The role of counterions in the membrane-disruptive properties of pH-sensitive lysine-based surfactants

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ABSTRACT

Surfactants are among the most versatile and widely used excipients in pharmaceuticals. This versatility, together with their pH-responsive membrane-disruptive activity and low toxicity, could also enable their potential application in drug delivery systems. Here we studied five anionic lysine-based surfactants that differ in the nature of their counterion. We examined their capacity to disrupt the cell membrane under a range of pH values, concentrations and incubation times using a standard hemolysis assay as a model for endosomal membranes. The surfactants showed pH-sensitive hemolytic activity and improved kinetics at the endosomal pH range. Low concentrations resulted in negligible hemolysis at physiological pH and high membrane lytic activity at pH 5.4, which is in the range characteristic of late endosomes. With increasing concentration, the surfactants showed an enhanced capacity to lyse cell membranes, and also caused significant membrane disruption at physiological pH. This observation indicates that at high concentrations surfactant behavior is independent of pH. We addressed the mechanism of surfactant-mediated membrane destabilization, and also performed scanning electron microscopy studies to evaluate the effects of the compounds on erythrocyte morphology as a function of pH. The *in vitro* cytotoxicity of the surfactants was assessed by MTT and NRU assays with the 3T3 cell line. The influence of different types of counterion on hemolytic activity and the potential applications of these surfactants in drug delivery are discussed. The possibility of using pH-sensitive surfactants for endosome disruption could hold great promise for intracellular drug delivery systems in future therapeutic applications.

Keywords: Hemolysis; pH-sensitivity; membrane disruption; cytotoxicity; drug delivery

1. Introduction

Many therapeutic agents, such as proteins, peptides, DNA, and some drugs, act at intracellular sites, and thus their therapeutic efficacy depends on efficient intracellular trafficking pathways [1]. One of the challenges for the efficient intracellular delivery of therapeutic compounds is to manipulate or circumvent the non-productive trafficking from endosomes to lysosomes, where degradation may occur. This would allow delivery systems to escape endosomal compartments and consequently facilitate subsequent drug release into cytoplasm after internalization of the systems through endocytosis [2].

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 cytoplasm; however clinical use of these carriers is potentially limited by their antigenicity and toxicity, and the difficulties of large-scale production [3-5]. Safety issues have prompted the development of synthetic peptides structurally derived from viruses to specifically disrupt endosomal membranes [1]. However these peptides are also likely to be immunogenic *in vivo* [6,7]. To circumvent these problems, a variety of non-viral delivery vectors have been developed, such as synthetic polymers and surfactants. Polymerizable surfactants with tunable pH-sensitive amphiphilicity have recently been designed. These allow carriers to change their amphiphilic structure at endosomal-lysosomal pH, which results in the disruption of endosomal-lysosomal membranes [8,9]. The functionalization of one of these polymerizable pH-sensitive amphiphilic surfactants for the preparation of a peptide-directed siRNA delivery system has also been reported [10]. Moreover, cationic amino acid-based surfactants have been used to prepare novel biocompatible devices for the controlled encapsulation and release of DNA [11]. Cationic and anionic polymers with pH-sensitive activity, including non-biodegradable polymers and biodegradable poly(amino acid)s and pseudo-peptides

[2,7,12-18], have also been developed to promote endosomal escape. Cationic compounds have commonly been used to form stable cationic complexes; however, they show cytotoxicity and non-specifically adsorb serum proteins, thereby leading to rapid blood clearance as a result of the strong cationic surface charge [19-21]. In contrast, anionic pH-responsive polymers are considered of interest as drug carriers because they mimic the structure and pH-dependent membrane-lytic behavior of endosomolytic viral peptides [1]. Moreover, recharging cationic complexes with anionic compounds has been reported as a promising method to overcome the adverse effects of cationic complexes [22].

Considerable research effort is devoted to delivery systems that specifically destabilize endosomal membranes in mildly acidic conditions following endocytic uptake [23]. In this context, amino acid-based surfactants with pH-sensitive activity and low toxicity deserve particular attention and could be a promising choice for application in non-viral drug delivery systems. Here we selected N^{α},N^{ε} –dioctanoyl lysine derivatives, a class of amino acid-based surfactants synthesized as lecithin analogs, since homologs with eight carbon atom chains are the least hemolytic and show the least irritant activity, thus proving the most suitable for practical applications [24]. Moreover, earlier studies by our group demonstrated the biocompatibility and low *in vitro* toxicity of this series of anionic lysine-based surfactants [25-28].

Here we studied the membrane lytic properties as a function of pH of five anionic lysine-based surfactants differing in the nature of their counterion. To evaluate the potential applications in cytoplasmic delivery carriers, we examined the pH-sensitive cell membrane disruptive activity of these compounds using a standard hemolysis assay of rat erythrocytes at a range of pH values as a model for endosomal membranes. The mechanism involved in cell membrane disruption, the kinetic properties of each surfactant in the endosomal pH range, and their effects on erythrocyte morphology as a function of

pH are also presented, together with *in vitro* cytotoxicity assays in the 3T3 fibroblast cell line. Furthermore, to gain insight into the structure-dependent interaction of these compounds with membrane bilayers, the influence of the surfactant structure and counterions on hemolytic activity is also discussed.

2. Materials and Methods

2.1. Materials

L-lysine monohydrochloride, L-lysine, Tris, the bases NaOH, LiOH and KOH, sodium dodecyl sulfate (SDS), glutaraldehyde, NaCl, Na_2HPO_4 and KH_2PO_4 were purchased from Merck (Darmstadt, Germany). PEG-10,000, $_D$-glucose, dimethyl sulfoxide (DMSO), 2,5-diphenyl-3,-(4,5-dimethyl-2-thiazolyl) tetrazolium bromide (MTT) and neutral red dye (NR) were from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco´s modified Eagle´s medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), L-glutamine solution (200 mM), trypsin-EDTA solution (170,000 U l^{-1} trypsin and 0.2 g l^{-1} EDTA) and penicillin-streptomycin solution (10,000 U ml^{-1} penicillin and 10 mg ml^{-1} streptomycin) were obtained from Lonza (Verviers, Belgium). The 75 cm^2 flasks and 96-well plates were obtained from TPP (Trasadingen, Switzerland).

2.2. Surfactants tested

Five anionic amino acid-based surfactants derived from N^{α},N^{ε} –dioctanoyl lysine and with counterions of distinct chemical nature were evaluated: two salts with organic counterions - lysine salt (77KK) and tris(hydroxymethyl) aminomethane salt (77KT); and three salts with inorganic counterions - sodium salt (77KS), lithium salt (77KL) and potassium salt (77KP) (Fig. 1). These surfactants were synthesized in our laboratory as

previously described [25,29]. The commercial anionic surfactant sodium dodecyl sulfate (SDS) was used as reference compound.

2.3. Preparation of red blood cells suspensions

Rat blood was obtained from anaesthetized 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 ºC for 10 min, and washed three times in an isotonic phosphate buffer solution (PBS) containing 123.3 mM NaCl, 22.2 mM Na_2HPO_4 and 5.6 mM KH_2PO_4 in distilled water (pH 7.4; 300 mOsmol l^{-1}). The cell pellets were then suspended in PBS solution at a cell density of 8 x 10^9 cell ml^{-1}.

2.4. Hemolysis assay

The membrane-lytic activity of the surfactants was examined by hemolysis assay. PBS buffers in the pH range of 5.4 – 8.0 were prepared to be isosmotic to the inside of the erythrocyte and cause negligible hemolysis. The 25-µl aliquots of erythrocyte suspension were exposed to various concentrations (from 100 to 800 µg ml^{-1}) of the surfactants dissolved in PBS solution in a total volume of 1 ml. Two controls were prepared by resuspending erythrocyte suspension either in buffer alone (negative control) or in distilled water (positive control). The samples were incubated at room temperature under constant shaking for various periods up to 90 min, and then centrifuged at 10,000 rpm for 5 min. Supernatants were taken, the absorbance of the hemoglobin release 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 totally hemolyzed with distilled water. Dose-response curves were obtained from hemolysis results and the concentrations inducing 50% hemolysis (HC_{50}) were calculated.

2.5. Mechanism of cell membrane disruption

2.5.1. Osmotic Protection Experiment

To determine the effect of osmolytes with diverse molecular weights on the hemolytic activity of the surfactants, PBS buffers at the pH range studied were prepared by adding $_D$-glucose (180 Da) and PEG 10,000 (10 kDa) separately at a concentration of 10 mM, which is below the range at which these molecules alone induce hemolysis as a result of osmotic pressures [19]. Red blood cells were incubated with these buffers and exposed to a concentration of each surfactant that achieved approximately 100% hemolysis in PBS buffer alone (400 – 800 μg ml^{-1}). Hemolysis was determined after incubating the cells for 10 min at room temperature, following the procedure described above.

2.5.2. Red blood cell count

Red blood cells 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 (400 – 800 μg ml^{-1}) were determined relative to the total cell number in PBS buffer alone. The hemolysis experiment was performed following the above procedure at the same pH range, and each sample was diluted four times for cell counts.

2.6. Scanning Electron Microscopy (SEM) studies of rat erythrocytes morphology

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 (100 µg ml^{-1}) of each surfactant. After 10-min incubation, samples were fixed by adding 1 ml of 2.5% glutaraldehyde in PBS solution and incubation at 4 ºC 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 over 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 in an SEM coating system SC 510 (Fisons Instruments, East Grinstead, UK). Resulting specimens were examined in a Zeiss DSM 940A scanning electron microscope (Carl Zeiss SMT AG, Jena, Germany).

2.7. Cell culture

The murine Swiss albino 3T3 fibroblast cell line was grown in DMEM medium (4.5 g l^{-1} glucose) supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin at 37 ºC, 5% CO_2. The 3T3 cells were routinely cultured in 75 cm^2 culture flasks and were trypsinized using trypsin-EDTA when the cells reached approximately 80% confluence.

2.8. Cytotoxicity assays

The cytotoxic effect of the surfactants was measured by tetrazolium salt MTT assay [30] and neutral red uptake (NRU) assay [31]. 3T3 cells were seeded into the central 60 wells of a 96-well plate at a density of 8.5 x 10^4 cells ml^{-1}. After incubation for 24 h under

5% CO_2 at 37 ºC, the spent medium was replaced with 100 µl of fresh medium supplemented with 5% FBS containing 0.22-µm filter-sterilized surfactant solution at the required concentration range (150-300 µg ml^{-1}). After 24 h, the surfactant-containing medium was removed, and 100 µl of MTT in PBS (5 mg ml^{-1}) diluted 1:10 in medium without FBS and phenol red was then added to the cells. Similarly, 100 µl of 50 µg ml^{-1} NR solution in DMEM without FBS and phenol red was added in each well for the NRU assay. The plates were further incubated for 3 h, after which the medium was removed, and the cells were washed once in PBS. Thereafter, 100 µl of DMSO was then added to each well to dissolve the purple formazan product (MTT assay). Likewise, for the NRU assay 100 µl of a solution containing 50% ethanol absolute and 1% acetic acid in distilled water was added to extract the dye. After 10 min on a microtitre-plate shaker at room temperature, the absorbance of the resulting solutions was measured at 550 nm using a Bio-Rad 550 microplate reader. The effect of each treatment was calculated as a percentage of cell viability inhibition against the respective controls.

2.9. Statistical analysis

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 (S.E.M.). Statistical analyses were performed using one-way analysis of variance (ANOVA) to determine the difference between the sets of data, followed by Bonferroni's or Dunnett's *posthoc* tests for multiple comparisons, as indicated, using the SPSS® software (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered to denote significance.

3. Results and discussion

3.1. Concentration-dependent hemolysis

The hemolytic activity of the lysine surfactants at pH 8.0, 7.4, 6.5 and 5.4 was determined as a function of concentration, and the concentration-dependent curves at pH 7.4 (physiological) and 5.4 (pH at late endosomes) are shown in Fig. 2. In this experiment, hemolysis was determined at a fixed time (after 10 min of incubation) in the presence of a range of surfactant concentrations, which were defined on the basis of the hemolytic potency of each surfactant [26]. At pH 7.4 (Fig. 2a), all the surfactants studied were non-hemolytic or displayed low hemolysis throughout the first points of the concentration curves; however, they exhibited a gradually stronger disruption of membranes as concentration increased, reaching complete hemolysis in the later points of the hemolytic curve. Similar hemolytic activities were obtained at pH 8.0 and 6.5, with only a small shift of the lysis curve to the left for pH 6.5 and to the right for pH 8.0 with respect to that registered at pH 7.4. These observations indicate increased and reduced activity, respectively (data not shown). These profiles show that hemolysis is a relatively complex process, with an initial lag period in all the curves. In contrast, at pH 5.4 (Fig. 2b) the lag period was not observed and the concentration required to reach 100% hemolysis was considerably lower than that needed at the other pH values tested. Raising concentrations from 100 to 250 μg ml^{-1} for 77KP and 77KS, from 100 to 300 μg ml^{-1} for 77KK and 77KL, and from 100 to 400 μg ml^{-1} for 77KT caused complete disruption of erythrocyte membranes. Our results show that the hemolytic activity of all the compounds was strongly influenced by concentration. Likewise, several authors [18,32,33] have reported similar results for the membrane-lytic activity of the polymers poly(α-ethylacrylic acid) and poly($_L$-lysine iso-phthalamide), and for arginine-based gemini surfactants,

respectively. In the case of polymers, those authors argued that increasing compound concentration enhances the migration of the polymer molecules to lipid bilayer membranes, thereby resulting in an increase in the membrane destabilization. Moreover, it is widely reported that surfactants at high concentrations are hemolytic, and membrane solubilization is often observed [34,35].

At pH 5.4, the surfactants with an inorganic counterion were more hemolytic than the surfactants with an organic counterion, and the HC_{50} values showed statistically significant differences ($p<0.05$) in comparison with the other pH values tested (Table 1). All the HC_{50} values were below the critical micellar concentration (CMC) of each surfactant [28], thereby indicating that the monomers are responsible for the hemolytic activity, and that the presence of micelles is not a prerequisite for surfactant-mediated cell lysis.

3.2. pH-dependent hemolysis

Here we studied the capacity of amino acid-based surfactants with distinct counterions to disrupt lipid bilayer membranes. For this purpose, we used a hemolysis assay with the erythrocyte membrane serving as a model for the endosomal membrane [8,17]. The hemolytic activity of the surfactants was studied as a function of pH over a range of concentrations (100 to 800 μg ml^{-1}) depending on the product tested. At low concentrations (below the HC_{50} determined at pH 7.4 for each surfactant), the surfactants showed negligible hemolytic activity at pH 8.0 and 7.4 (Fig. 3a). When the pH was decreased to 6.5, the surfactants were also almost non-hemolytic, resulting in a maximum hemolysis value of 5.57%. At pH 5.4, the hemolytic activity of the surfactants increased considerably ($p<0.05$), reaching maxima of between 65.31% and 84.69%, with the following ranking for the HC_{50} values: 77KT < 77KK < 77KS < 77KP < 77KL. Endosomal

compartments have a pH of between 5.5 to 6.8, while the pH in the lysosome is about 5.0 [2,21,36]. Therefore, this pH-responsive membrane disruption around pH 5.4, which is in the pH range characteristic of late endosomes, is favorable for intracellular trafficking of therapeutic compounds. Lytic activity at the level of late endosomes should facilitate membrane destabilization, and allow surfactant-drug complexes to escape to the cytoplasm for efficient intracellular drug delivery. Gene delivery vehicles that exhibit specific membrane lytic activity at pH 5.5 have higher transfection efficiency than vehicles that destabilize membranes at pH 6.0-6.5 [21]. Moreover, surfactants with high hemolytic activity at pH 5.4 gave the best siRNA delivery efficiency in U87-luc cells [8]. Furthermore, it was observed that the commercial surfactant SDS showed no pH-sensitive activity, and thus did not facilitate endosomal destabilization.

At concentrations in the range of HC_{50}, surfactants presented higher hemolytic activity at pH 8.0 and 7.4, with a concomitant marked rise at pH 6.5, where almost total hemolysis was recorded, demonstrating that at high concentrations the surfactants do not retain pH-responsive behavior (Fig. 3b). These findings show that an increased in surfactant concentrations also caused membrane disruption within the pH range characteristic of early endosomes. However, these concentrations may present toxic effects, as determined by the cytotoxicity assays performed in the 3T3 fibroblast cell line (data shown in section 3.6), and also as reported in previous cytotoxicity studies in 3T6 fibroblasts and NCTC 2544 keratinocyte cell lines [25].

A strong correlation has been reported between hemolytic activity and endosomal disruption by membrane-disruptive agents [6]. The lysine-based anionic surfactants with pH-sensitive properties were studied on the basis of the hypothesis that pH-responsive membrane lytic activity facilitates the escape of drug-delivery systems from endosomal compartments. Numerous amphiphilic materials have been found to mediate material

transport across cell membranes, such as amphiphilic peptide sequences used by viruses [37], and amphiphilic lipids, such as DOPE [38]. However, virus peptides may have problems of immunogenicity, and lipids may not have the capacity of selective membrane disruption at the endosomal pH. A useful intracellular drug delivery system is required to have low lytic activity at physiological pH and high destabilizing activity in the mildly acidic conditions found in the endosomes, which will cause only selective endosomal membrane disruption [8,15].

Noteworthy is the significant increase in the hemolytic activity observed at pH 5.4 for all the surfactants independently of their counterions. This increase could be explained by a modification in the hydrophobic/hydrophilic balance of these compounds at this pH. The pKa value of the carboxylic group of the lysine amino acid is reported to be of the order of 2.2 [39] and, therefore, it was initially assumed that in the pH range tested, there would be no changes in the protonation of this group nor in the hydrophobicity of the molecule. However, taking into account the enhanced membrane lysis at pH 5.4, it was assumed that the carboxylic group of the amino acid lysine undergoes an increase in its pKa value when it is included in the surfactant molecule. This increase could lead to changes in the protonation state of the surfactants at the late endosome pH range, and thus further increase binding to the membrane and enhance the hemolytic activity. This supposition is based on the studies by Pinazo et al. [40], in which the guanidine group of the amino acid arginine was observed to undergo a strong alteration in its original pKa value when included in the surfactant molecule. Moreover, the enhanced hemolysis at pH 5.4 could also be attributed to modifications in the surface potential of the lipid bilayer after cell exposure to low pH [41]. These altered properties could enhance the interaction of the surfactants with such a modified bilayer.

Moreover, compounds containing counterions differ in their capacity to interact with biological and model membranes [42], and the type of counterion is one of the factors that determine the efficiency of the interaction of a compound with membranes [43,44]. Counterions within the polar domains of phospholipids have been shown to modulate lipid dynamics in numerous models [45,46]. Therefore, given that the lysine surfactants have the same chemical structure, differing only in the type of counterion, the characteristics of the counterion may be crucial to the pH-sensitive membrane disruptive activity. Hemolysis studies of several surfactants reported that hemolytic effects are dependent both on the structure (polar head dimension and alkyl chain length of compound), and form (the kind of the counterion) of the compound [47]. As demonstrated above, the inorganic counterions facilitated the interaction with the membrane and produced stronger hemolysis at the late endosome pH range. The difference in the effects of the surfactants with inorganic counterions with same valence could be explained by their different binding to charged surfaces resulting from their distinct polarizability or hydration and mobility [44]. Among the surfactants with organic counterions, the higher hemolytic activity of 77KK could be attributed to the presence of two protonatable amino groups in the lysinium counterion compared to only one in the Tris counterion, which could favor the amphiphilicity of this compound upon reduction of pH below the pKa ranges. When more amino groups are protonated, the surfactants become more amphiphilic, which results in stronger hemolysis [8,10]. Thus it has been demonstrated that the pH-sensitive membrane disruptive activity of these surfactants can be tuned by varying the nature of the counterion.

3.3. Time-dependent hemolysis

Induction of hemolysis by the surfactants was also determined as a function of time at the pH range characteristic of early and late endosomes (pH 6.5 and 5.4, respectively). The concentrations evaluated (200 to 500 μg ml^{-1}) were in the range of those that present specific pH-dependent activity, and were slightly adjusted to achieve time-dependent hemolysis. All the surfactants showed improved kinetic hemolytic activity in the endosomal pH range (Fig.4). At pH 6.5 (Fig. 4a), the presence of the compounds caused a low degree of hemolysis up to 17% after 10 min of incubation, followed by a sharp increase in the degree of hemolysis to a maximum between 56% and 100% after 90 min. At pH 5.4 these concentrations resulted in high degree of hemolysis after 10 min of incubation. As it was not possible to evaluate the time-dependent hemolysis with the same concentration range, we assessed low concentrations (150 to 200 μg ml^{-1}) (Fig. 4b). Despite the reduced kinetic hemolytic activity, time-dependent hemolysis was also observed, reaching a maximum (between 37% and 82%) after 90 min of incubation. This significant hemolytic kinetics indicates that one or more previous steps are required before the erythrocyte membrane becomes permeable to hemoglobin. Among these previous steps, the most important could be the formation of pores or channels, which lead to the efflux of low molecular weight solutes, as discussed in the section 3.4.1. The commercial surfactant SDS did not show a significant time-dependent hemolysis in the endosomal pH range. This observation thus confirms the non pH-responsive activity of this surfactant.

The molecules taken up by endocytosis are trafficked from early endosomes to lysosomes within several hours [16]. Thus, given the improved kinetics of the hemolytic activity shown by the lysine-based surfactants, these compounds could enable the disruption of 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.

3.4. Mechanism of cell membrane disruption

3.4.1. Osmotic Protection

Two processes have been proposed to explain cell lysis by surfactants. Hemolysis may be caused by direct disruption of the membrane through complete or partial solubilization of membrane lipids and proteins by the formation of mixed micelles (which usually occurs at high surfactant/membrane ratios), or by the intercalation of the compounds into the membrane and consequently the change in its permeability (which is characteristic of low surfactant concentrations, under the CMC) [48]. As the concentration range studied here was below the surfactant CMC [28], it was assumed that cell lysis and hemoglobin leakage could occurred through the colloid osmotic mechanism. To corroborate this hypothesis, an osmotic protection experiment was conducted in suspensions of PBS solutions that contained molecules of varying sizes. The rationale behind this experiment was that since sufficiently large molecules do not permeate through the cell membrane, their presence would counteract the effect of the macromolecules inside the cell (specially hemoglobin) and diminish water penetration into it, and consequently, cell swelling [19].

PEG-10,000 significantly inhibited the hemolysis by the surfactants throughout the pH range studied, thereby indicating that defects such as pores or channels are formed in the erythrocyte membrane (Fig. 5). Collapse of transmembrane potential and osmotic swelling are consequences of pore formation and lead to red blood cell lysis [1]. The resulting osmotic imbalance was restored by adding high molecular weight PEG 10,000, which does not diffuse through the pores or channels formed in the membrane, thus leading to a marked decrease in the hemolysis. This decrease was considered evidence of the presence of the osmotic mechanism [49], and as a further indication that the membrane

lysis is a complex process and that one or more previous steps, such as pore formation and the efflux of low molecular weight solutes, are required before the erythrocyte membrane becomes permeable to hemoglobin. Corroborating the osmotic mechanism, the low molecular weight D-glucose had no effect on the osmotic imbalance caused by the hemoglobin trapped inside the erythrocyte cytoplasm, therefore no 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. Osmotic protection at pH 5.4 was less significant than at the other pH values tested (Fig. 5d). This observation indicates that in this case the osmotic cell swelling is not the only mechanism involved in the membrane lysis, and a more complex mechanism is operating. Given the enhanced hemolytic activity observed at pH 5.4, cell membrane disruption could also be due to partial solubilization of membrane lipids and proteins through micellization caused by extensive surfactant adsorption. Moreover, it is also feasible that the opening of large pores is sufficient to release hemoglobin molecules [49]. Previous studies on the commercial surfactant SDS suggested that this compound opens large pores sufficiently to allow hemoglobin release, or even that it can cause direct disruption of the membranes. Thus SDS does not present the colloid-osmotic mechanism of hemolysis [48,49].

3.4.2. Red blood cell count

Red blood cell counts were performed in order to study whether hemoglobin passes through the cell membrane, or whether it is released into the extracellular medium after the cell membrane rupture. It has been noted that the overall cell numbers were reduced significantly after 10 min of incubation with a hemolytic concentration of each surfactant throughout the pH range tested, and the percentages of lysed erythrocytes are in agreement with the hemolytic activity results (Fig. 6). These results indicate that hemoglobin is

released into the PBS buffer after lysis of cell membranes, and is not related to pore formation or increased cell membrane permeability.

3.5. Scanning Electron Microscopy (SEM) studies of rat erythrocyte morphology

The capacity of human erythrocytes to maintain their biconcave disk shape is governed by structural properties of the membrane [41]. A variety of phenomena can induce normal discocytes to undergo transformation to crenated (echinocytic) or cupped (stomatocytic) shapes, for example, ATP depletion, changes in pH, exposure to glass surfaces, Ca^{2+} loading, metabolic depletion, or incubation with certain amphiphilic compounds [50]. For a better understanding of the effect of the pH of lysine-based surfactants on the erythrocyte membrane structure and cell lysis, we performed SEM studies of rat erythrocytes. Erythrocytes were treated with the sub-lytic concentration of 100 μg ml^{-1} of the surfactants in the 5.4 – 8.0 pH range. The results indicated that the surfactants altered the normal biconcave morphology of the cells (Fig. 7). Control erythrocytes incubated in PBS solution at the pH values studied (8.0, 7.4, 6.5 and 5.4) were also evaluated and were found to be discoid or slightly echinocytic. This shape is considered normal in erythrocytes isolated in buffer and in the absence of albumin [51,52]. Figure 7a shows the control, which consisted of erythrocytes incubated with PBS alone at pH 7.4.

Regardless of the varying capacity of the surfactants to exert membrane lytic activity, both those with organic and those with inorganic counterions induced the same changes in erythrocyte morphology throughout the pH range studied. According to the bilayer couple hypothesis [50], the changes induced in erythrocytes shape by foreign molecules are due to the differential expansion of their two monolayers. The SEM micrographs shown in Fig.7 correspond to 77KK and 77KS, the surfactants with organic

and inorganic counterions, respectively. A morphological analysis revealed that the erythrocytes underwent alteration after treatment with these two surfactants at pH 7.4 (Fig. 7b and 7e, respectively). These compounds changed the discoid shape of cells (Fig. 7a) to echinocytic, an altered condition in which the erythrocytes show a spiny configuration, exhibiting blebs or protuberances on their surfaces. At pH 8.0, the compounds also induced an echninocyte type of deformation (data not shown). Echinocytes are induced when the compound added is inserted in the outer monolayer of the membrane [50]. This morphological change may be the result of the incapacity of the amphiphats to cross the bilayer. Our results are consistent with the bilayer hypothesis [50], which proposes that, general speaking, anionic amphiphiles induce echinocytes at physiological pH as a result of the electric repulsion between the negative charge of the molecule and the acidic phospholipids, the latter having negative charges under the physiological conditions and localizing in the inner layer of the lipid bilayer.

In contrast, in mildly acidic media, the surfactants interacted with the phospholipid bilayer in another way. At pH 6.5, they induced a stomatocyte-type deformation, causing the erythrocytes to show a cup-shaped form with evagination of one surface and a deep invagination of the opposite face. These morphological changes are shown in Fig. 7c and 7f, for 77KK and 77KS, respectively. Stomatocytes arise when the compound accumulates in the inner monolayer. This accumulation may support the slight increased hemolytic potency shown by the surfactants at this pH, given that a deeper penetration into the membrane took place. Finally, for 77KK and 77KS, respectively, at pH 5.4, most of the cells showed swollen forms (spherocytes), which comprised echinocytes and stomatocytes, by increasing the incorporation of the products into the membrane [50] (Fig. 7d and 7g). These findings indicate that these compounds were equally located in the outer and the inner moieties of the red cell membrane, supporting our findings regarding the

significantly increased hemolysis at pH 5.4, as a higher interaction (both in the outer and inner moieties of the bilayer) of the surfactants within the cell membrane could be associated with the enhanced hemolytic response. Moreover, one can assume that at mildly acidic condition the surface potential of the lipid bilayer is positive and favors the electrostatic attraction of the anionic compound, thus also inducing its intercalation in the inner layer of this modified bilayer [41].

Therefore, in addition to the physico-chemical characteristics of the surfactants, the difference in the transbilayer distribution and mobility are crucial for hemolytic potency. Of note, the increase in negative charges on the lipid bilayer as a result of the distribution of the surfactants may modify the electrostatic and hydration properties of the phospholipid molecules, which determine the correct function of the membrane. The location of the surfactants in the bilayer generates a reorganization of water molecules, which in turn affects phospholipid packing. These observations may also explain the changes in the normal biconcave shape of the red blood cells [53]. The hypothesis that the surfactants bind on the membrane surface was also confirmed by the results obtained by Martínez et al. [54] using fluorescence anisotropy measurements. That study demonstrated that almost all the lysine-based surfactants disturbed the external region of the erythrocyte membrane at physiological pH. In addition to the phospholipid bilayer interactions, surfactants may disrupt the structure of the cytoskeleton, a protein responsible for the maintenance of the biconcave shape of erythrocytes [55]. Finally, it has been proposed that the anion-exchange protein band 3 plays a critical role in determining erythrocyte shape [56,57]. Thus, surfactant-band 3 could be involved in the morphological changes undergone by the erythrocytes in response to surfactants.

3.6. Cytotoxicity assays

The cytotoxic effects of the lysine-based surfactants were evaluated with colorimetric assays that measure the capacity of live cells to take up the NR dye (NRU assay) and to metabolize a tetrazolium colourless salt to a blue formazan (MTT assay) as indirect measurements of cell viability. The cytotoxicity assays were performed in the concentration range in which the surfactants presented pH-sensitive membrane lytic activity (150 – 300 μg ml^{-1}). The compounds showed some cytotoxicity towards 3T3 cells, which displayed viability in the range of 96.64% to 46.44% by the MTT assay (Fig. 8a) and 107.61% to 16.93% by the NRU assay (fig. 8b) at the surfactant concentration range tested. We previously reported (section 3.2) the concentrations at which each surfactant presents specific pH-dependent activity (Fig. 3a). For 77KK and 77KL, these concentrations (250 and 200 μg ml^{-1}, respectively) induced only mild cytotoxicity (80.51% and 87.87% cell survival by the MTT assay, and 85.03% and 91.34% cell survival by the NRU assay, respectively), and thus could be considered suitable for drug delivery systems. The MTT assay showed that the surfactants 77KT, 77KS and 77KP presented more significant cytotoxic effects with cell viability falling below 70% within the concentrations that demonstrated marked pH-responsive activity. In contrast, by the NRU assay it was observed that 77KS and 77KP also displayed viabilities of ~ 85% with the 200 μg ml^{-1} concentration, while higher concentrations showed lower cell viabilities in comparison to the MTT assay. These results suggest different toxicity mechanisms for each surfactant as the two cytotoxicity assays are based on different endpoints. In order to avoid overestimation or underestimation of the toxicity, the combination of cytotoxicity assays are important to increase the reliability of the results.

4. Conclusions

The lysine-based surfactants showed concentration-dependent and pH-sensitive hemolytic activity, with a significant increase in the hemolysis at pH 5.4. The range of membrane-lytic profiles demonstrated that the counterions make a critical contribution to the interaction of these compounds with the phospholipid bilayer. The improved kinetics of the hemolytic activity of the surfactants supports their capacity to disrupt endosomal membranes before vesicular evolution from endosomes to lysosomes. Furthermore, the decrease in the rate of hemolysis by PEG 10,000 was considered evidence of the colloid osmotic mechanism in cell membrane disruption. SEM studies on rat erythrocytes showed that the surfactants interacted with the phospholipid bilayer to induce shape changes, forming echinocytes at pH 7.4, stomatocytes at pH 6.5, and spherocytes at pH 5.4. The surfactants 77KK and 77KL were also well tolerated by 3T3 cells at surfactant concentrations that present specific pH-dependent activity, and thus appear promising as endosomal disruptive agents with specificity for late endosome stages. This study on anionic lysine-based surfactants provides a foundation on which to further study the therapheutic applications of these lysine-based surfactants. On the basis of recent studies in our group that reported the preparation of biocompatible devices with amino acid-based surfactants for the controlled encapsulation and release of DNA [11], our research is now focused on the development of biocompatible lysine-based surfactants conjugates as potential drug delivery systems for pharmaceutical applications.

Acknowledgements

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Figure captions:

Fig. 1. Molecular structure of lysine-based anionic surfactants with the different counterions. The codes P, L, S, K, T represent potassium, lithium, sodium, lysine and Tris, respectively.

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

Fig. 3. Surfactant-induced hemoglobin release from rat erythrocytes as a function of pH. Surfactants were added from 10 mg ml^{-1} or 1 mg ml^{-1} (SDS) stock solutions to give final concentrations of: **(a)** 77KK 250 µg ml^{-1}, 77KT 300 µg ml^{-1},77KP, 77KS and 77KL 200 µg ml^{-1}, or SDS 30 µg ml^{-1}; and **(b)** 77KK 400 µg ml^{-1}, 77KT 650 µg ml^{-1}, 77KP and 77KS 300 µg ml^{-1}, 77KL 450 µg ml^{-1}, or SDS 50 µg ml^{-1}. Each point represents the mean of three independent experiments ± S.E.M. (error bars).

Fig. 4. Time dependence of the lysine-based surfactant-induced hemolysis at pH 6.5 and 5.4. Surfactants were added at time zero from a 10 mg ml^{-1} or 1 mg ml^{-1} (SDS) stock solutions to give final concentrations of: **(a)** pH 6.5: 77KK and 77KL 300 µg ml^{-1}, 77KT 500 µg ml^{-1}, 77KP and 77KS 200 µg ml^{-1}, or SDS 20 µg ml^{-1}; and **(b)** pH 5.4: 77KK, 77KP, 77KS and 77KL 150 µg ml^{-1}, 77KT 200 µg ml^{-1}, or SDS 20 µg ml^{-1}. Each point represents the mean of three independent experiments ± S.E.M. (error bars).

Fig. 5. Effects of osmotic protectants PEG-10,000 (10 kDa) and $_{D}$-glucose (180 Da) on the lysine-based surfactant-induced hemolysis of rat erythrocytes. **(a)** pH 7.4; **(b)** pH 8.0; **(c)** pH 6.5; and **(d)** pH 5.4. PBS alone (striped), PBS + $_{D}$-glucose (blank) and PBS + PEG-10,000 (black). Surfactants were added at a range of final concentrations: 77KL 600 µg ml^{-1}, 77KS 400 µg ml^{-1}, 77KP 400 µg ml^{-1}, 77KK 600 µg ml^{-1}, and 77KT 800 µg ml^{-1}. The data represent the mean of three independent experiments. All the hemolysis results obtained in the presence of the osmolyte PEG 10,000 were significantly different from the control in PBS solution alone (Dunnett's *posthoc* test, $p < 0.05$). The data represent the mean of three independent experiments ± S.E.M. (error bars).

Fig. 6. Comparison between the hemolysis (line) and the percentage of lysed rat erythrocytes (column) by the lysine-based surfactants at the pH range tested. Surfactants

were added at the following range of final concentrations: (a) 77KK 600 μg ml^{-1}, (b) 77KT 800 μg ml^{-1}, (c) 77KL 600 μg ml^{-1}, (d) 77KP 400 μg ml^{-1}, and (e) 77KS 400 μg ml^{-1}. The data represent the mean of three independent experiments ± S.E.M. (error bars).

Fig. 7. Effect of the lysine-based surfactants on rat erythrocytes morphology. SEM images of control in PBS pH 7.4 (a), and incubated with the surfactant with organic counterion 77KK at pH 7.4 (b), pH 6.5 (c) and pH 5.4 (d); and with the surfactant with inorganic counterion 77KS at pH 7.4 (e), pH 6.5 (f) and pH 5.4 (g). The erythrocytes were incubated for 10 min with a concentration of 100 μg ml^{-1} of each surfactant. Scale bars correspond to 5μm.

Fig. 8. Surfactant cytotoxicity on 3T3 cells as a function of concentration, as determined by MTT (a) and NRU (b) colorimetric assays at (from left to right) 150 μg ml^{-1} (blank), 200 μg ml^{-1} (striped), 250 μg ml^{-1} (black), and 300 μg ml^{-1} (squared). The data represent the mean of three independent experiments ± S.E.M. (error bars).

Figure(s)

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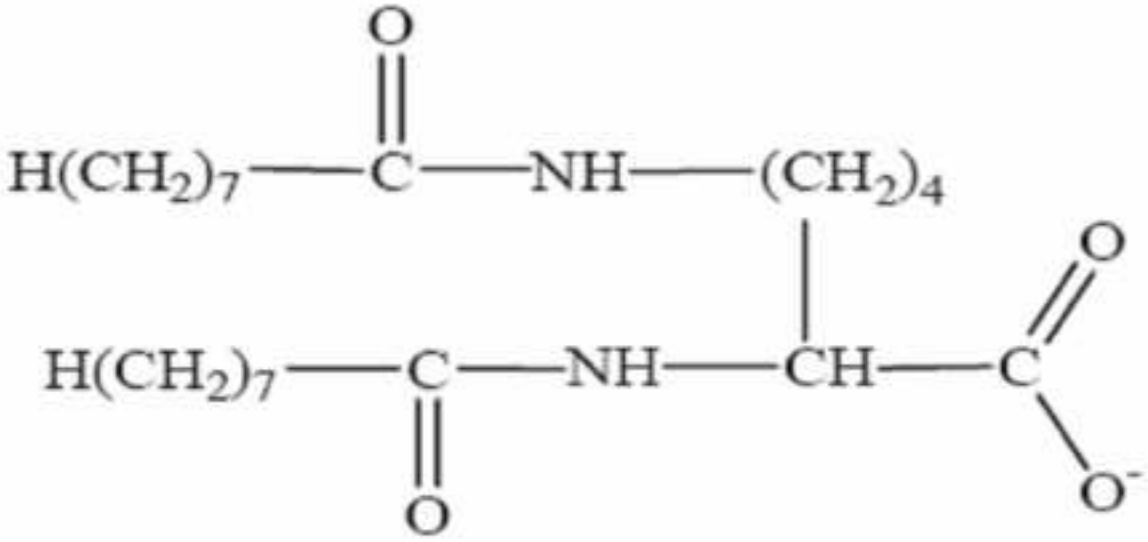

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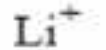

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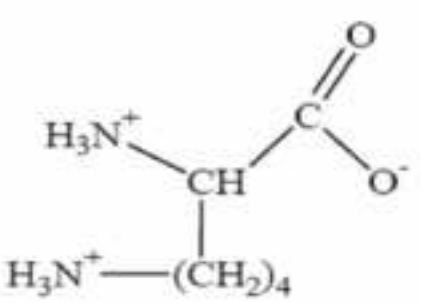

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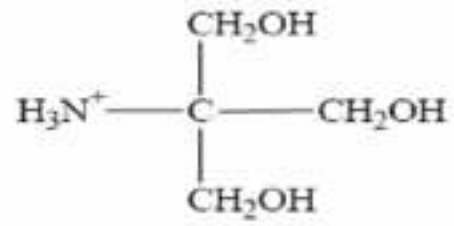

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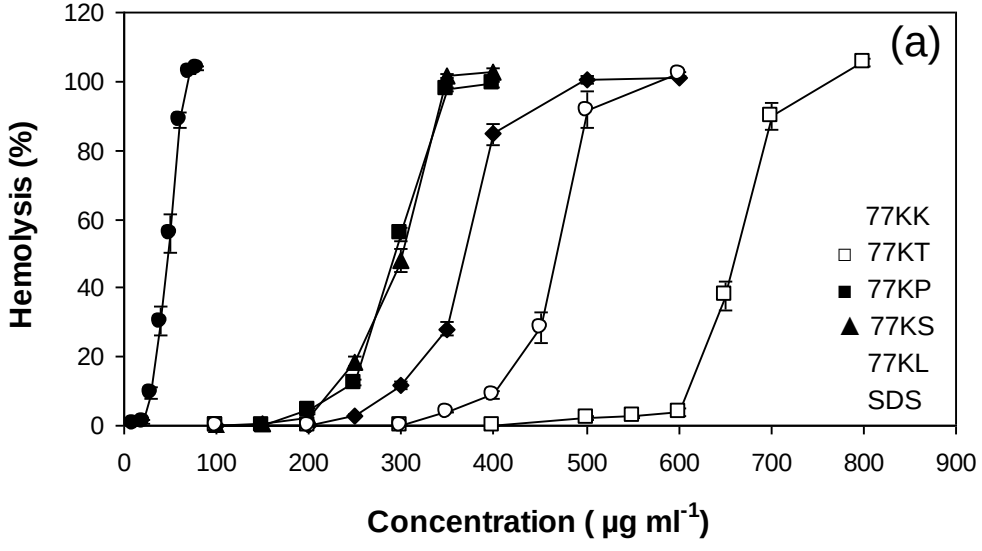
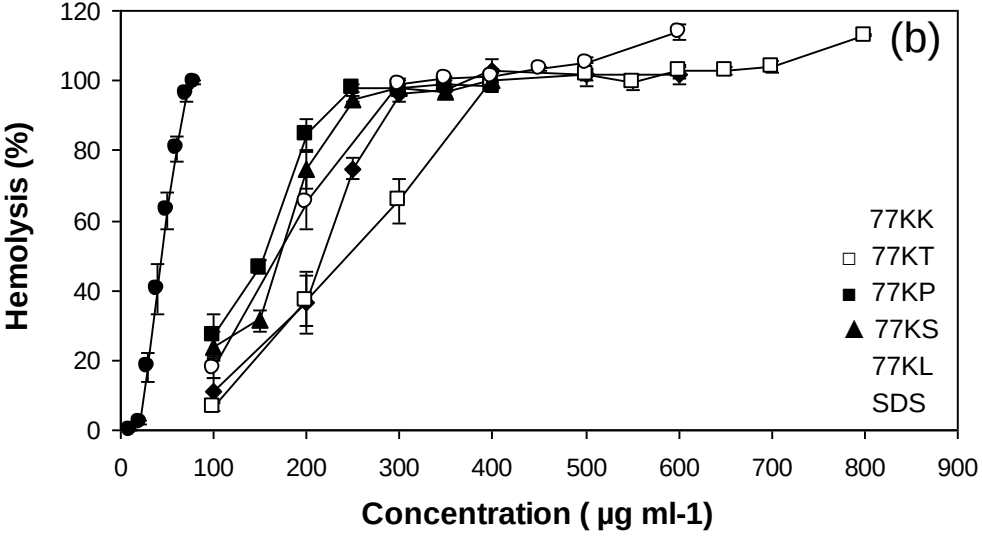
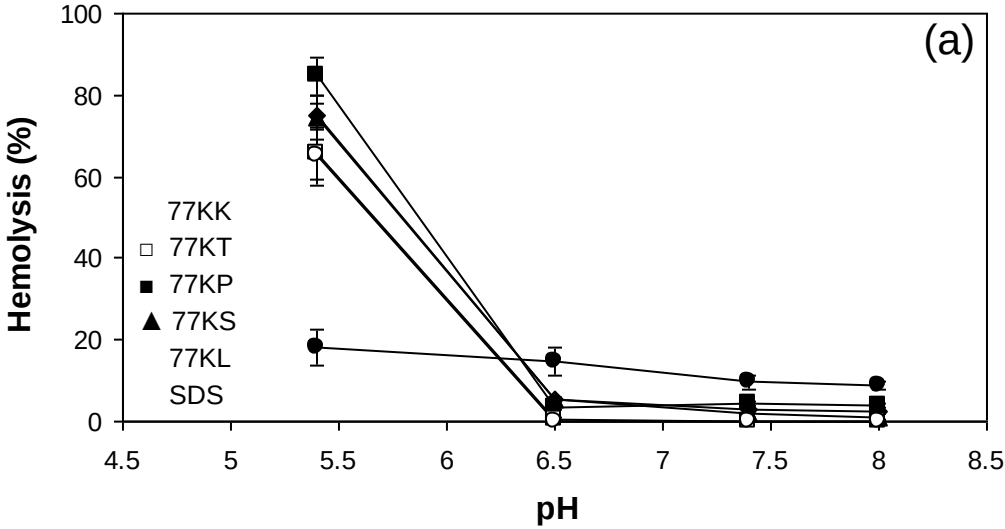

(a)
Hemolysis (%)
Concentration (µg ml-1)
77KK
77KT
77KP
77KS
77KL
SDS


(b)
Hemolysis (%)
Concentration (µg ml-1)
77KK
77KT
77KP
77KS
77KL
SDS

Figure(s)


(a)
Hemolysis (%)
100
80
60
40
20
0
77KK
77KT
77KP
77KS
77KL
SDS
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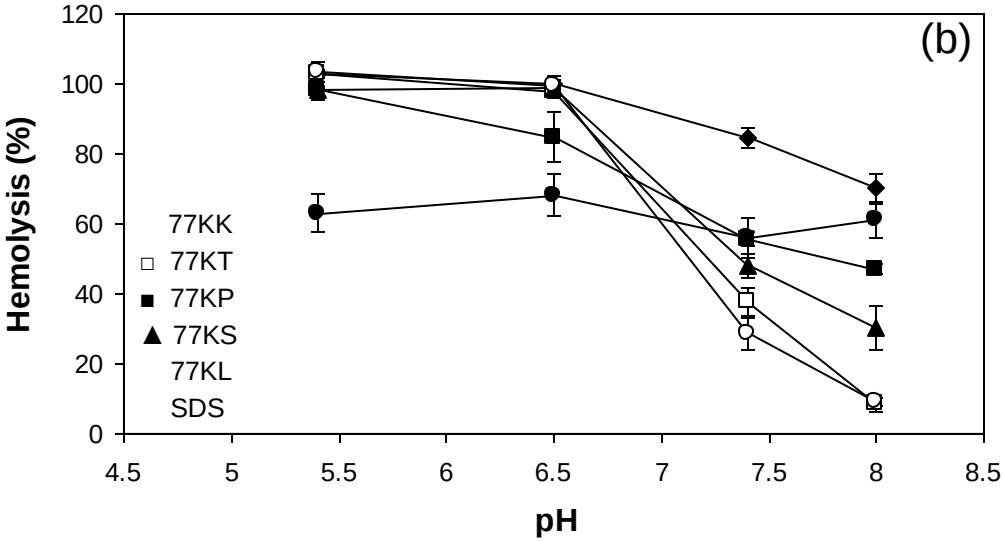
5
5.5
6
6.5
7
7.5
8
8.5
pH


(b)
Hemolysis (%)
120
100
80
60
40
20
0
77KK
77KT
77KP
77KS
77KL
SDS
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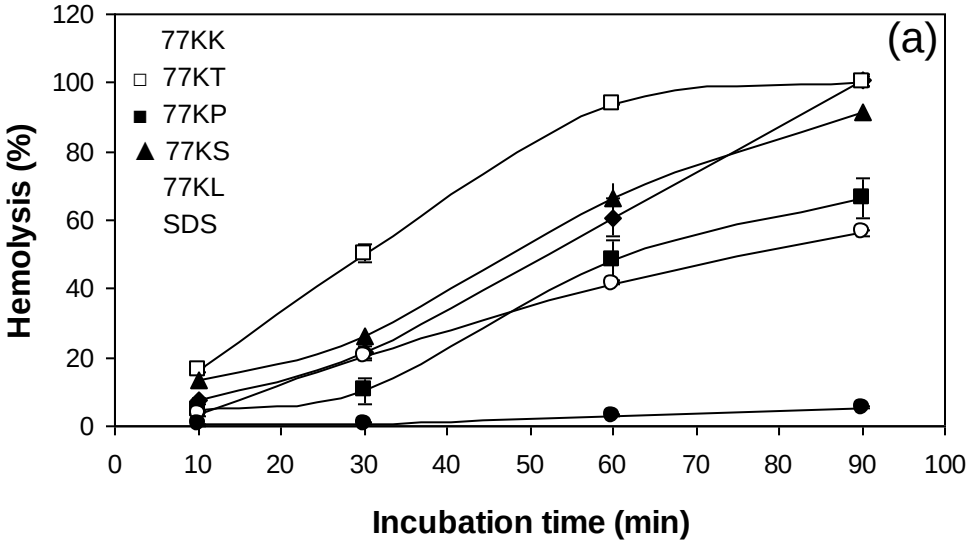
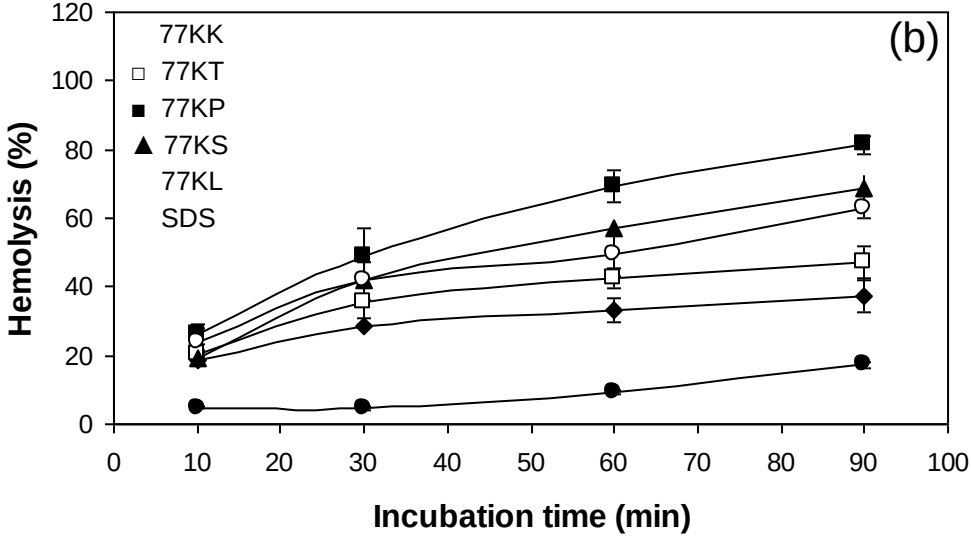
6.5
7
7.5
8
8.5
pH

Figure(s)


(a)
77KK
77KT
77KP
77KS
77KL
SDS
Hemolysis (%)
Incubation time (min)
0
20
40
60
80
100
120
0
10
20
30
40
50
60
70
80
90
100


(b)
77KK
77KT
77KP
77KS
77KL
SDS
Hemolysis (%)
Incubation time (min)
0
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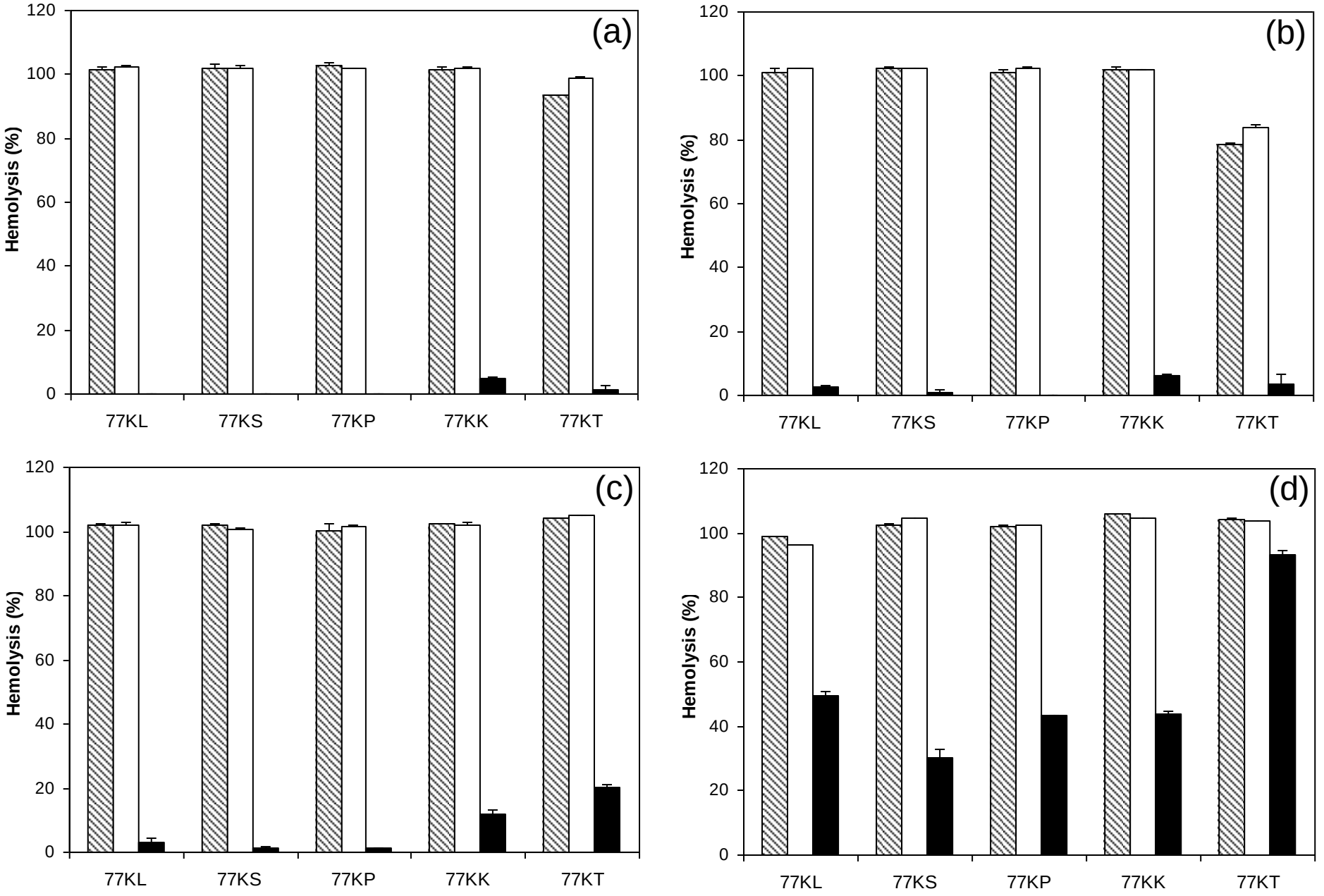
100
120
0
10
20
30
40
50
60
70
80
90
100

Figure(s)



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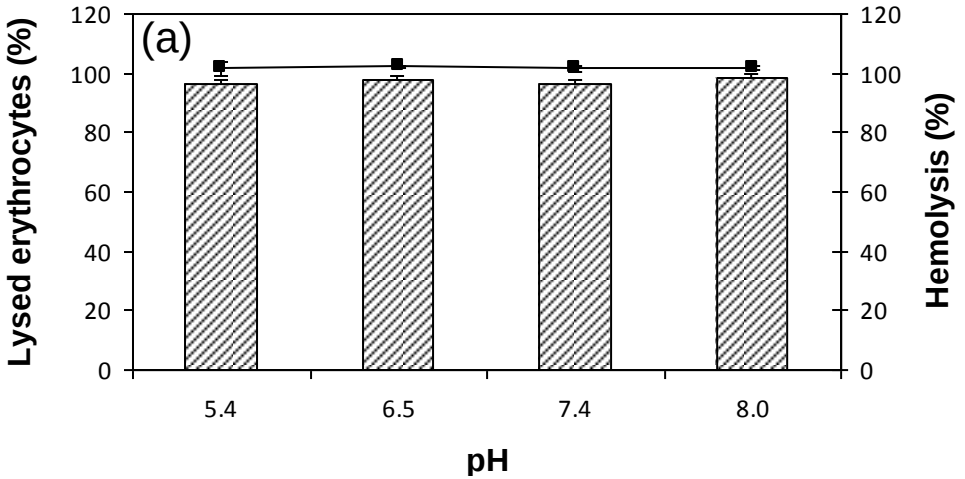
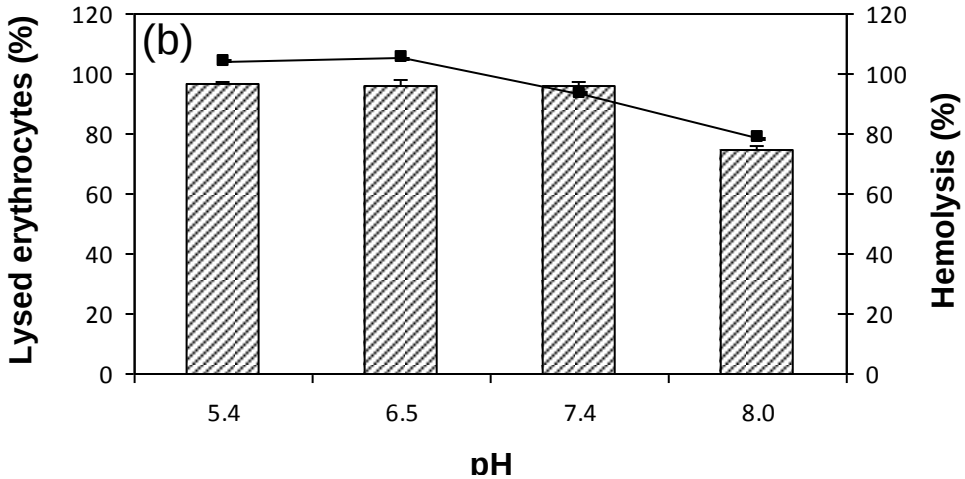

(a)
Lysed erythrocytes (%)
Hemolysis (%)
120
100
80
60
40
20
0
5.4
6.5
7.4
8.0
pH


(b)
Lysed erythrocytes (%)
Hemolysis (%)
120
100
80
60
40
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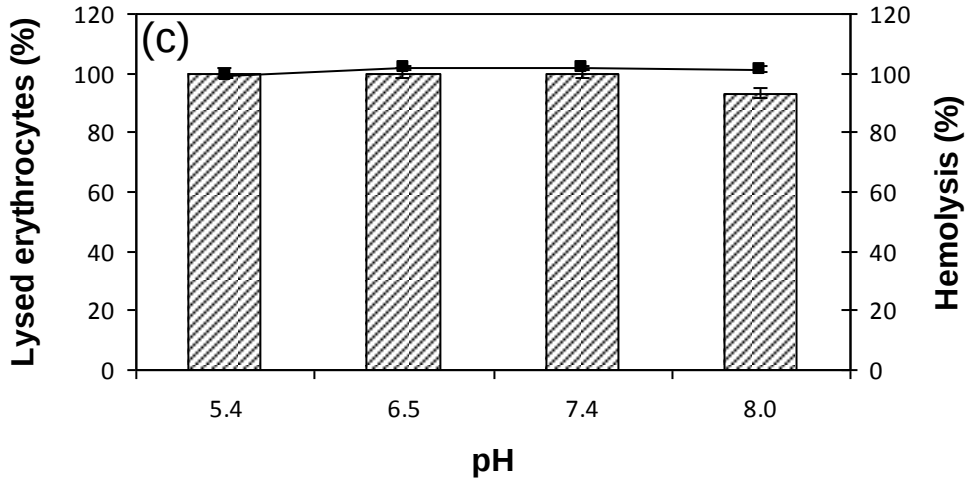
0
5.4
6.5
7.4
8.0
pH


(c)
Lysed erythrocytes (%)
Hemolysis (%)
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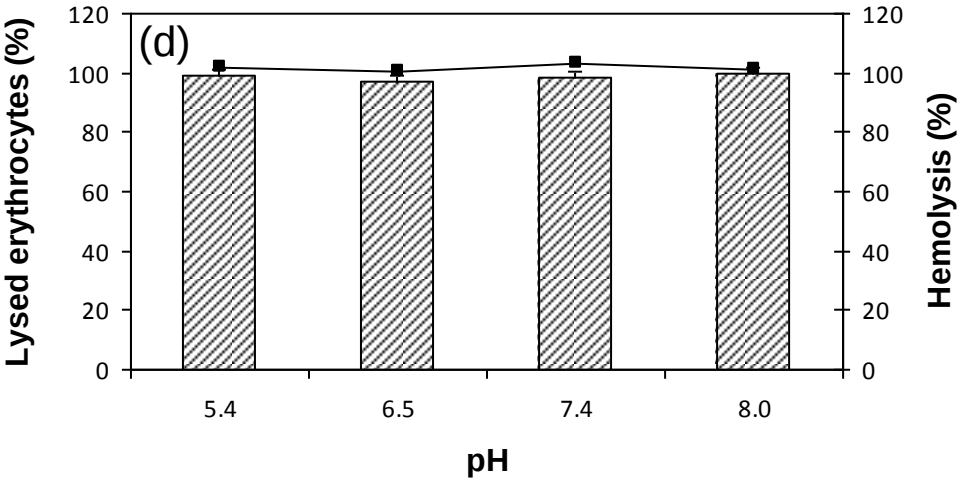
0
5.4
6.5
7.4
8.0
pH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Hemolysis (%)
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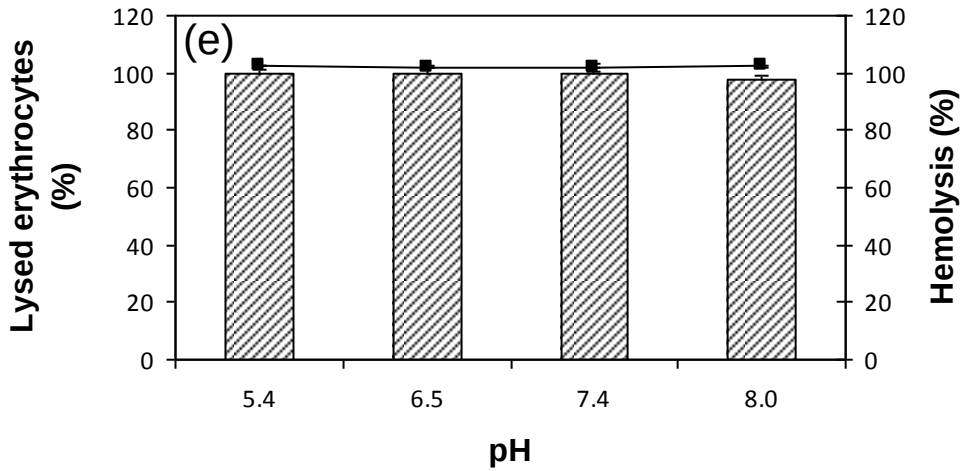
7.4
8.0
pH


(e)
Lysed erythrocytes (%)
Hemolysis (%)
120
100
80
60
40
20
0
5.4
6.5
7.4
8.0
pH

Figure(s)

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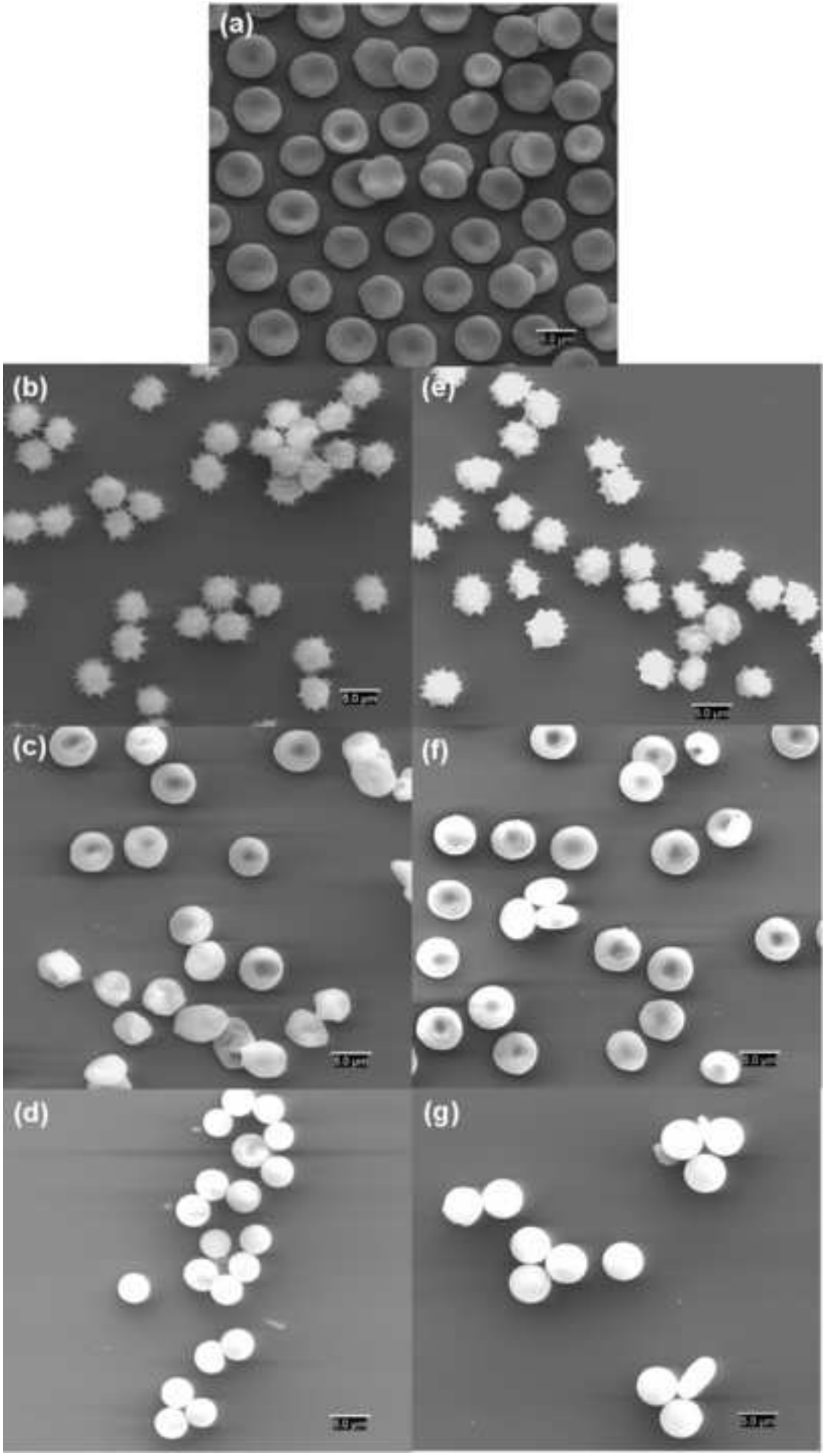
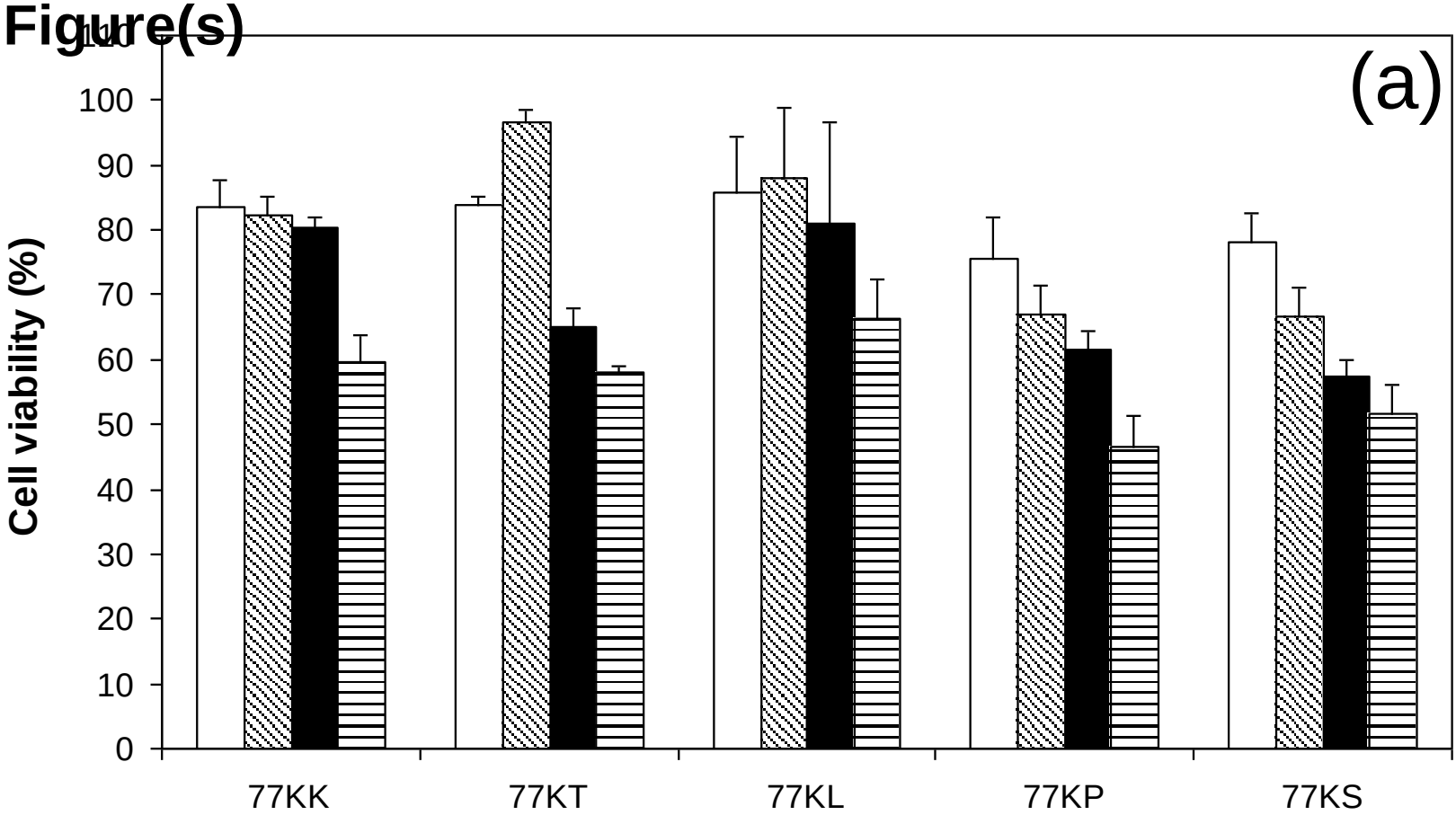
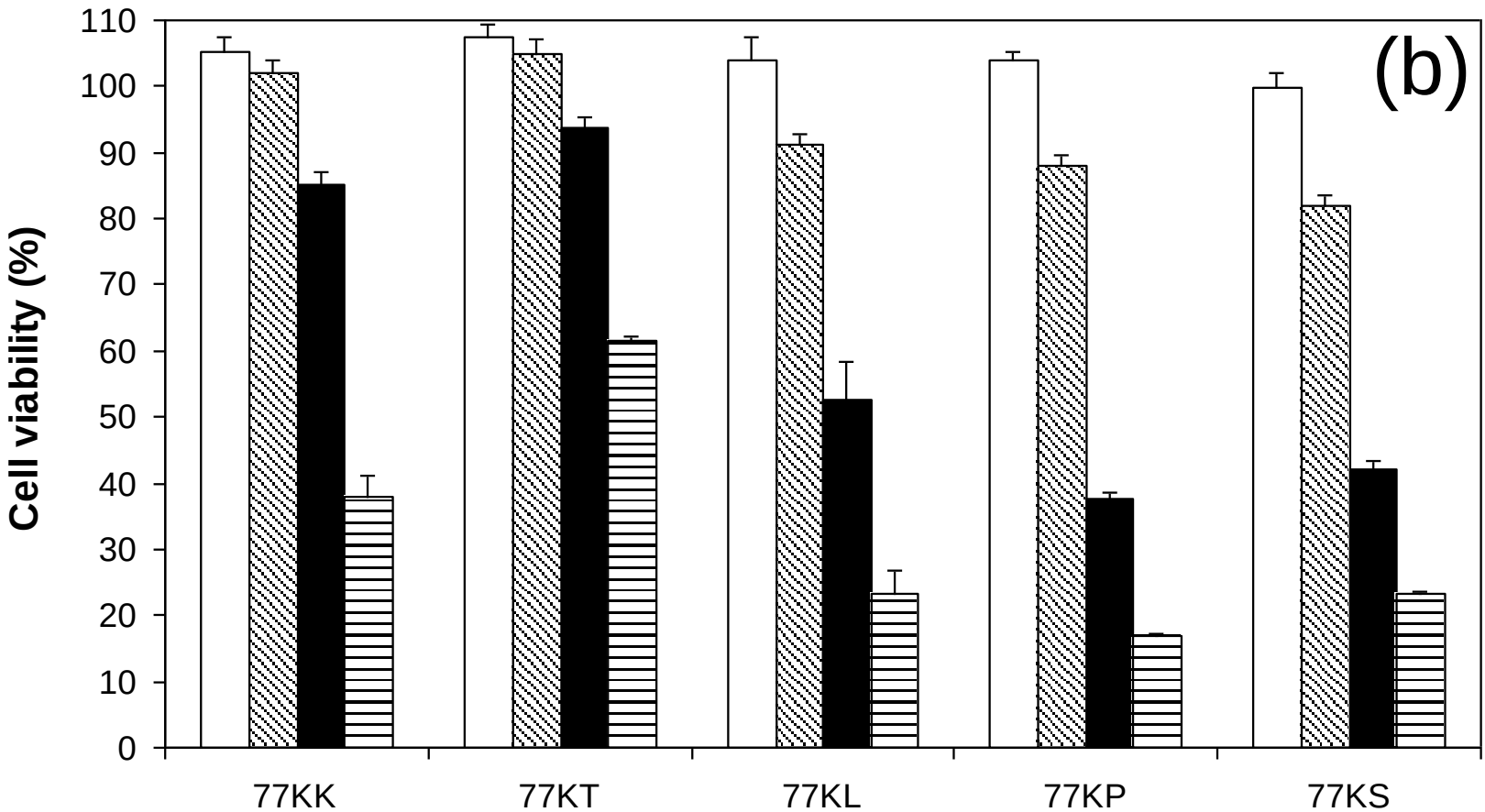


Figure(s)


(a)
Cell viability (%)
0
10
20
30
40
50
60
70
80
90
100
77KK
77KT
77KL
77KP
77KS


(b)
Cell viability (%)
0
10
20
30
40
50
60
70
80
90
100
110
77KK
77KT
77KL
77KP
77KS

Table(s)

Table 1. HC_{50} values of the surfactants at the different pHs under investigation.

	HC_{50} (μg ml^{-1}) (mean[a] ± SEM[b])			
Surfactants (MW)	pH 8.0	pH 7.4	pH 6.5	pH 5.4
77KK (545.7)	370.99±15.84	361.22±9.34	344.51±13.51	211.00±10.35[b,c,d]
77KT (519.7)	729.54±15.61	651.53±2.55	593.96±5.99[c]	290.32±11.69[b,c,d]
77KP (437.6)	285.07±0.69	269.01±10.51	276.51±11.81	145.38±11.83[b,c,d]
77 KS (421.5)	298.90±7.79	284.10±13.01	250.89±6.17	158.51±1.43[b,c,d]
77KL (405.6)	489.22±20.39	440.12±10.23	382.23±3.81	139.68±8.77[b,c,d]
SDS (288.4)	44.82±1.54	45.53±1.48	43.01±2.31	44.93±3.00

[a] Mean ± SEM of three experiments
[b] Significantly different from the pH 7.4 condition (Bonferroni`s posthoc test, $p < 0.05$)
[c] Significantly different from the pH 8.0 condition (Bonferroni`s posthoc test, $p < 0.05$)
[d] S ignificantly different from the pH 6.5 condition (Bonferroni`s posthoc test, $p < 0.05$)