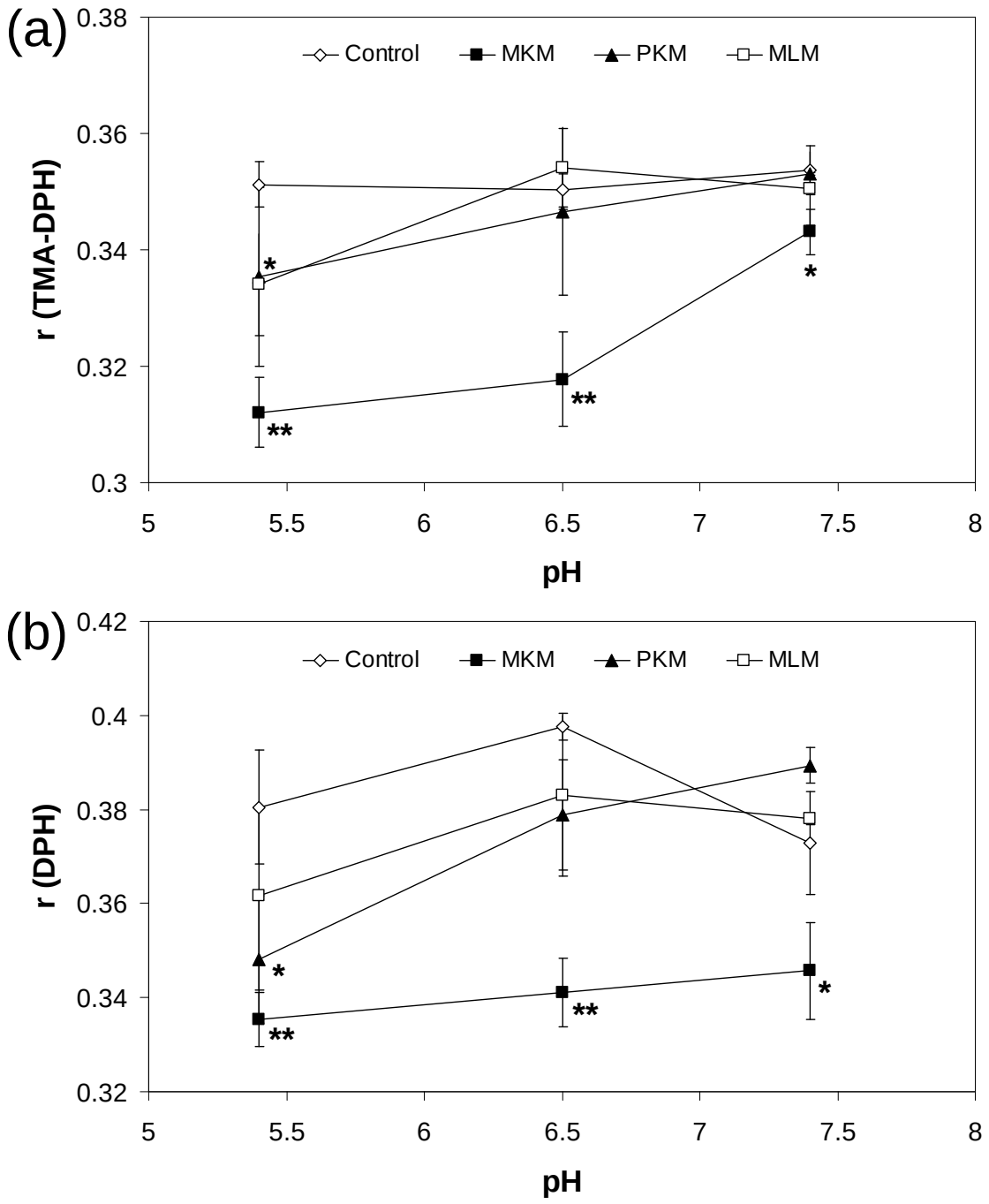


Figure 5. The effect of surfactants on the membrane fluidity of rat erythrocytes as a function of pH. (a) TMA-DPH and (b) DPH fluorescence probes. Surfactants were added at the following final concentrations: 25 μg/ml MKM and PKM, 15 μg/ml MLM. The fluorescence anisotropy (r) values were compared to the control (untreated cells) by ANOVA followed by Dunnett's *post hoc* test. * $p < 0.05$ and ** $p < 0.005$ denote significant differences. The data represent the mean of three independent experiments ± S.E.M. (error bars).

The effect of the surfactants on membrane fluidity was also evaluated in the hypo-osmotic medium, and the results were compared to those obtained in isotonic medium with the same final concentrations. The concentrations assessed were those that showed the greatest antihemolytic activity in rat erythrocytes. Surfactant treatment in hypotonic conditions increased membrane fluidity significantly ($p < 0.005$) in both the core and external region of the bilayer (Table 2). Moreover, significant changes ($p < 0.05$ and $p < 0.005$) in the lipid bilayer fluidity was observed in comparison to the same surfactant concentration assessed in physiological condition (with the exception of MLM (DPH) and HTAB (TMA-DPH)), corroborating that the hypotonicity of the medium enhances the effect of the surfactants in the membrane fluidity. These results can be directly correlated to the antihemolytic activity of the surfactants and might be one of its mechanisms (see section 3.1).

Table 2. Steady-state fluorescence anisotropy of the probes DPH and TMA-DPH incorporated into erythrocyte membrane in isotonic and hypotonic conditions.

Samples (μg/ml)	(r) DPH (mean ± SE)	(r) TMA-DPH (mean ± SE)
Control	0.3772 ± 0.0081	0.3593 ± 0.0038
Isotonic medium		
MKM (37.5)	0.3480 ± 0.0005 [a]	0.3251 ± 0.0104 [a]
PKM (50)	0.3683 ± 0.0005	0.3546 ± 0.0022
MLM (15)	0.3594 ± 0.0059	0.3509 ± 0.0014
HTAB (5)	0.3708 ± 0.0005	0.3541 ± 0.0024
Hypotonic medium		
MKM (37.5)	0.2980 ± 0.0061 [a, c]	0.2982 ± 0.0053 [a, b]
PKM (50)	0.3211 ± 0.0094 [a, c]	0.3147 ± 0.0036 [a, c]
MLM (15)	0.3420 ± 0.0075 [a]	0.3305 ± 0.0013 [a, b]
HTAB (5)	0.3328 ± 0.0036 [a, c]	0.3362 ± 0.0040 [a]

r values = anisotropy measurements
[a] $p < 0.005$ when compared to control PBS (Dunnett's *post hoc* test)
[b] $p < 0.05$ and [c] $p < 0.005$ when compared to isotonic medium (Tukey's *post hoc* test)

3.3. Electrophoresis of membrane proteins

SDS-PAGE experiments were run to assess whether surfactant-induced membrane lytic activity is associated with alterations in the erythrocyte membrane proteins and damage to them. We performed the electrophoresis experiments with human erythrocytes, as the preparation of ghost membranes was not very effective with rat erythrocytes. Moreover, this technique is widely performed for the same purpose with human erythrocytes.[46,48,49]

Figure 6 shows the electrophoretic profile of the erythrocyte membrane proteins. The well-established normal distribution of the major membrane cytoskeletal proteins is shown in the lane 2, which contains untreated erythrocytes ghosts. The effect of the surfactants on the membrane proteins was evaluated after treatment under hypotonic conditions (at the cAH_{max} concentrations) and in isotonic conditions varying the pH of the medium (at concentrations of about HC_{50} for each pH). The treatments with surfactants in hypotonic medium led to a significant loss of band 3 (36.21%, 39.89% and 11.88% for MKM, PKM and MLM, respectively, as determined by densitometry analysis) (Figure 6; lanes 3, 4 and 5). This corroborates a previous report that suggested that band 3, an anion exchange protein in human erythrocytes, participates in hypotonic hemolysis.[50] In contrast, as shown in Figure 6 and revealed by the densitometric analysis, the surfactants did not cause noticeable changes in the electrophoretic pattern of erythrocyte membrane proteins when the treatments were in isotonic medium at pH 7.4 and 6.5 (Figure 6, lanes 6 to 11). These results indicated minimal interaction with the bilayer proteins during surfactant-induced membrane lysis under physiological conditions and at the pH range characteristic of the early endosomes, respectively. However, when the treatments were performed at pH 5.4, all tested surfactants induced significant loss of band 3 (34.10%, 44.55% and 40.34% for MKM, PKM and MLM, respectively), ankyrin (band 2.1) (73.53%, 56.14% and 45.66% for MKM, PKM and MLM, respectively) and band 6 (81.80%, 68.53% and 74.44% for MKM, PKM and MLM, respectively) (Figure 6, lanes 12, 13 and 14), indicating that these compounds caused degradation of some membrane proteins before the onset of cell lysis or that the proteins were

segregated from the mother cell into shed microvesicles.[51,52] These microvesicles are enriched with membrane proteins[53] and, their release might depends on the level of membrane fluidity.[54] In summary, the alterations observed in some membrane proteins indicate that the surfactants change lipid-protein interactions in the bilayer, which can be a biophysical mechanism directly related to membrane lysis. These data suggest that an efficient bioactive compound in an intracellular drug delivery system might has the ability to interact with both membrane lipids and proteins to prompt the destabilization of the endosome membrane and release of the active content inside the cell.

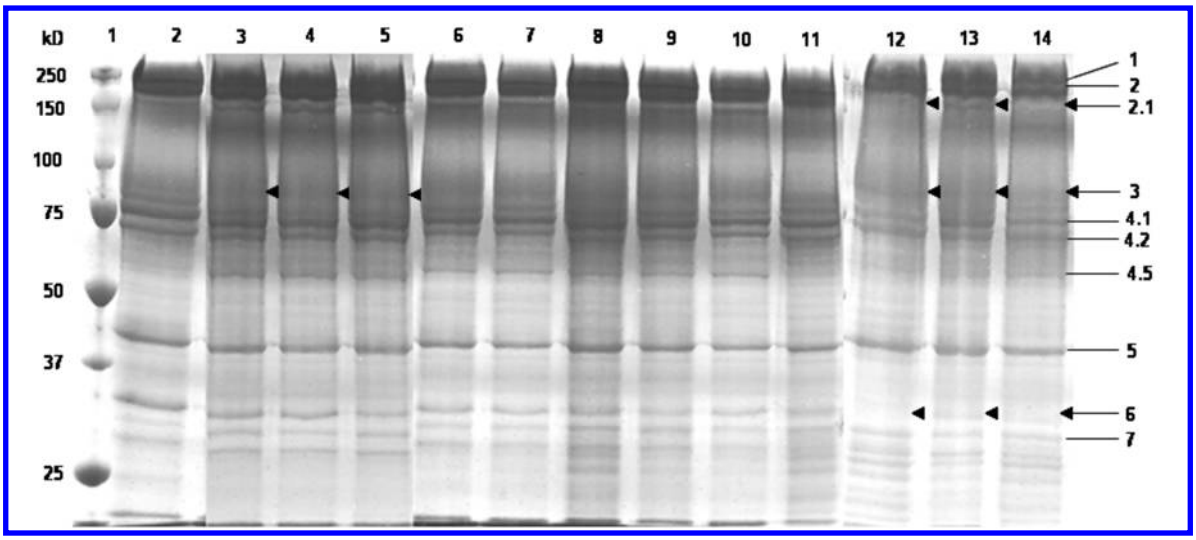


Figure 6. Effect of surfactants on human erythrocyte membrane skeletal proteins, as determined by SDS-PAGE. Following the pre-incubation of the erythrocytes for 10 minutes in the absence or presence of the surfactants, membranes were separated and washed as described in Section 2.8. The names of the major cytoskeletal proteins follow the classification of Fairbanks et al.[22] and are given on the right of the gel. Arrowheads indicate the differences in the protein banding pattern between control and surfactant treatments. Each lane corresponds to a different treatment: (1) Protein standard, (2) Untreated membrane proteins (control); treatment of surfactants in hypo-osmotic medium at their cAH_{max}: (3) 100 μg/ml MKM, (4) 150 μg/ml PKM, (5) 20 μg/ml MLM; treatment of surfactants at concentrations about their HC_{50}: (6) 300 μg/ml MKM at pH 7.4, (7) 300 μg/ml PKM at pH 7.4, (8) 35 μg/ml MLM at pH 7.4, (9) 50 μg/ml MKM at pH 6.5, (10) 200 μg/ml PKM at pH 6.5, (11) 100 μg/ml MLM at pH 6.5, (12) 50 μg/ml MKM at pH 5.4, (13) 50 μg/ml PKM at pH 5.4 and (14) 75 μg/ml MLM at pH 5.4.

3.4. SEM studies of erythrocyte morphology

SEM experiments were conducted in order to better understand the interaction of cationic lysine-based surfactants with the lipid bilayer. From the changes in cell morphology we assessed how the surfactants interact with the membrane, whether it is with the outer or inner layer of the lipid bilayer. Rat erythrocytes were treated with each surfactant in isotonic medium and

physiological pH at the sub-lytic concentration of 10 μg/ml.[16] The effect of pH on erythrocytes' shape changes induced by these surfactant treatments was described previously by our group,[16] results that are complementary and correlate directly with the membrane-related properties studied here.

SEM examinations corroborated that the surfactants interacted with the lipid bilayer by altering the normal biconcave morphology of the cells (Figure 7). Control erythrocytes incubated in PBS solution were found to be discoid (Figure 7a). Despite varying membrane lytic activity of MKM and MLM, erythrocytes underwent similar morphological alterations after treatment with these surfactants, indicating that morphological changes are not affected by the position of the cationic charge, unlike hemolytic activity, which is strongly affected. MKM and MLM changed the discoid shape of cells at pH 7.4 to stomatocytes (Figures 7b and 7c, respectively), which is consistent with the bilayer hypothesis.[47] Stomatocytes are formed when the compounds interact with the inner layer of the bilayer and thus, for MKM, the increased membrane fluidity in the core of the bilayer (see section 3.2) could also be related to this type of cell deformation due to the deeper incorporation of the surfactant. The stomatocyte-type deformation induced by MLM (Figure 7c) seems to show also endovesicle formation in the membrane, which can be related to torocyte-like endovesicles.[55] Torocyte endovesicles seem to be formed in a process in which an initially stomatocyte invagination loses volume whilst maintaining a large surface area. Moreover, this type of deformation might be attributable to the interaction of the surfactant with both the outer and the inner layers of the lipid bilayer,[56] which could be directly related to the greater hemolytic activity of MLM than of MKM that we found.

However, PKM (with a longer alkyl chain) induced in general a leptocyte-type deformation (Figure 7d), but also prompt the morphological change of some cells to the shape characteristic of the first stage of echinocytosis. Echinocytes are induced when the compound added is inserted in the outer monolayer of the membrane, which, therefore, might substantiate the greater effect of PKM on the membrane fluidity in the external leaflet of the bilayer (see section

3.2). Leptocyte is a flattened cell with decreased volume that also has an unusually large membrane in proportion to its contents, which could be attributed to the greater incorporation of PKM into the lipid bilayer, thus giving an appearance of "excess" plasma membrane. This hypothesis of enhanced incorporation did not lead to increased hemolytic activity or membrane fluidity (see sections 3.1 and 3.2, respectively), possibly because PKM may increase the packing degree of the lipid bilayer and thus exert a protective effect at sublytic concentrations.[5]

Finally, as we have demonstrated no noticeable change in the membrane proteins' profile after surfactant treatments at physiological pH, the surfactant-protein interaction might not be involved in the morphological changes undergone by the erythrocytes in this condition. Our findings contradict studies of some authors [35,57] that reported the proteins of the membrane skeleton and integral membrane proteins as responsible for the shape of erythrocytes. However, our results obtained under acidic conditions are in accordance with these reports, as we demonstrated significant alterations in the membrane proteins' profile (see section 3.3) and the change in cell morphology to spherocytes.[16]

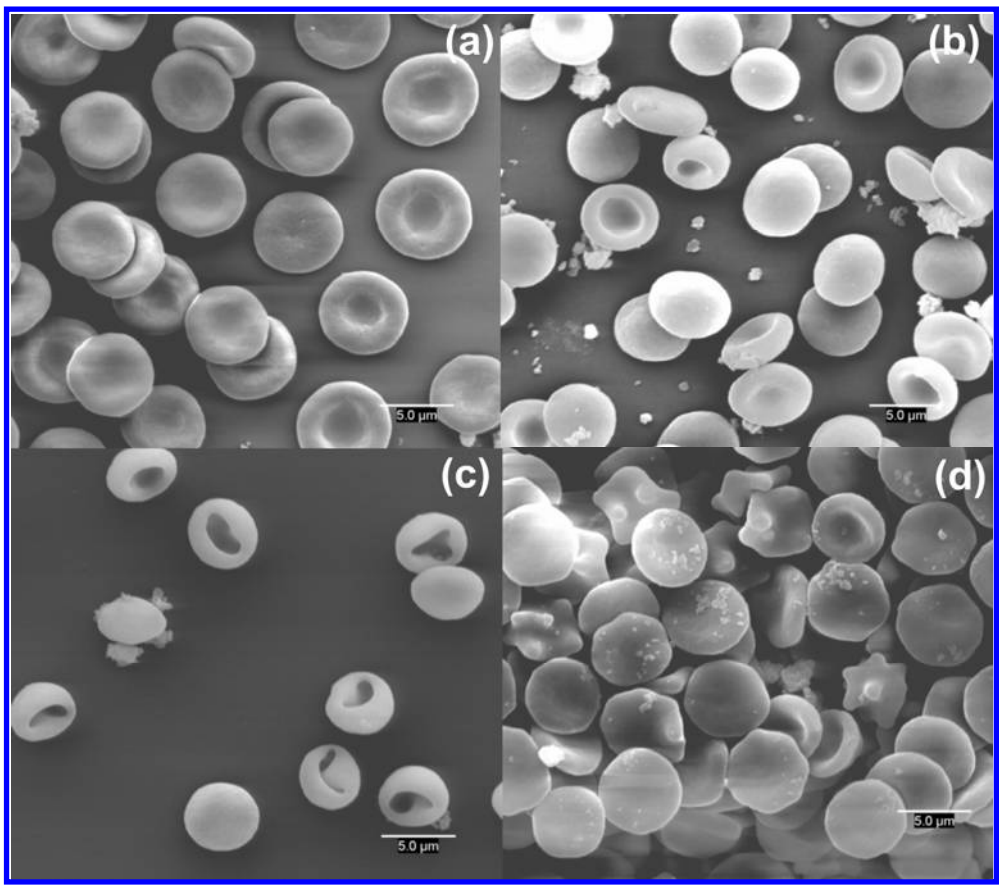


Figure 7. Effect of the cationic lysine-based surfactants on rat erythrocyte morphology. SEM images [16] of (a) control in PBS pH 7.4, and after incubation with the surfactants at pH 7.4: (b) MKM, (c) MLM and (d) PKM. The erythrocytes were incubated for 10 minutes at the sub-lytic concentration of 10 μg/ml of each surfactant. Scale bars correspond to 5μm.

3.5. Structure-activity relationship

Studies on surfactant-induced biomembrane lysis were performed in an attempt to gain some mechanistic understanding and insights into the structure-activity relationship of this new class of cationic lysine-based amphiphiles. The main found structure-activity relationships were summarised in Table 3, in which is shown that, for molecules with similar hydrophobic character, the position of the charged group and, thus, the density of charge, play an important role in the overall surfactant's activity. The cationic charge on the α-amino group of lysine determines the pH-sensitive membrane-lytic activity of the amphiphiles.[16] This specific activity can be explained by the increasing protonation state of the surfactants with decreasing pH and, beyond that, can be mechanistically evidenced by the increased disturbance in the packing of lipid bilayer and by the prominent changes on the membrane protein pattern at acidic environment. To substantiated this proposed mechanism, it is worth to note that the normal lipid-protein interactions are required for the maintenance of the overall membrane function.[44] In turn, the positive charge on the ε-amino group of lysine gives to the surfactant a greater hemolytic activity at physiological pH (due to the increased charge density), together with a prominent antihemolytic potency. This behavior might be because the highly hemolytic compounds immediately insert themselves into the erythrocyte lipid bilayer, likely increasing its permeability or even its lateral expansion ability, and hence initially conferring hypotonic protection.[58] Moreover, for this specific class of lysine-based surfactants, the longer is the alkyl chain, the lower is the overall membrane-disturbing activity. Surprisingly, the enhanced hidrophobicity did not directly increase the phospholipid bilayer perturbing-effects. Finally, we can conclude that the combination of structural parameters such as shorter alkyl chain (C14) and positive charge on the α-amino group of lysine gives to the surfactant the overall much greater effects on membrane.

Table 3. Summary of the structure-activity relationship of the cationic lysine-based surfactants.

Surfactant	**MKM**		**PKM**		**MLM**	
Charge position	α-amino		α-amino		ε-amino	
Alkyl chain length	14C		16C		14C	
Hemolysis ***pH 7.4***	+		+		+ + +	
pH 6.5 [a]	+ + +		+ +		+	
pH 5.4 [a]	+ + +		+ + +		+	
Antihemolytic potency	+ + +		+		+ + +	
Volume expansion	+ +		-		+ +	
Membrane fluidity	***DPH***	***TMA-DPH***	**DPH**	**TMA-DPH**	**DPH**	**TMA-DPH**
Increasing concentrations	+ + +	+ + +	+ +	+ + +	-	+ +
pH 7.4	+ +	+	-	-	-	-
pH 6.5	+ +	+ +	-	-	-	-
pH 5.4	+ + +	+ + +	+ +	+	-	-
Hypotonic medium	+ + +	+ + +	+ +	+ +	+	+
Membrane proteins						
pH 7.4	-		-		-	
pH 6.5	-		-		-	
pH 5.4	+ + +		+ + +		+ + +	
Hypotonic medium	+		+		+	
Cell morphology						
pH 7.4	Stomatocyte (+)		Leptocyte (+)		Stomatocyte (+)	
pH 6.5 [a]	Spherostomatocyte (+ +)		Stomatocyte (+)		Spherostomatocyte (+ +)	
pH 5.4 [a]	Spherocyte (+ + +)		Spherocyte (+ + +)		Spherocyte (+ + +)	

[a] Nogueira et al. [16]
Effect level: (+) low, (+ +) medium, (+ + +) high

4. CONCLUSIONS

This study revealed some important characteristics of surfactant-biomembrane interactions. The mechanism of biomembrane lysis was shown to be associated with lipid bilayer disorganization, through interaction with the lipids and proteins of the membrane. We also showed that the phospholipid bilayer-perturbing properties of the surfactants depend on their structural features. A tendency might be established: the longer the alkyl chain, the lower is its power to protect the cell against lysis in hypotonic medium. In addition, the compounds having the positive

charge on the α-amino group of the lysine (MKM and PKM) prompted higher disturbance in the lipid bilayer packing at acidic conditions. The SDS-PAGE experiments showed that the main interaction of the surfactants with the membrane proteins occurred at pH 5.4, while minimal interaction was at physiological pH. These findings can be interpreted as necessary steps in the overall process of pH-sensitive membrane-lytic activity of MKM and PKM, which might be directly attributable to their higher charge densitiy at low pH. Furthermore, SEM studies corroborated the interaction of the surfactants with the lipid bilayer, as demonstrated by the changes in the cell shape. On the basis of our overall results, we conclude that the pH-sensitive surfactants affect the structural and dynamic properties of the biomembranes especially at acidic environment. At the molecular level, they operate mainly by increasing the fluidization of the phospholipid bilayer and, to a lesser extent, by interacting with the proteins of the membrane. Finally, the results obtained here, which mechanistically corroborated the pH-sensitive activity of MKM and PKM previously demonstrated by our group,[16] suggested that these amphiphiles could be promissing as a new class of multifunctional bioactive excipients for active intracellular drug delivery. Substantial efforts are still needed to fully understand the functions of surfactant-like biomolecules and to optimize the numerous biomedical and technical applications of these compounds rationally. Therefore, the insights into the biological processes of surfactant-induced changes in membrane pattern given in this paper are of great importance in increasing our knowledge of the phospholipid bilayer-perturbing properties of novel bioactive excipients and, thus, in developing new efficient and specific drug delivery devices.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

ACKNOWLEDGMENTS

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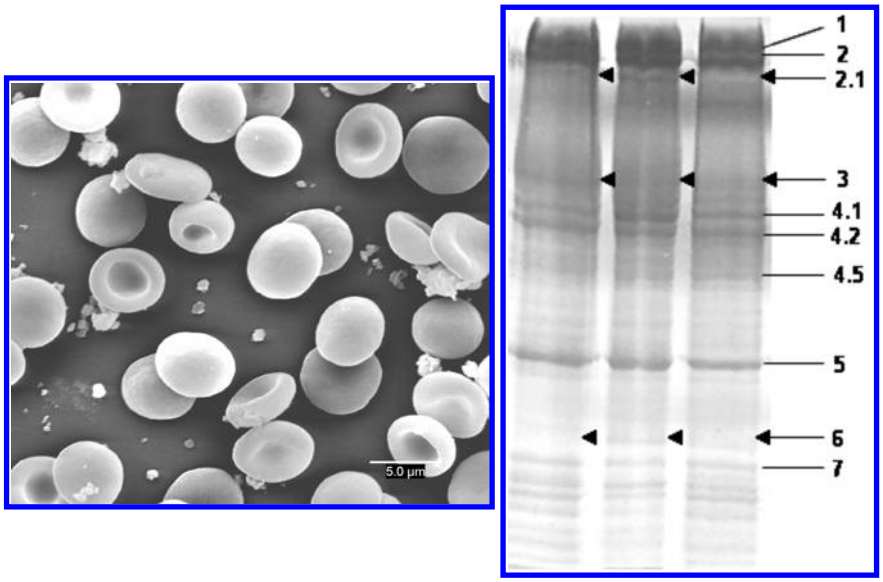
TABLE OF CONTENTS GRAPHIC

Phospholipid bilayer perturbing-properties underlying lysis induced by pH-sensitive cationic lysine-based surfactants in biomembranes



To gain better mechanistic understanding of surfactant-induced membrane destabilization, it was assessed here the phospholipid bilayer-perturbing properties of pH-sensitive cationic lysine-based surfactants using erythrocytes as biomembrane models. This study leads with the characterization of the main mechanisms of surfactant-biomembrane interactions and also with the role of the structural parameters of the cationic amphiphiles on their lytic activity. The insights into the biological processes of surfactant-induced changes in membrane pattern given in this work are of great importance in finding and understanding new multifunctional bioactive excipients for active intracellular drug delivery.