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ORIGINAL ARTICLE

Membrane-destabilizing activity of pH-responsive cationic lysine-based surfactants: role of charge position and alkyl chain length

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Abstract Many strategies for treating diseases require the delivery of drugs into the cell cytoplasm following internalization within endosomal vesicles. Thus, compounds triggered by low pH to disrupt membranes and release endosomal contents into the cytosol are of particular interest. Here, we report novel cationic lysine-based surfactants (hydrochloride salts of N^{ε}- and N^{α}-acyl lysine methyl ester) that differ in the position of the positive charge and the length of the alkyl chain. Amino acid-based surfactants could be promising novel biomaterials in drug delivery systems, given their biocompatible properties and low cytotoxic potential. We examined their ability to disrupt the cell membrane in a range of pH values, concentrations and incubation times, using a standard hemolysis assay as a model of endosomal membranes. Furthermore, we addressed the mechanism of surfactant-mediated membrane destabilization, including the effects of each surfactant on erythrocyte morphology as a function of pH. We found that only surfactants with the positive charge on the α-amino group of lysine showed pH-sensitive hemolytic activity and improved kinetics within the endosomal pH range, indicating that the positive charge position is critical for pH-responsive behavior. Moreover, our results showed that an increase in the alkyl chain length from 14 to 16 carbon atoms was associated with a lower ability to disrupt cell membranes. Knowledge on modulating surfactant-lipid bilayer interactions may help us to develop more efficient biocompatible amino acid-based drug delivery devices.

Keywords Lysine-based surfactants · Hemolysis · pH-sensitivity · Membrane disruption · Drug delivery

Abbreviations

CMC	Critical micellar concentration
HC_{50}	Surfactant concentration that induces 50% hemolysis
HTAB	Hexadecyl trimethyl ammonium bromide
MKM	N^{ε}-myristoyl lysine methyl ester
MLM	N^{α}-myristoyl lysine methyl ester
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PKM	N^{ε}-palmitoyl lysine methyl ester
SEM	Scanning electron microscopy
SEM	Standard error of the mean

Introduction

Advances in strategies for treating a wide variety of diseases require the efficient delivery of the active compound into the cytosol or nucleus of target cells (Hu et al. 2007). Therapeutic agents, such as proteins, peptides, DNA and antitumor drugs, act at intracellular sites, thus their therapeutic efficacy depends on efficient intracellular trafficking (Plank et al. 1998). Cells usually take up drug carriers via endocytosis that confines the internalized active compounds to vesicles (endosomes). Therefore, one of the

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challenges for efficient intracellular delivery of therapeutic compounds is to facilitate their release into the cytosol by destabilizing endosomal membranes under mildly acidic conditions (Chen et al. 2009; Stayton et al. 2000). This would manipulate or circumvent the non-productive trafficking from endosomes to lysosomes, thereby avoiding degradation at a pH as low as 4.6 (Mellman 1996).

Over the past two decades, considerable research has focused on delivery systems that specifically destabilize endosomal membranes following endocytic uptake (Christie and Grainger 2003). Carriers based on attenuated viruses have been studied extensively as pH-dependent membrane-disruptive components in gene delivery systems to enhance transport from endosomes to the cytosol. However, clinical use of these carriers is limited by their antigenicity and toxicity (Gordon and Anderson 1994; McTaggart and Al-Rubeai 2002; Temin 1990). Safety issues have prompted the development of synthetic peptides structurally derived from viruses to specifically disrupt endosomal membranes (Plank et al. 1998), but these peptides are also likely to be immunogenic in vivo (Sandhu et al. 1997). To overcome these limitations, a variety of non-viral delivery vectors has been studied, such as synthetic surfactants and polymers. Anionic lysine-based surfactants exhibit pH-responsive membrane-lytic activity in the late endosomal pH range, thus showing promise for intracellular drug delivery systems (Nogueira et al. 2011). Polymerizable surfactants with tunable pH-sensitive amphiphilicity have been designed and tested as multifunctional delivery devices for systemic and targeted delivery of therapeutic siRNA (Wang et al. 2007, 2008, 2009). Moreover, cationic amino acid-based surfactants have been used to prepare biocompatible devices for the controlled encapsulation and release of DNA, where the surfactants form stable complexes with the oppositely charged DNA through electrostatic interactions (Morán et al. 2010). Cationic and anionic polymers with pH-sensitive activity, including derivatives of poly (acrylic acid) (Jones et al. 2003; Kusonwiriyawong et al. 2003; Kyriakides et al. 2002), methacrylic acid copolymers (Yessine et al. 2003), imidazole-containing polymers (Seo and Kim 2010) and pseudo-peptidic polymers (Chen et al. 2008, 2009), have also been developed to promote endosomal release. Cationic polymers have been described to enhance membrane lysis at low pH via electrostatic interactions between protonated amines and the negatively charged membranes (Yessine et al. 2003).

Surfactants are one of the most widely applied excipients in the pharmaceutical industry due to their surface and interface activities (Paulsson and Edsman 2001). Those derived from amino acids usually present biocompatible properties and low cytotoxicity, and are therefore of great interest for pharmaceutical applications, especially in the field of novel non-viral drug delivery devices (Morán et al. 2010; Pérez et al. 2009). As surface properties (hydrophobicity and surface charge) have a major impact on cellular uptake of particulate drug delivery systems, the incorporation of charged surfactants into these carriers might improve targeting to specific cells (Schöler et al. 2001). Furthermore, surfactants with pH-responsive membrane-disruptive activity may further destabilize endosomal compartments (Nogueira et al. 2011; Wang et al. 2007). The physicochemical and biological properties of cationic amino acid-based surfactants, as well as their synthesis, have been widely reported by our group (Colomer et al. 2011a; Lozano et al. 2011; Infante et al. 2010; Pérez et al. 2002, 2009). Therefore, we selected biocompatible cationic surfactants from the amino acid lysine (hydrochloride salts of N^{ε}- and N^{α}-acyl lysine methyl ester) since they display lower toxicity potential and are classified as biodegradable (Pérez et al. 2009), and thus suitable for practical applications.

Here, we studied the membrane-destabilizing properties as a function of pH of three cationic lysine-based surfactants that differ in the position of the positive charge and the length of the alkyl chain. To evaluate their potential application in intracellular drug delivery systems, we examined the pH-dependent cell membrane-disruptive activity of these compounds using a standard hemolysis assay of rat erythrocytes as a model of endosomal membranes. The hemolysis dependence on the concentration and the kinetic properties of the surfactants at the endosomal pH range were also evaluated. Furthermore, we investigated the mechanisms involved in cell membrane disruption, including the effects of each surfactant on erythrocyte morphology at varying pH values. To gain insight into the structure-dependent interaction of these compounds with membrane bilayers, the influence of the charge position and alkyl chain length on hemolytic activity was also discussed.

Materials and methods

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). Polyethylene glycol (PEG)-10,000, D-glucose and hexadecyl trimethyl ammonium bromide (HTAB) were from Sigma-Aldrich (St. Louis, MO, USA).

Surfactants

Three biocompatible amino acid-based surfactants derived from N^{ε} or N^{α}-acyl lysine methyl ester salts with one lysine

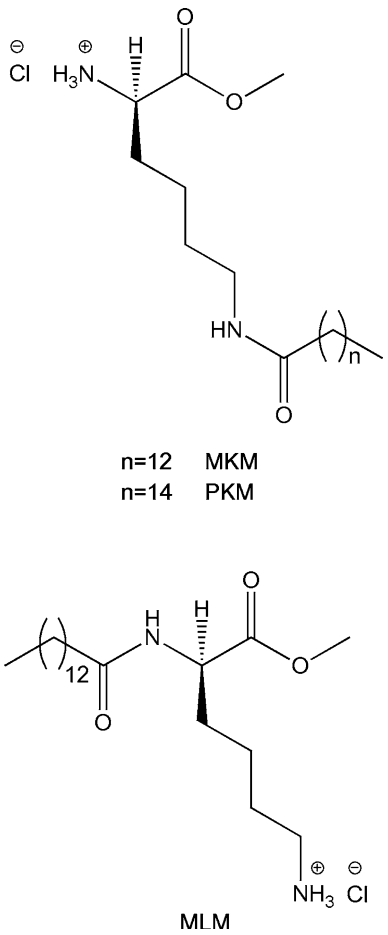

Fig. 1 Molecular structures of the cationic lysine-based surfactants

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 (Fig. 1). The commercial cationic surfactant hexadecyl trimethyl ammonium bromide (HTAB) was used as the reference compound. These lysine-based surfactants were synthesized in our laboratory as previously described (Colomer et al. 2011a; Pérez et al. 2009) and made from natural fatty acid and amino acid organic building blocks. The chemical structure of these compounds was checked by nuclear magnetic resonance and their purity, higher than 99%, was confirmed by elemental analysis and high-performance liquid chromatography. In all cases, all building blocks were linked by amide bonds to form biodegradable molecules. See Table 1 for the physicochemical properties and analytical data.

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 experimentation. Red blood cells were isolated by centrifugation at 3,000 rpm at 4°C 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 8×10^9 cells/ml.

Hemolysis assay

The membrane lytic activity of the surfactants was examined by hemolysis assay. PBS buffers in the pH range of 5.4–7.4 were prepared to be isosmotic inside the erythrocyte and cause negligible hemolysis. 25-µl aliquots of erythrocyte suspension were exposed to different surfactant

Table 1 Physicochemical properties and analytical data for the cationic lysine-based surfactants

Surfactant	MW (g/mol)	CMC[a] (µg/ml)	p*Ka*[a]	Number of alkyl chains	Length of alkyl chain	Elemental analysis (%) (% calculated)[b]		
						C	H	N
MKM	406.66	650[c]	5.3[c]	1	C14	61.77 (62.02)	10.78 (10.56)	6.73 (6.88)
PKM	434.66	260[c]	4.5[c]	1	C16	63.61 (63.49)	11.20 (10.81)	6.37 (6.44)
MLM	406.66	765[d]	8.1[d]	1	C14	61.76 (62.02)	10.42 (10.56)	6.62 (6.88)

MW Molecular weight

[a] Determined in water

[b] Pérez et al. (2009)

[c] Colomer et al. (2011b)

[d] Determined as described in "Hemolysis assay" and "pH-dependent hemolysis" sections

concentrations based on preliminary studies (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 solution in a total volume of 1 ml. The samples were incubated at room temperature for 10 min. Two controls were prepared by resuspending erythrocyte suspension either in buffer alone (negative control) or in distilled water (positive control). The kinetic experiments were performed with concentrations associated with initial low hemolytic in the pH range of endosomal compartments (pH 6.5 and 5.4). The samples were incubated at room temperature under constant shaking for various periods up to 90 min. In all these hemolysis experiments, the samples were centrifuged at 10,000 rpm for 5 min at the end of each incubation time. Absorbance of the hemoglobin release in supernatants was measured at 540 nm using a Shimadzu UV-160A spectrophotometer (Shimadzu, Kyoto, Japan) and the percentages of hemolysis 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.

Osmotic protection experiments

To gain further insight into the mechanism of cell membrane disruption, the effect of osmolytes with different molecular weights on the membrane-disruptive activity of the surfactants was investigated. For this, PBS buffers at the pH range studied were prepared by adding D-glucose (180 Da) or polyethylene glycol (PEG)-10,000 (10 kDa) at a concentration of 10 mM, which is below the range at which these molecules alone induce hemolysis as a result of osmotic pressure (Murthy et al. 1999). Red blood cells were incubated with these buffers and exposed to a concentration of each surfactant that achieved significant hemolysis in PBS buffer alone (500 μg/ml for MKM and PKM, and 60 μg/ml for MLM). Hemolysis was determined after incubating the cells for 10 min at room temperature, following the procedure described above. Controls with only the osmolytes were prepared to ensure that no hemolysis occurred without the surfactants.

Erythrocyte count

Erythrocytes were counted in a Zeiss Axioskop optical microscope (Zeiss, Jena, Germany) using a Bürker counting chamber (Brand, Wertheim, Germany). The percentages of lysed erythrocytes at the required concentration of each surfactant (500 μg/ml for MKM and PKM, and 60 μg/ml for MLM) were determined relative to the total cell number in PBS buffer alone. The hemolysis experiment was performed following the procedure above at the same pH range and each sample was diluted four times for cell counts.

Studies of rat erythrocyte morphology by scanning electron microscopy (SEM)

Interaction of the surfactants with the erythrocyte membrane in the pH range under study was determined by incubating intact cells with a sub-lytic concentration (10 μg/ml) of each surfactant. After a 10-min incubation, samples were fixed by adding 1 ml of 2.5% glutaraldehyde in PBS solution and incubated at 4°C for 2 h. The samples were then centrifuged (1,500 rpm for 5 min), the supernatant was discarded, and 500 μl of 1.25% glutaraldehyde in PBS was added. Fixed samples were washed with PBS solution, postfixed with 1% osmium tetroxide, placed on a glass coverslip, dehydrated in an ascending series of ethyl alcohol (50–100%), air-dried by the critical point drying method using a CPD 7501 apparatus (Polaron, Watford, UK), and finally mounted on an aluminium stub and gold-coated by an SEM coating system SC 510 (Fisons Instruments, East Grinstead, UK). Resulting specimens were examined under a Zeiss DSM 940A scanning electron microscope (Carl Zeiss SMT AG, Jena, Germany).

Statistical analyses

Each hemolysis experiment was performed at least three times using three replicate samples for each surfactant concentration tested. Results are expressed as mean ± standard error of the mean (SEM). 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 Bonferroni's or Dunnett's post hoc tests for multiple comparisons using the SPSS® software (SPSS Inc., Chicago, IL, USA). $P < 0.05$, $P < 0.01$ and $P < 0.005$ were considered significant. Pearson's correlation coefficients (r) between the HC_{50} and CMC values were also calculated by linear regression analysis.

Results and discussion

Hemolysis assay

We studied the disruption of lipid bilayer membranes by cationic lysine-based surfactants. Erythrocytes are considered a simple cellular model and are therefore used as a convenient cell membrane system to study surfactant–membrane interactions (Sánchez et al. 2007). We used a hemolysis assay with the erythrocyte membrane as a model of the endosomal membrane (Chen et al. 2009; Wang et al. 2007). Early endosomal compartments have a pH from 6.5

to 6.8, while the lumen of late endosomes has a lower pH, of about 5.5 (Moore et al. 2008; Stayton et al. 2000). The lysosome has a pH as low as 4.6 to 5.0 (Mellman 1996). Therefore, we explored the membrane lytic activity of the class of compounds at the pH range of 5.4–7.4, mimicking the environment that the surfactant molecules are expected to encounter when incorporated into a drug delivery device translocating through the endocytic pathway.

The hemolytic activity of the surfactants was determined as a function of concentration at pH 5.4, 6.5, and 7.4. The concentration-dependent curves in Fig. 2 demonstrate that the membrane-disruptive activity of the surfactants was strongly influenced by concentration. At pH 7.4 (Fig. 2a), all the surfactants displayed almost a linear increase in hemolysis as a function of concentration. Moreover, the surfactants MKM and PKM were considerably less hemolytic than MLM and the commercial surfactant HTAB. MLM and HTAB (used as the reference compound) were nearly 9 and 30-fold more active in disrupting cell membranes at physiological conditions, respectively, as demonstrated by the HC_{50} values calculated from these curves (Table 2).

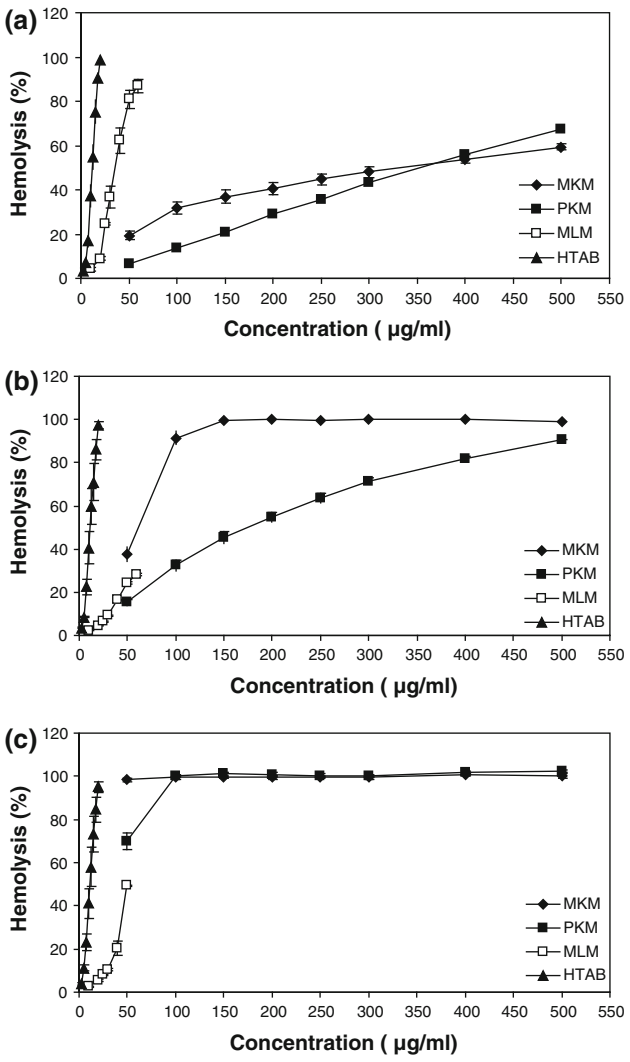

Fig. 2 Dependence of rat erythrocyte hemolysis on surfactant concentration. **a** pH 7.4, **b** pH 6.5 and **c** pH 5.4. Each point represents the mean of three independent experiments ± SEM (*error bars*)

When the effect of concentration on hemolytic potency was evaluated at pH 6.5 (a very early stage of endosomal acidification), the surfactants showed different profiles (Fig. 2b). MKM and PKM displayed higher degrees of hemolysis than at physiological conditions (pH 7.4) throughout the concentration range tested, and the differences in HC_{50} values were significant ($P < 0.05$) (Table 2). MKM was much more potent in disrupting the plasma membrane at pH 6.5, showing 100% membrane lysis at 150 µg/ml, while only 36.91% hemolysis was observed at pH 7.4. PKM was 3.5-fold less efficient than MKM in disrupting plasma membrane on the basis of HC_{50}, since almost complete disruption of erythrocyte membranes was only achieved gradually with an increasing concentration from 50 to 500 µg/ml. In contrast, MLM was 2.5-fold less hemolytic at pH 6.5 than at pH 7.4, reaching maximum hemolysis of only 28.24% at 60 µg/ml while a maximum of 86.86% was reached at physiological conditions.

Finally, at pH 5.4 (a late stage of endosomal acidification) (Fig. 2c), the membrane lytic activity of MKM and PKM increased dramatically, reaching 100% hemolysis at low concentrations of 50 and 100 µg/ml, respectively. In contrast, MLM did not show increased activity at pH 5.4. Hemolysis was greater here than at pH 6.5 but still lower than in physiological conditions (Table 2). The commercial surfactant HTAB displayed the same concentration-dependent hemolytic activity in the endosomal and physiological pH range and differences in HC_{50} values were not significant ($P > 0.05$).

Table 2 HC_{50} values of the surfactants in the pH range studied

Surfactants	HC_{50} (µg/ml) (mean[a] ± SEM)		
	pH 7.4	pH 6.5	pH 5.4
MKM	340.86 ± 14.03	56.45 ± 12.10[b]	<50[c]
PKM	356.27 ± 13.68	199.84 ± 24.54[b]	<50[c]
MLM	38.88 ± 3.51	98.58[d] ± 1.53[e]	75.51[d] ± 20.73
HTAB	11.61 ± 0.88	11.48 ± 1.57	11.49 ± 1.79

[a] Mean ± SEM of three experiments

[b] Significantly different from the pH 7.4 condition (Student *t* test, $P < 0.05$)

[c] The lowest concentration tested displayed more than 50% hemolysis

[d] Estimated value (out of experimental curve range)

[e] Significantly different from the pH 7.4 condition (ANOVA followed by Bonferroni's post hoc test, $P < 0.05$)

Despite the individual differences within the pH range tested, the hemolytic activity of all the compounds was strongly affected by concentration. In previous studies, we reported similar results for the membrane lytic activity of arginine-based gemini surfactants and anionic lysine-based surfactants (Mitjans et al. 2003; Nogueira et al. 2011). These results were as expected, since it has been widely reported that surfactants at high concentrations are hemolytic and membrane solubilization is often observed (Jones 1999; Maher and Singer 1984). Here, the enhanced hemolysis with increasing surfactant concentration could also be due to increased ionic interactions between the negatively charged lipid membranes and positively charged amino groups of the surfactant molecule (Seo and Kim 2010). Furthermore, surfactant properties, such as alkyl chain length, position of the cationic charge and head group hydrophobicity can significantly affect surfactant interaction with cell membranes (Colomer et al. 2011a). Our results here showed that the compounds with the positive charge in the α-amino group (MKM and PKM) had lower disruptive potency at physiological pH and higher membrane lytic activity in the late endosomal pH range. Regarding the surfactants with the charge in the same position (MKM and PKM), concentration-dependent hemolytic activity decreased with increasing length of the hydrophobic tail. Likewise, several authors have reported similar results for the hemolysis of hydrogels of amino acid-based cationic amphiphiles (Roy and Das 2008) and of partially fluorinated pyridinium bromides (Vyas et al. 2006). This behavior contrasts with the typically hemolytic activity described for cationic surfactants, where longer alkyl chains are associated with higher membrane lytic activity (Rasia et al. 2007; Benavides et al. 2004).

It is worth mentioning that the HC_{50} values of MKM and MLM were below the critical micellar concentration (CMC) across the entire pH range studied here, indicating that the monomers are responsible for hemolytic activity and that micelles are not needed for surfactant-mediated cell lysis (Table 1). In contrast, PKM disrupted membranes at micellar concentrations in physiological conditions and in its monomer form in the pH range of endosomal compartments. The CMC was determined by conductivity measurements at 25°C of each surfactant aqueous solution at the adequate concentration range, following the procedure described by Colomer et al. (2011a). The CMC values of MKM and PKM were previously reported by our research group (Colomer et al. 2011b). We found no significant correlations ($P > 0.05$) between the CMC and the HC_{50} values at pH 7.4 ($r = 0.7209$) and 6.5 ($r = 0.8756$). Several studies have been performed to demonstrate a correlation between hemolytic activity and the CMC of surfactants; however, there are no clear conclusions in the literature (Pérez et al. 2009; Preté et al. 2002; Spengler et al. 2011).

pH-dependent hemolysis

To corroborate the pH-dependent hemolytic activity of MKM and PKM in contrast to MLM and HTAB, specific studies on the effects of pH were performed. At low concentrations (Fig. 3a), the surfactants showed low or negligible hemolytic activity at pH 7.4. When the pH was decreased to 6.5, MKM had increased membrane lytic activity and so did PKM but to a lesser extent, resulting in a maximum hemolysis of 37.51 and 15.23%, respectively. At pH 5.4, the hemolytic activity of MKM and PKM increased considerably ($P < 0.01$), reaching maxima of 98.26 and 69.92%, respectively. The pK50 (pH at which 50% hemolysis is obtained) was also calculated for each surfactant from the pH-response curves showed in Fig. 3a (Lee et al. 2010), with a value of approximately 6.2 for MKM, while the hemolysis response curve of PKM shifted toward lower pH and had an apparent pK50 of 5.6. These results support the early lytic activity of MKM at mildly acidic conditions (pH 6.5). MLM and HTAB showed no pH-sensitive activity throughout the endosomal pH range and thus, did not facilitate endosomal destabilization.

At concentrations near the HC_{50} values (Fig. 3b), MKM and PKM exhibited a concomitant rise in hemolytic activity at pH 7.4 and 6.5, reaching approximately 40–50% hemolysis at physiological conditions, while 71.5 and 100%

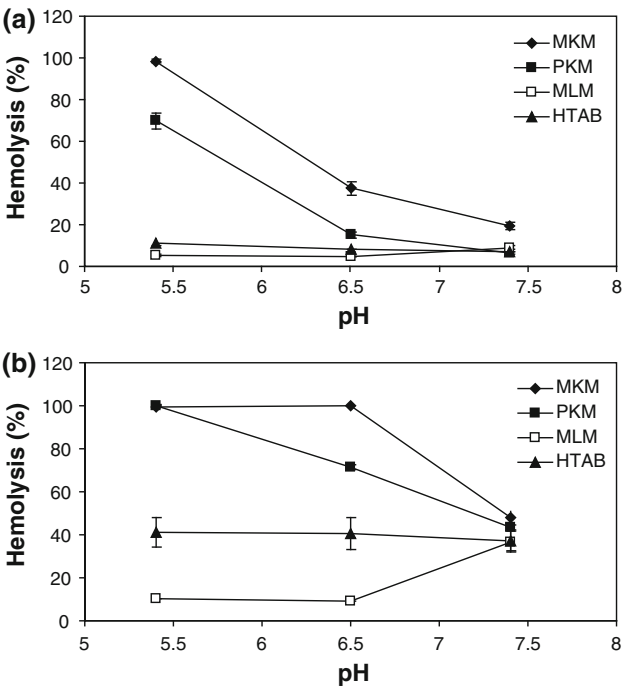

Fig. 3 Surfactant-induced hemoglobin release from rat erythrocytes as a function of pH. Surfactants were added from concentrated solutions to give final concentrations of **a** 50 μg/ml MKM and PKM, 20 μg/ml MLM, or 5 μg/ml HTAB; and **b** 300 μg/ml MKM and PKM, 30 μg/ml MLM, or 10 μg/ml HTAB. Each point represents the mean of three independent experiments ± SEM (*error bars*)

hemolysis were recorded at pH 6.5 for PKM and MKM, respectively. At pH 5.4, MKM maintained the approximately 100% lysis observed at lower concentrations and PKM also reached total hemolysis. These observations demonstrated that surfactants at high concentrations do not retain the specific pH-responsive behavior, as significant hemolysis was also observed at physiological conditions. Surprisingly, higher concentrations of MLM displayed lower membrane-disruptive activity in acidic conditions than at physiological pH, while HTAB did not show any significant changes in its hemolytic potency throughout the pH range evaluated, confirming the inability to disrupt endosomal membranes.

Noteworthy was the significant rise in the hemolytic activity of MKM and PKM at pH 5.4 and to a lesser extent at pH 6.5, while MLM demonstrated no pH-dependent responses. The difference between MKM and MLM lies in the polar group (position of the cationic charge), while MKM and PKM differ in the length of the hydrophobic tail. It has been suggested that protonation and pH-dependent activity may be governed by the overall p*K*a of the head group of the surfactants (Wang et al. 2007). Lysine is a dibasic amino acid consisting of two amino groups with different basicity since the α-amino group has a p*K*a = 8.9 and the ε-amino group a p*K*a = 10.5. However, to estimate the protonation state of the surfactant molecule in the pH range studied, we determined the apparent p*K*a values of the polar groups when they were included in the surfactant molecule. The p*K*a value of each compound was estimated by potentiometric titration, as described by Tabohashi et al. (2001). The titration was carried out at 25°C by adding increments of the NaOH solution to the surfactant solution with constant magnetic stirring, and the p*K*a value was obtained from the inflection point in the titration curve. The experimental p*K*a values obtained from pH measurements in aqueous medium were found to be considerably lower than those of the original lysine amino acid: 5.3 and 4.5 for MKM and PKM (cationic charge in the α-amino group of the lysine), respectively (Colomer et al. 2011b), and 8.1 for MLM (cationic charge in the ε-amino group of the lysine). Given that the only difference between MKM and MLM is the position of the cationic charge, the p*K*a associated with the protonated amino group may be crucial for pH-sensitive membrane-disruptive activity. Consistent with these values, the lack of pH-responsive activity of MLM is related to the fact that no significant changes in the protonation state of the molecule took place in our experimental conditions, as the polar head should remain almost completely protonated throughout the studied pH range (which means an average charge of approximately 1). In contrast, MKM and PKM have p*K*a values lower or in the order of the low pH range studied and therefore different protonation states can be achieved. The increased membrane-disruptive activity of these compounds in acidic conditions could be explained by a modification in the hydrophobic/hydrophilic balance. At pH 5.4, the lysine in MKM is approximately 50% protonated (which means an average charge of 0.5), while PKM has a protonation state lower than 50% (average charge < 0.5) and hence, a lower ability to disrupt the lipid bilayer. This higher lytic activity of MKM is in accordance with the study reported by Chen et al. (2003), in which pH-sensitive acyloxyalkylimidazoles reached the maximum rate of hemolysis when about 50% of the molecule was protonated. The same reasoning is valid for the surfactant lytic activity at pH 6.5, in which MKM has a more protonated polar group and is thus more hemolytic than PKM (even though both compounds are less than 50% protonated). More protonable amino groups add more charges to the polar head, which consequently increases membrane-lytic activity. At neutral pH, these compounds predominantly exist as unprotonated species, which explains the lower hemolysis achieved at physiological conditions. To summarize, surfactant membrane lytic activity changes with the pH because of the variation in the net charge at the studied pH range. Furthermore, concerning the surfactants that have the charge in the same position but differ in the alkyl chain length (MKM and PKM), our results showed that increasing the number of carbon atoms from 14 to 16 did not affect the pH-responsive behavior, but decreased membrane lytic activity in the endosomal pH range. In conclusion, the pH-sensitive membrane-disruptive activity of these surfactants can be fine-tuned mainly by varying the position of the cationic charge (by adjusting the p*K*a) and, to a lesser extent, by altering the length of the alkyl chain.

A strong correlation has been reported between hemolytic activity and endosomal disruption by membrane-disruptive agents (Plank et al. 1994). The cationic lysine-based surfactants with pH-sensitive properties were studied based on the hypothesis that pH-responsive membrane lytic activity in endosomes should facilitate membrane destabilization and allow surfactant-drug complexes to escape to the cytoplasm for efficient intracellular drug delivery. Numerous amphiphilic materials can mediate material transport across cell membranes, such as amphiphilic peptide sequences used by viruses (Janshoff et al. 1999) and amphiphilic lipids (Wasungu and Hoekstra 2006). However, virus peptides may have problems of immunogenicity and lipids may not selectively disrupt membranes at the endosomal pH. Our results demonstrated that MKM and PKM have specific pH-responsive membrane disruption at pH 5.4, the final most acidic endosomal pH, which may help to circumvent the non-productive trafficking of therapeutic compounds from endosomes to lysosomes, where degradation may occur. In summary, a useful intracellular drug delivery system should have low lytic activity at physiological pH and high destabilizing

activity in the mildly acidic conditions found in the endosomes to elicit only selective endosomal membrane disruption (Wang et al. 2007; Yessine et al. 2003). Low concentrations of PKM and MKM induced membrane lysis only at the endosomal pH range and there was no significant hemolysis at blood stream pH (7.4), supporting these surfactants as potential biocompatible materials in novel non-viral drug delivery systems.

Kinetics of hemolytic activity

Surfactant-mediated hemolysis was also determined as a function of time in the pH range characteristic of early and late endosomes (pH 6.5 and 5.4, respectively). The concentrations evaluated (25 μg/ml MKM, 50 μg/ml PKM, 20 μg/ml MLM and 5 μg/ml HTAB) were in the range of those that displayed specific pH-dependent activity and/or low degree of hemolysis after the initial 10 minutes of incubation. MKM and PKM showed improved kinetics of hemolytic activity at both stages of endosomal acidification. At pH 6.5 (Fig. 4a), MKM caused 100% of hemolysis after 30 min of incubation, which corresponded to a membrane-disruptive activity approximately 17-fold higher than that observed after 10 min of incubation. PKM was less effective than MKM, as it showed a lag time of 30 min and achieved around 80 and 95% hemolysis only after 60 and 90 min of incubation, respectively. The presence of a lag time implies the requirement of a minimum threshold amount of surfactant that has to accumulate in the lipid bilayer before hemoglobin leakage (Chen et al. 2003). At pH 5.4 (Fig. 4b), both compounds caused relatively weak hemolysis after 10 minutes of incubation, followed by a sharp increase to a maximum of about 80% (MKM) and 100% (PKM) after 90 minutes. The membrane lytic activity of MLM and the reference surfactant HTAB did not show any dependence on incubation time at the two pH values assessed, confirming their inability to disrupt endosomal membranes.

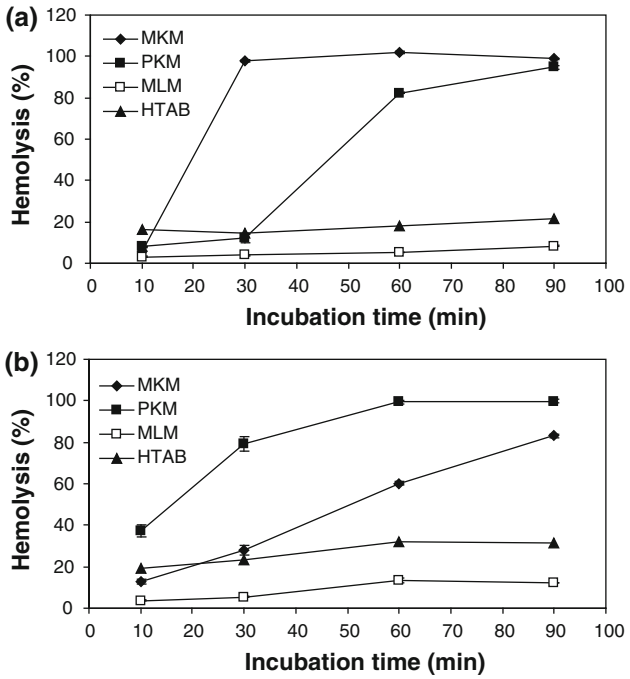

Fig. 4 Kinetics of hemolytic activity of the lysine-based surfactants at **a** pH 6.5 and **b** pH 5.4. Surfactants were added at time zero from concentrated solutions to give final concentrations of 25 μg/ml MKM, 50 μg/ml PKM, 20 μg/ml MLM and 5 μg/ml HTAB. Each point represents the mean of three independent experiments ± SEM (*error bars*)

The significant hemolytic kinetics shown by MKM and PKM indicate that one or more previous steps are required before the erythrocyte membrane becomes permeable to hemoglobin. Among these steps, the most important could be the formation of pores or channels that lead to the efflux of low molecular weight solutes (see "Osmotic protection"). The molecules taken up by endocytosis are trafficked from early endosomes to lysosomes within several hours (Pack et al. 2005). Thus, timely permeabilization of the endosomal membrane is a prerequisite for cytosolic translocation of drugs in order to exert their pharmacological effect (Asokan and Cho 2005). Given the improved hemolytic kinetics and pH-responsive membrane activity of PKM and MKM, these compounds could disrupt endosomal membranes before fusion of the endocytic vesicles with lysosomes, thus avoiding non-productive intracellular trafficking, a critical feature for potential intracellular drug delivery applications.

Mechanism of cell membrane disruption

Osmotic protection

To assess whether membrane lysis occurs through the colloid osmotic mechanism (after formation of transient defects or pores in the membrane due to surfactant treatment), an osmotic protection experiment was conducted in PBS solutions that contained molecules of varying sizes. The rationale behind this was that since sufficiently large molecules do not permeate through the cell membrane, their presence would counteract the osmotic pressure of the macromolecules inside the cell (especially hemoglobin), diminishing water penetration and consequently cell swelling (Murthy et al. 1999).

PEG-10,000 significantly ($P < 0.05$) inhibited surfactant-mediated hemolysis throughout the pH range studied, thereby indicating that defects such as pores or channels are formed in the erythrocyte membrane (Fig. 5). In the presence of PKM and MKM at pH 7.4, and MLM at pH 6.5, PEG-10,000 produced less pronounced inhibition of

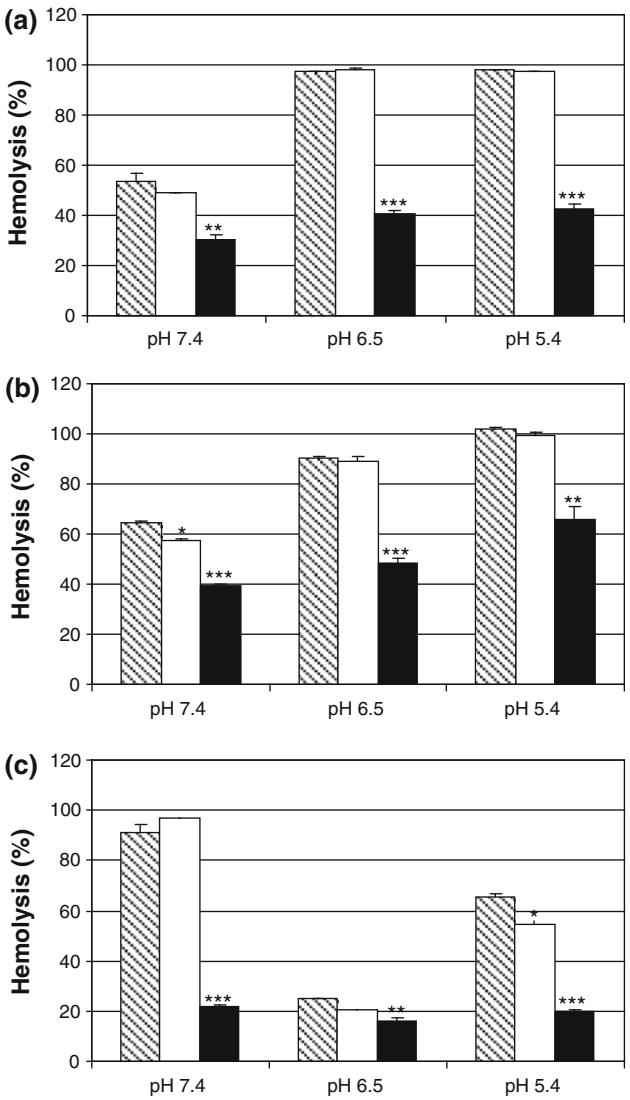

Fig. 5 Effects of the osmotic protectant PEG-10,000 (10 kDa) and D-glucose (180 Da) on lysine-based surfactant-induced hemolysis of rat erythrocytes. Surfactants were added at the following range of final concentrations **a** 500 μg/ml MKM, **b** 500 μg/ml PKM, and **c** 60 μg/ml MLM. PBS alone (*striped bars*), PBS + D-glucose (*blank bars*) and PBS + PEG-10,000 (*black bars*). The hemolysis results obtained in the presence of the osmolytes D-glucose and PEG-10,000 were compared to the control in PBS solution alone by ANOVA followed by Dunnett's post hoc test. $*P < 0.05$, $**P < 0.01$ and $***P < 0.005$ denote significant differences. The data represent the mean of three independent experiments ± SEM (*error bars*)

hemolysis, achieving maximum protection of 39.15, 43.26, and 35.96%, respectively. The highest protection degrees mediated by PEG-10,000 addition were 46.33, 58.19, and 75.83% for PKM and MKM at pH 6.5, and MLM at pH 7.4, respectively. Despite the significant degrees of protection against hemolysis after the addition of PEG-10,000, this osmolyte only partially avoided cell membrane disruption (generally by less than 50%) within the pH range studied. This means that the surfactants induced pores in the lipid bilayer that were smaller than the hydrodynamic radius of PEG-10,000 (hence, not permeable to this osmolyte, thereby decreasing hemolysis), as well as larger pores that were permeable to this protectant. The osmotic imbalance resulting from the diffusion of low molecular weight solutes out of the cell cannot be balanced when these large pores are formed and consequently, cell lysis occurs. The overall observations of this experiment indicated that osmotic cell swelling was not the only mechanism involved in membrane lysis and other more complex mechanisms may also contribute. It is also feasible that cell membrane disruption could be due to partial solubilization of membrane lipids and proteins through micellization caused by extensive surfactant adsorption (Chernitsky and Senkovich 1998). Regardless of the partial inhibition of cell lysis and the complexity of the mechanisms involved, the significant protection against hemolysis ($P < 0.05$) can be considered as evidence of the presence of an osmotic mechanism (Chernitsky and Senkovich 1998). Corroborating the osmotic mechanism, the low molecular weight D-glucose had no effect on the osmotic imbalance caused by the hemoglobin trapped in the erythrocyte cytoplasm (Fig. 5). Negligible or very small protection was observed and the degree of hemolysis did not differ significantly ($P > 0.05$) from that caused by the same surfactant concentration in PBS alone, except for PKM at pH 7.4 ($P = 0.018$) and MLM at pH 5.4 ($P = 0.01$). For comparison, our previous studies on anionic lysine-based surfactants showed that PEG-10,000 almost completely protected against cell lysis at pH 7.4 and 6.5, while partial protection was only observed at pH 5.4 (Nogueira et al. 2011).

Erythrocyte count

Erythrocyte counts were performed to study whether hemoglobin passes through the cell membrane or whether it is released into the extracellular medium after cell membrane rupture (Chen et al. 2009; Nogueira et al. 2011). The overall cell numbers declined significantly after 10 minutes of incubation with the hemolytic concentration of each surfactant throughout the pH range tested and the percentages of lysed erythrocytes were in agreement with the hemolytic activity results (Fig. 6). These observations indicate that hemoglobin is released into the PBS buffer after cell membrane lysis and is not related to increased cell membrane permeability or the opening of large pores sufficient to release hemoglobin molecules.

SEM studies of rat erythrocyte morphology

To better understand the interaction of cationic lysine-based surfactants with the lipid bilayer, we assessed

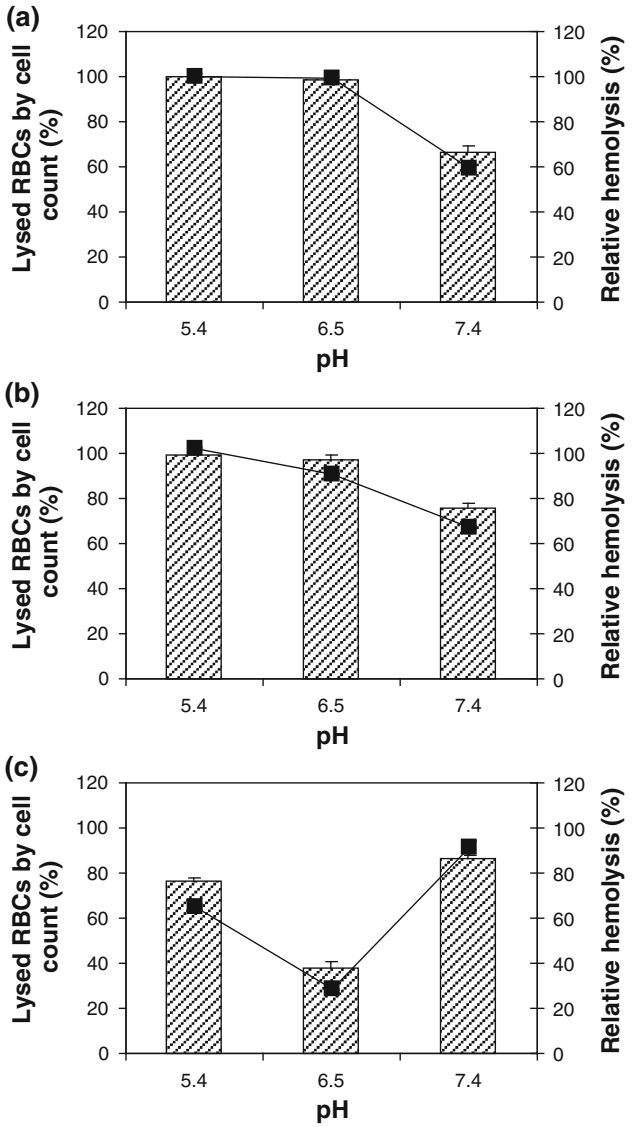

Fig. 6 Comparison between the hemolysis (*line*) and the percentage of lysed rat erythrocytes (*columns*) caused by the cationic lysine-based surfactants at the pH range tested. Surfactants were added at the following range of final concentrations **a** 500 μg/ml MKM, **b** 500 μg/ml PKM, and **c** 60 μg/ml MLM. The data represent the mean of three independent experiments ± SEM (*error bars*)

morphological changes produced in rat erythrocytes by SEM after treatment with the surfactants at the sub-lytic concentration of 10 μg/ml at pH ranging from 5.4 to 7.4. The results indicated that the surfactants interacted with the lipid bilayer and altered the normal biconcave morphology of the cells (Fig. 7). Control erythrocytes incubated in PBS solution at the pH values studied (7.4, 6.5, and 5.4) were also evaluated and found to be discoid or slightly echinocytic (Fig. 7a). This shape is considered normal in erythrocytes isolated in buffer and in the absence of albumin (Dubnicková et al. 2000; Rasia et al. 2007).

Despite varying membrane lytic activity of MKM and MLM, erythrocytes underwent similar morphological alterations after treatment with these surfactants throughout the pH range studied, 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 (Fig. 7b, e, respectively), producing a cup-shaped form. This morphological change was induced when the compound was inserted into the inner monolayer of the membrane, expanding it relative to the outer layer. Our results are consistent with the bilayer hypothesis (Sheetz and Singer 1974), which proposes that cationic amphiphiles induce stomatocytes at physiological pH as a result of the electrostatic attraction between the positive polar head group of the molecule and the acidic phospholipids, the latter having negative charges under physiological conditions and localizing in the inner layer of the lipid bilayer. Furthermore, in mildly acidic media (pH 6.5), these two surfactants induced a spherostomatocyte-type deformation (Fig. 7c, f, respectively). The spherostomatocytes are formed from stomatocytes increasing the incorporation of compounds into the membrane, and are considered to be the previous stage before transformation to spherocytes. Finally, at pH 5.4, the cells displayed swollen forms (spherocytes), which can be assumed to be the last stage of the morphological change with maximum accumulation of the compound at the membrane before cell lysis (Fig. 7d, g, respectively). This increased accumulation at both pH 6.5 and 5.4 supports the significantly increased membrane lytic activity of MKM, since more interactions of the surfactants with the cell membrane could enhance hemolysis (Nogueira et al. 2011). In contrast, the higher incorporation of MLM into the erythrocyte membrane did not reflect increased hemolysis, suggesting that the accumulation of this compound in the lipid bilayer may play a protective role against hemolysis.

In contrast, PKM (with a longer alkyl chain) interacted with the phospholipid bilayer in a slightly different way. At physiological pH, it induced a leptocyte-type deformation (Fig. 7h), with the erythrocyte having a larger surface area than normal cells. This morphological change could be attributed to the higher incorporation of this surfactant into the lipid bilayer, thus giving an appearance of "excess" plasma membrane. A possible explanation is that the most common phospholipid in the bilayer is 16–18 carbons long, which could favor the incorporation of this compound (with 16 carbon atoms) into the membrane (Martínez et al. 2007). This hypothesis of increased incorporation was not reflected in its hemolytic activity, possibly because this surfactant may rearrange the lipid bilayer and thus exert a protective effect at low concentrations. At pH 6.5, PKM also induced stomatocytes (Fig. 7i), but at an earlier stage

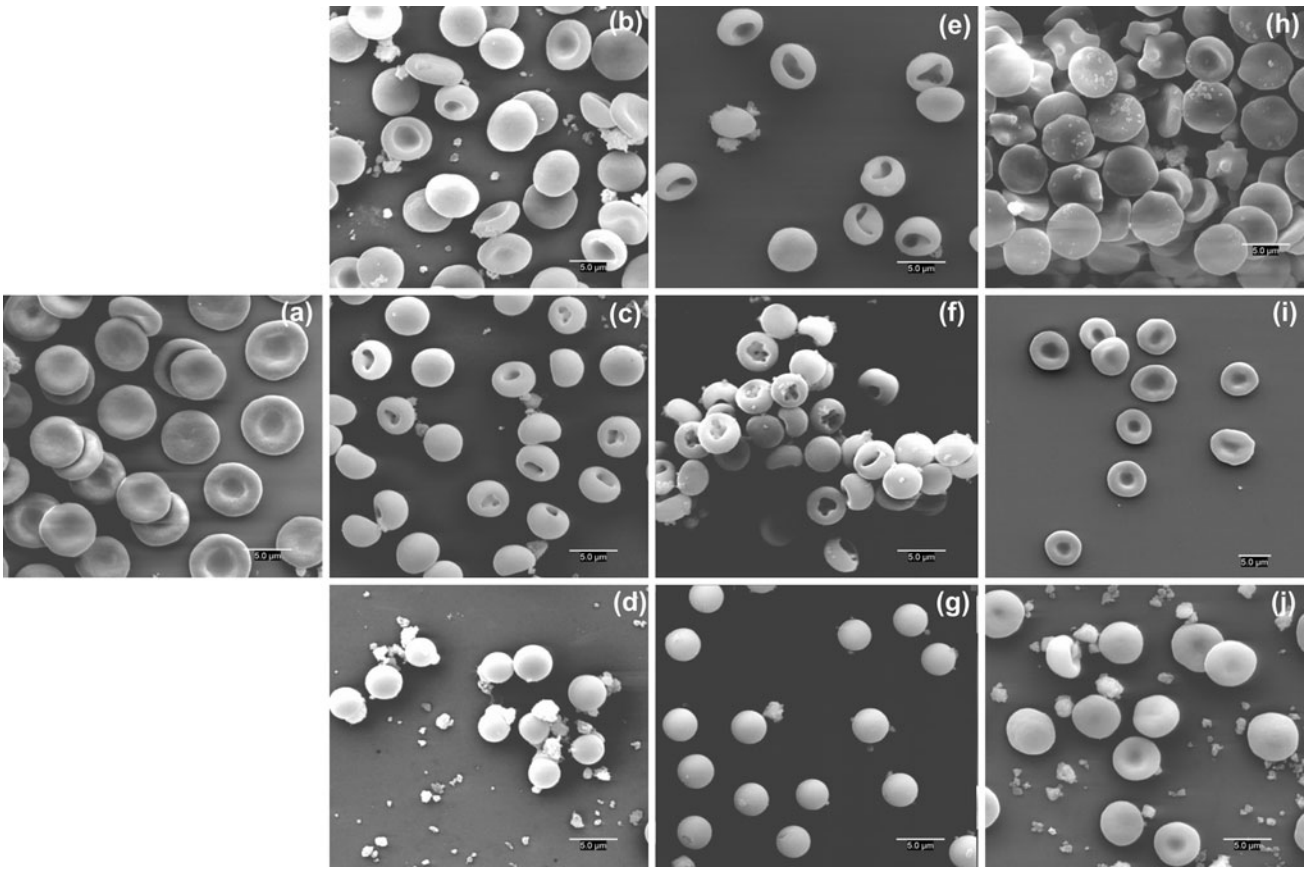

Fig. 7 Effect of the cationic lysine-based surfactants on rat erythrocyte morphology. SEM images of **a** control in PBS pH 7.4, and after incubation with the surfactants: MKM at **b** pH 7.4, **c** pH 6.5 and **d** pH 5.4; MLM at **e** pH 7.4, **f** pH 6.5 and **g** pH 5.4; and PKM at **h** pH 7.4, **i** pH 6.5 and **j** pH 5.4. The erythrocytes were incubated for 10 min at a concentration of 10 μg/ml of each surfactant. *Scale bars* correspond to 5 μm

than that observed with MKM and MLM. The less pronounced stomatocyte-type of deformation was consistent with the lower hemolytic activity of PKM at mildly acidic conditions. At the pH range of late endosomes (pH 5.4), PKM caused spherocytes and some spherostomatocytes (Fig. 7j), which could also explain the significant rise in its membrane lytic activity (see discussion above).

The same set of experiments was carried out across the pH range of 5.4–7.4 in the presence of PEG 10,000, an osmotic protectant against surfactant-induced hemolysis. We observed that PEG only partially protected against cell lysis (generally less than 50% protection), which is in agreement with the effects observed on the morphology of erythrocytes (data not shown). PEG did not recover the initial discocyte shape and only small changes were observed at pH 5.4 from spherocytes to stomatocytes for PKM and MKM, and at pH 7.4 from leptocytes to an early stage of stomatocytes for PKM. Therefore, no clear protective role of PEG was observed, corroborating the fact that osmotic cell swelling is not the only mechanism involved in the surfactant's membrane-disruptive activity. In contrast to our results, some authors have reported that the presence of PEG induced significant changes in cell morphology (Zaragoza et al. 2010).

The overall results showed that in addition to the physicochemical characteristics of the surfactants, differences in the transbilayer distribution and mobility are crucial for hemolytic potency. Of note, the increase in positive charges at the lipid bilayer due to the distribution of the surfactants might modify phospholipid packing, which determines the correct function of the membrane. This observation may also explain the changes in the normal biconcave shape of the red blood cells (Manrique-Moreno et al. 2010).

Conclusions

The membrane-disruptive activity of novel cationic lysine-based surfactants was assessed using erythrocytes as a model of an endosomal membrane. Although all the surfactants enhanced hemolysis with increasing concentrations, only the compounds with the positive charge on the α-amino group of lysine (MKM and PKM) showed

pH-responsive hemolytic activity. Moreover, an increase in the alkyl chain length from 14 to 16 carbon atoms lowered the ability to disrupt cell membranes. Hence, the pH-dependent membrane-disruptive activity of these surfactants can be fine-tuned mainly by varying the position of the cationic charge and, to a lesser extent, by altering the length of the alkyl chain. The overall hemolysis results suggest that MKM and PKM might achieve maximum membrane lytic activity in the late endosomes, and the improved hemolytic kinetics demonstrate their ability to disrupt endosomal membranes before vesicular evolution from endosomes to lysosomes. The partial decrease in the rate of hemolysis by PEG-10,000 implies that osmotic cell swelling is not the only mechanism involved in membrane lysis and that a more complex mechanism may operate. SEM studies on rat erythrocytes showed that the surfactants interacted with the phospholipid bilayer and induced shape changes, forming mainly stomatocytes at pH 7.4 and 6.5, and spherocytes at pH 5.4. Based on our results, we conclude that the biocompatible surfactants MKM and PKM, but not MLM, have potential applications as a new class of bioactive excipients in drug delivery systems. These insights into the membrane-disruptive properties and mechanisms by which pH-sensitive surfactants facilitate the delivery of membrane-impermeant molecules into the cell cytoplasm may help in the design of specific endosome-destabilizing compounds. Furthermore, knowledge on the modulation of the physicochemical properties of novel surfactants may lead to the development of many more efficient amino acid-based drug carriers. Current studies are focusing on the development of lysine-based surfactant conjugates as potential biocompatible drug delivery systems for pharmaceutical applications.

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Conflict of interest The authors declare that they have no conflict of interest.

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