Mixed monolayer of DPPC and Lysine based cationic surfactants: An investigation into the antimicrobial activity

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Abstract

In this paper we report studies which aim to elucidate the mechanisms involved in the antimicrobial activity of three cationic lysine-based surfactants: LLM, LALM and C_6 $(LL)_2$. To this end, a simple membrane model (DPPC) was used to explore the monolayer properties at the air/liquid interface. Compression π-A isotherms of mixtures of DPPC-lysine surfactants at different pH showed an expansion of the DPPC monolayer suggesting cationic lysine surfactants-DPPC interactions which strongly depend on surfactant structure and hydrophobic interactions. Antimicrobial activity of the three surfactants has also been assessed with Transmission Electron Microscopy, observing the effects on *Staphylococcus aureus* and *Escherichia coli*. The three surfactants caused various kinds of damage to the bacteria tested such as structural alterations, leakage of internal material and cell destruction.

INTRODUCTION

Antimicrobial resistance to antibiotics is today a major global public health threat, thus development of new compounds with antimicrobial activity is of great significance in medicinal chemistry.[1-3] Cationic surfactants adsorb at solid/liquid interphases and interact with cellular membranes of microorganisms. As a result, they exhibit antimicrobial activity and for many years they have been used as disinfectants in hospitals and in the food and pharmaceutical industries. [4,5] Recently, it has been shown that cationic amphiphiles have great potential in new therapeutic biomedical applications as cationic vesicles to encapsulate RNA or DNA for cellular transfer in gene therapy, [6-8] as vehicles for certain drugs, [9,10] and as modifiers of the physicochemical and biological properties of biomaterials.[11] These types of therapeutic applications require the use of stable cationic surfactants under sterilization conditions that do not present hemolytic or cytotoxic activities. Additionally, the antimicrobial activity of amphiphilic compounds can be of great interest for some of these applications.

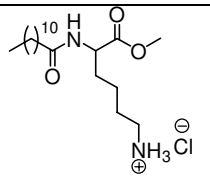
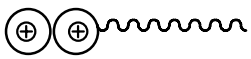
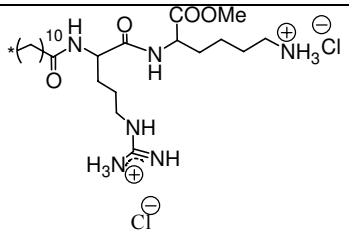
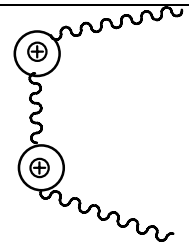
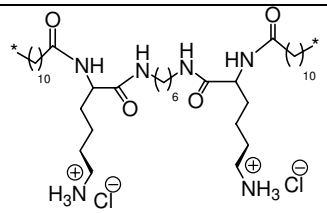
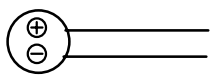
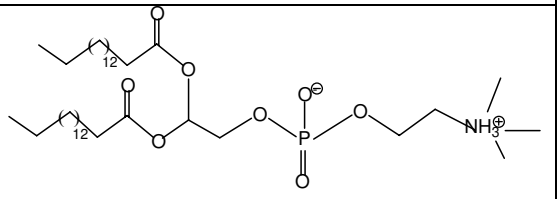
Over the last years, our group has synthesized cationic surfactants from different amino acids to search for new antimicrobial agents. Amino acid based surfactants can be prepared from renewable raw materials and are characterized by their high biodegradability and low toxicity against aquatic microorganisms.[12,15] Regarding toxicity against human cells, these compounds show moderate toxic levels. The toxicity depends on the alkyl chain length and the structure of the molecule.[16,17,18]

Recently, we have reported the synthesis and studies on toxicity[19] of ten different cationic lysine derivatives where the type of polar head group, the spacer character and the type and number of cationic charges on the head group region were systematically varied. The cationic charges of these surfactants are located on an amino protonated group. In aqueous solutions, the amino protonated group can dissociate, losing the cationic charge. pKa values of the dissociation equilibrium for each compound depend on the structure, the hydrophobicity, the number of cationic charges, as well as the density of the charge. [20,21] We also should mention the complexity of the polar head of these surfactants which contains both acceptor and donor hydrogen bonding groups which can give rise to intra- and inter-molecular interactions. These parameters play an important role in the compounds´ toxicity and influence surfactant behavior in a different way depending on the type of chemical architecture and on the type of cell line used for evaluating toxicity. For this reason, predicting the toxicity of cationic

surfactants is a rather difficult task and a long way is still ahead in order to establish their mechanism of action.

Among the ten lysine based surfactants synthesized, we select three compounds to further study their antimicrobial behavior. Their molecular structures are included in Scheme 1. The first row shows a monocatenary surfactant (LLM) with one alkyl chain and the lysine amino acid as the polar group. The surfactant in the second row (LALM) has one alkyl chain and arginine and lysine polar groups. The third row corresponds to gemini surfactants ($C_6(LL)_2$) which are made up from the corresponding monomers of the first row. The alkyl chain of these surfactants always includes twelve carbon atoms.

Scheme1. Molecular structures and acronyms of lysine based surfactants and DPPC

Surfactant Type	Acronyms	Structure
	LLM	
	LALM	
	$C_6(LL)_2$	
	DPPC	

To develop a plausible approach to the antimicrobial mechanism, in this paper we report the results obtained after considering the interactions of the three new lysine-based surfactants

with a simple membrane model (DPPC) and microscopic observation of bacteria before and after they get in contact with the new surfactants.

MATERIALS AND METHODS

Materials

Commercial phospholipid 1,2-dipalmitoyl-*sn*-phosphatidylcholine (DPPC) was purchased from Sigma-Aldrich with a purity of 99% and used as received. Sodium chloride (> 99.5% by weight) was purchased from Fluka. Water was obtained using a Synergy Ultrapure water system from Milipore (resistivity 18.2 MΩ). Spreading solutions were prepared using hexane and ethanol both HPLC grade supplied by Merck and Panreac respectively. Mueller-Hinton broth (MHB) was purchased from Difco Laboratories (USA). The lysine based surfactants were synthesized and purified according to our previously published procedure.[19] Purity of synthetic surfactants was higher than 96%, as determined by ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR), High-Performance Liquid Chromatography (HPLC) and Elemental Analysis. The structures of all lysine based surfactants are shown in Scheme 1.

Acid-Base Titration

Apparent pKa values were determined by potentiometric titration with a pH electrode (model 8102 ROSS Thermo Orion, Beverly, USA) at 25ºC under nitrogen gas atmosphere and magnetic stirring. Two series of surfactant solutions were titrated. In one series the solvent was water and in the other series the solvent was an aqueous solution of NaCl 0.5M. In both series, titration was carried with a solution of NaOH where the concentration of surfactant and NaOH were the same. Apparent pKa values were figured out as the corresponding semi equivalent point in the titration curves.

π-A Compression Isotherms

To obtain surface pressure versus mean molecular area (π-A) isotherms at room temperature, a computer controlled Langmuir Balance (KSV Instruments Ltd. Helsinki) with a 364 mm length and a 75 mm width was used. The instrument has a Teflon trough with two symmetrical moving barriers which can vary the surface area from 22.0 to 242.25 mm^2. The surface pressure was measured with a Wilhelmy plate made of filter paper (Whatman ash less, 70 mm Ø). The uncertainty of the Langmuir balance is ±0.1 mN/m. Before spreading, the surface is compressed and the top layer of the subphase aspirated with a Pasteur pipette. We verified that

the surface was clean by checking that the pressure raised between full expansion and full compression was < 0.1 mN/m. The samples were DPPC, Lysine surfactants, or DPPC/Lysine mixtures and they were prepared by weight in a concentration of 1 mg/mL in hexane/methanol (9:1) solvent. Aliquots of 20 μL were spread on to the surface of saline aqueous solutions of 0.5 M NaCl at pH 2 (HCl), pH 12 (NaOH) and free pH (6.7), with a micro syringe (Hamilton, 50 ± 1 μL). Symmetric compression of the monolayer after 20 min for solvent evaporation was started with a 20 mm/min rate. The pressure was monitored by means of the weight of the plate. To ensure reproducibility, each isotherm was measured at least three times. The reproducibility of the surface pressure was better than 0.4 $mNm.^{-1}$

Images of the monolayer were captured by means of a Brewster Angle Microscope (BAM) with a KSV MicroBAM (KSV Instruments) equipped with a 30mV laser emitting p-polarized light at 659 nm wavelength which was reflected off the air/water interface at the 53.1º Brewster angle.

Antimicrobial Activity

MIC Determinations

Antimicrobial activities were determined "in vitro" on the basis of the minimum inhibitory concentration (MIC) values,[22] defined as the lowest concentration of antimicrobial agent which inhibits the development of visible cellular growth after 24 hours of incubation at 37 ºC. The compounds tested were dissolved in Muller Hinton Broth (MHB, Oxoid, Basingstoke, UK, pH 7.3) in the concentration range of 0.1-256 μg/mL (no precipitate was observed at the highest concentration of surfactant). The MHB was prepared according to the manufacturer's instructions. Then 10 μL of an overnight culture of each bacterial strain was used as inoculum to achieve a final concentration of ca. 5×10^{-4}-5×10^{-5} colony forming units (cfu) per mL. The cultures were incubated overnight at 37 ºC. Inoculated MHB served as control. The growth of the microorganisms was determined visually after incubation for 24 hour at 37 ºC. The lowest concentration of antimicrobial agent at which no visible turbidity was observed was taken as the minimum inhibitory concentration. For MIC determinations, Gram-negative bacteria such as *Escherichia coli* ATCC 27325, and Gram-positive bacteria such as *Staphylococcus aereus* ATCC 25178 were used.

Viability Curves

The viability curves of *Staphylococcus aureus* and *Escherichia coli* have been followed for three compounds. The curves have been carried out at two concentrations: MIC and 2/3 of the MIC. Suspensions of microorganisms were obtained by growing the bacteria overnight at 30 ºC in trypticase soy agar plates (TSA, Pronadisa, Barcelona, Spain). The respective cell suspensions were prepared inoculating bacteria in 75 mL of sterilized peptone buffered water solutions (reference) (pH 7) to obtain a cell density of about 10^8-10^7 cfu/mL. An appropriate volume of 25 mL of the respective bacterial suspensions was taken and then 1 mL of surfactant solution was added in order to obtain the corresponding surfactant concentrations (MIC and 2/3 the MIC). The inoculated flasks were kept at room temperature and after a different contact period (0, 30, 60, 90 and 120 minutes) the antimicrobial action was immediately blocked by dilution (1/10) with sterile Ringer solution (Scharlau, Barcelona, Spain).Viable counts (cfu/mL) were carried out on trypticase soy agar, TSA and incubated at 30 ºC for 24 hours. Cell counting was performed in triplicate.

Electron Microscopy Analysis

Electron microscopy analysis was carried out by High-Pressure Freezing and Freeze substitution method and subsequent sectioning. Samples of 5 mL were taken after contact time, and centrifuged at 4500 x *g* for 30 min. The bacterial pellets were rinsed with 0.1 M phosphate buffer (pH 7.4) and centrifuged again. Samples were transferred to planchettes (1.5 mm in diameter and 200 μm deep) and immediately cryoimmobilized using a Leica EMpact high-pressure freezer (Leica, Vienna, Austria) in the absence of cryoprotectants or freezing solutions. Planchettes were then stored in liquid nitrogen until further usage. Frozen samples were freeze substituted in a Leica EM AFS (automatic freeze substitution system, Leica Vienna, Austria), where the substitution was performed in pure acetone containing 2% (w/v) osmium tetroxide and 0.1% (w/v) uranyl acetate at – 90 ºC for 72 h. Temperature was gradually raised (Δt = 5 ºC/h) to 4 ºC, held constant for 2 h, and then finally raised to room temperature and maintained for 1 h. Samples were washed for 1 h in acetone at room temperature and infiltrated in glacial series of Epon-acetone mixtures: 1:3 for 2 h, 2:2 for 2 h, 3:1 for 16 h, and pure Epon 812 (Ted Pella, Inc., USA) for 30 h. Samples were embedded in fresh Epon and polymerized at 60 ºC for 48 h. Ultrathin sections were cut with a Leica UCT ultra microtome and mounted on Formvar carbon-coated copper grids. Sections were post-stained with 2% aqueous uranyl acetate and lead citrate and examined by a Tecnai Spirit

electron microscope (FEI Company, Netherlands) at an acceleration voltage of 120 kV and a computer program analySIS (Soft Imagine System, Switzerland).

RESULTS AND DISCUSSION

Acid-Base Titration

The surfactants studied in this work, LLM, LALM and $C_6(LL)_2$ have a cationic charge on the protonated ε-amino group of lysine. LALM has an additional cationic charge on the protonated guanidine group. These surfactants present acid-base equilibrium in aqueous solution.[20]

As stated above, pKa values in aqueous solution have been determined from the pH value at the point of semi equivalence in the titration curves. LALM and $C_6(LL)_2$ yielded titration curves with two inflection points corresponding to two different apparent pKa: pKa_1 and pKa_2. The titration curve of LLM shows just one inflection point. Results are show in Table 1.

Table1. Apparent pKa values of lysine based surfactants

Compound	CMC* (mM)	Surfactant Concentration (mM)	Na Cl Concentration (M)	pKa_1	pKa_2
LLM	7.2	0.5	0	7.7	
		0.5	0.5	7.6	
		1	0	7.1	
		1	0.5	7.2	
		5	0	8.8	
LALM	25	2	0	9.7	10.5
		2	0.5	9.8	10.0
		30	0	9.4	10.4
$C_6(LL)_2$	0.74	0.5	0	7.8	≈8.4
		0.5	0.5	7.9	
		5	0	7.4	8.2

*Values from reference 19. A possible variability in the cmc values can be observed depending on the technique used for their determination.

The apparent pKa values are substantially lower than those of free lysine (pKa_1=9.1, pKa_2 = 10.5) or arginine (pKa_1= 9.0, pKa_2=12.5) amino acids, which can be attributed to the presence

of long hydrophobic chains which induce aggregation. This behavior is common in amino acid based surfactants,[21, 23] as well as in other cationic surfactants in which the cationic charge is placed on a protonated amino group.[24, 25]
The differences on the apparent pKa values for the same lysine protonated amino group can be attributed to the different structures of the compounds which in turn affect the critical micellar concentration.[20]

Values in Table 1 show that apparent pKa values for premicellar concentrations of LALM and $C_6(LA)_2$ are higher than for postmicellar concentrations. This shift in apparent pKa values when molecules aggregate has been extensively studied.[21,26,27] Two main contributions to the apparent pKa shift have been identified, one comes from the virtual charge effect caused by a discontinuity in the dielectric constant at the micellar surface. The second contribution comes from polar head interactions. In the surfactants we have studied, the apparent pKa shift can be attributed to both contributions. The apparent pKa of LLM obtained at 5mM is higher than that obtained at lower concentrations. This result does not agree with standard behaviors reported.[21,28,29] So far we have not found a rationale for this fact.

Since the monolayer studies have been conducted in saline aqueous solution, the apparent pKa values were also measured in 0.5 M NaCl to check whether the presence of salt affects their values. Results are shown in Table 1. No noticeable differences between apparent pKa values measured in water and those measured in presence of NaCl were observed. This suggests that the ionic strength has a minor effect on the apparent pKa values for the surfactants studied.

The three compounds studied have apparent pKa values greater than 7 thus they should have weak acidic properties, consequently, in aqueous solutions at pH close to 7 they should remain as cationic surfactants.

Surface Pressure-area Isotherms

Single Component Systems

It has been reported [30] that lipid multilamellar vesicles are suitable membrane models for studying interactions of biologically active molecules with lipid membranes. However, they suffer from several limitations. For example, the range over which the lipid composition can

be varied without modifying the surface curvature and phase is limited. Besides, the degree of lipid packing is not uniform along the bilayer and the physical state of compositionally identical bilayer dispersions depends on the method of preparation.[31] Lipid monolayers can overcome these limitations, being considered as an excellent model system to study the interactions of active compounds with lipids at the air/water interface. DPPC was select as a model for the major components of membranes.

Although practically all studies on π-A isotherms involve amphiphiles that are almost insoluble in the subphase, soluble surfactant monolayers can also be compressed in some cases. [32, 33] In our case, the compounds that we have synthesized can be classified as soluble or partially soluble. To enhance adsorption at the interface we used as subphase a 0.5 M of aqueous NaCl solution (free pH = 6.7) and a compression speed of 20 mm/min.

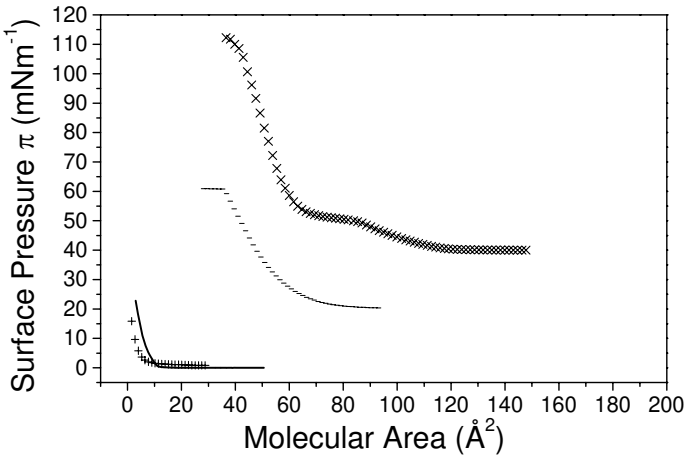


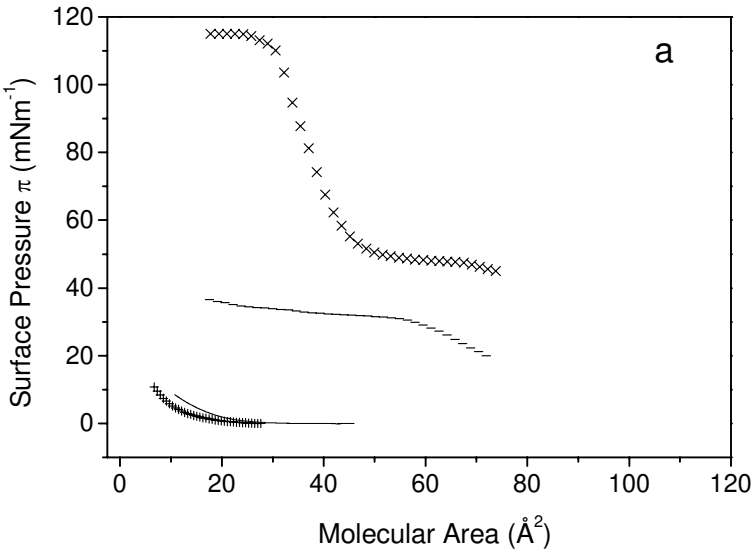
Figure 1. π-A Compression isotherms for pure lysine based surfactants and DPPC. DPPC (x), LLM (+), LALM (Straight line), C_6 $(LL)_2$ (Bar -). DPPC surface pressure values have been shifted 40 units, surface pressure of C_6 $(LL)_2$ has been shifted 20 units. Subphase was NaCl 0.5M (pH = 6.7).

The π-A isotherm at 25 ºC for pure DPPC is shown in Figure 1. When the monolayer was compressed, the area decreased to 98 $Å^2$/molecule and the gaseous monolayer state changed to liquid expanded state. With further area compression, the monolayer state changed to liquid condensate and solid states and finally the monolayer collapsed when the surface pressure

reached 65 mN/m. The isotherm observed for pure DPPC agrees with previously published curves with a pattern similar to those found in phospholipids with intermediated chain lengths.[34]

The shape of the π-A isotherms of our synthetic surfactants with single hydrocarbon chains (Figure 2) shows a profile characteristic of surfactants with a relatively high solubility and the spread monolayers of these compounds were almost not compressible. As expected, the shorter alkyl chain, the larger the tendency to form expanded phases, and only gas and expanded liquid are present (plateau is not observed). Gemini lysine based surfactants form stable monolayers at the air-water interface, as evidenced in Figure 2. Monolayers were compressible showing a collapse plateau. The area per molecule extrapolated from the collapse monolayer in $Å^2$/mol was 61.84. The collapse pressure (π_C) is defined as the point of the isotherm where the steep part of the curve begins to bend. The π_C in mNm^{-1} was 41.6.

Given the acidic properties of the surfactants studied, we can assume that when pH is higher than 10.5, surfactants lose the cationic charge and become in part nonionic, whereas at low pH values they remain as cationic surfactants. To figure out how monolayer profiles change depending on whether surfactant species are cationic or nonionic, monolayers at pH = 12 and pH = 2 were studied. Results are show in Figure 2a and 2b.



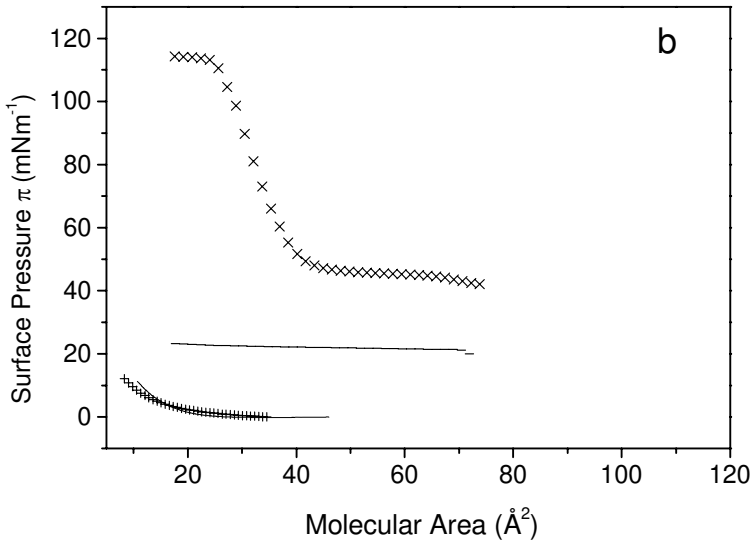


Figure 2. π-A compression isotherms for pure lysine based surfactants and DPPC. DPPC (x), LLM (+), LALM (Straight line), C_6 $(LL)_2$ (Bar -). DPPC surface pressure values have been shifted 40 units. Surface pressures of C_6 $(LL)_2$ have been shifted 20 units. Subphase was always NaCl 0.5M. (a) pH12, (b) pH 2.

Results in Figure 2 show that at the pH values studied, DPPC monolayers are slightly changed by the subphase pH. These results agree with the results reported by Brzozowska et al. [35] The π-A isotherms of LLM and LALM (Figure 2 a, b) show a rather flat profile which is characteristic of surfactants with high solubility and not compressible spread monolayers. Gemini surfactants showed several global trends upon variation of the pH. The profiles of the π-A isotherms at pH = 12 (Figure 2a) and pH = 2 (Figure 2b) are clearly different. At pH = 12, the π-A isotherms show constant pressure collapse for molecular areas lower than 60 $Å^2$/mol whereas at pH = 2 the monolayer is not compressible. A tentative explanation for the observed π-A isotherms is as follows. As has been said before, at pH = 2 we can expect that most surfactant molecules at the interface would be cationic while at pH = 12 they would be nonionic. Consequently, at pH = 2 the presence of fully charged molecules with high solubility and thus not compressible should result in a flat isotherm. However, at pH = 12 gemini molecules are basically nonionic and insoluble thus spread monolayers should be compressible. As a result, the isotherm should show a decrease in the area per molecule as the pressure increases. Besides, the high hydrophobic character of gemini surfactants sets a limit to the monolayer compressibility giving rise to a plateau in the isotherm.

To further check the behavior of the monolayers generated by LLM, LALM and C_6 $(LL)_2$ we observed their monolayers with BAM microscopy. Since monolayers generated by LLM and LALM at any pH, and gemini surfactants $C_6(LL)_2$ at pH = 2 are not compressible, BAM microscopy of their monolayers shows a bare interface (Figure 3a). At pH = 12, BAM images of $C_6(LA)_2$ monolayer at a pressure corresponding to the isotherm plateau show fibrous structures forming multilayer assemblies, associated with fracture into multilayer structures that coexist with a bare interface. It appears that at this pH, the nonionic head group allows hydrophobic interactions between the fatty acid chains to dominate, and hence to drive the aggregation behavior. This phenomenon has been also found in fatty acids where the interface was essentially uncharged[36, 37]

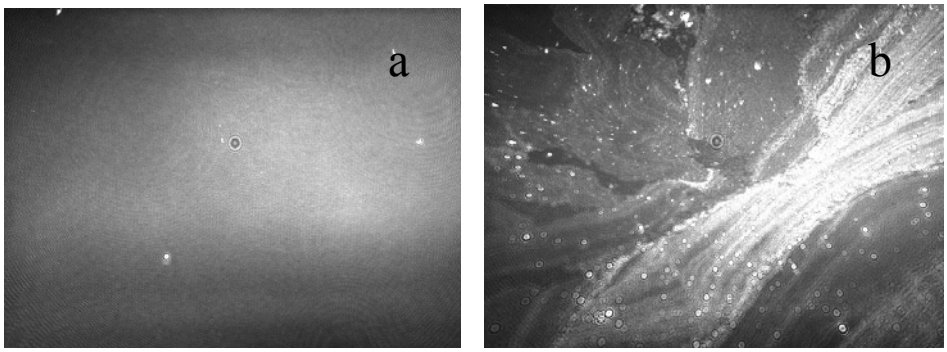
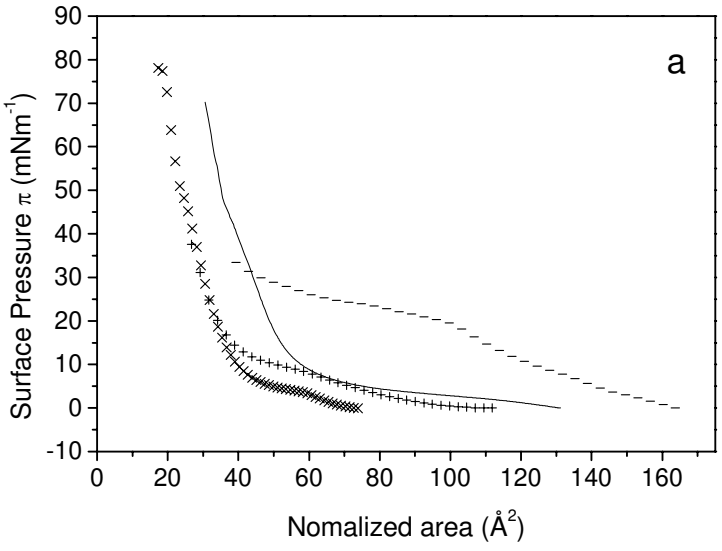


Figure 3. $C_6(LA)_2$ BAM images at 15 mNm^{-1}surface pressure. (a) pH = 2. (b) pH = 12.

Binary Systems

The compression isotherm of amphiphilic compounds shows the extent to which the compound is forced into the bulk subphase. In mixtures of surfactants with phospholipids, if the surfactant desorbed completely as the monolayer was compressed, the resulting isotherm would match that of a pure phospholipid. Thus any deviation from this behavior can be attributed to incomplete desorption of the surfactant.

To investigate the behavior of monolayers of DPPC when either LLM, LALM or $C_6(LL)_2$ is present, we have studied the π-A compression isotherms of DPPC/LLM, DPPC/LALM and DPPC/$C_6(LL)_2$ 50/50 mixtures. Since we are also interested in knowing how the presence of cationic or nonionic species in the interface affect the monolayer, we have conducted three series of measurements, at pH = 2, pH = 12 and in saline aqueous solution (pH=6.7). NaCl 0.5 M was always present in the subphase. First we discuss the results obtained at pH = 12 shown in Figure 4a.



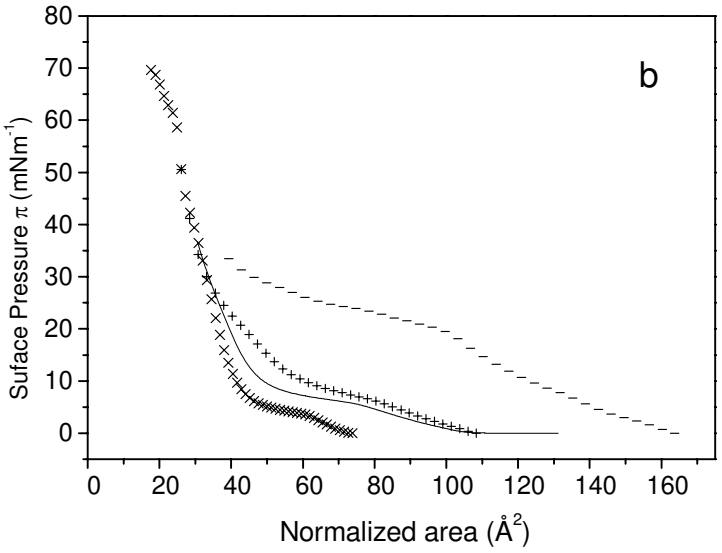


Figure 4. π-A compression isotherms for binary mixtures (50/50) of DPPC/lysine surfactants. (a) pH12. (b) pH 2. DPPC (x), DPPC/LLM (+), DPPC/LALM (Straight line), DPPC/C_6 $(LL)_2$ (Bar -).

Clearly, the DPPC/LLM monolayer profile is similar to that of DPPC (NaCl 0.5 M, pH=6.7) in Figure 1, but shifted toward slightly larger molecular areas. This shift suggests some kind of interaction between DPPC and LLM. Since at pH = 12 most of the LLM molecules are nonionic, the DPPC-LLM monolayer should present hydrophobic interactions between the alkyl chains combined with hydrogen bonding between the -NH_2 lysine groups and the phosphate group of DPPC.

For the DPPC/LALM mixture, the monolayer profile is again similar to that of DPPC but shifted toward molecular areas slightly larger. However, the liquid-condensed phase does not show up. At the pH = 6.7 (NaCl 0.5M), LALM has two cationic charges. In general, up to three different LALM ions can be present: nonioinic molecules, ionic molecules with one cationic charge and molecules with two cationic charges. Since the apparent pKa of LALM is 9.8, nonionic molecules are favored at pH = 12, therefore we would expect most of the interactions to be hydrophobic. However, we can expect that some ionic interaction between polar heads would also take place.

The DPPC/$C_6(LL)_2$ mixture monolayer reminds that of $C_6(LL)_2$ at pH=12 (compare the corresponding plots in Figure 4a and Figure 2a). The monolayer is shifted toward larger

molecular areas and shows a clear plateau after collapse. As in the LALM surfactant case, $C_6(LL)_2$ has two different apparent pKa values, both close to 8 and the rationale applied to LALM concerning the species present in the interface applies here. For the $C_6(LL)_2$ at pH 12 (Figure 2a), the plateau spanned from about 60 down to about 40 $Å^2$ per molecule. Now, when mixed in DPPC, the plateau spans from 100 down to 60 $Å^2$ per molecule (Figure 4a). The presence of DPPC favors hydrophobic interactions, thus the plateau shows up at larger molecular areas.

Next we consider the experiments conducted at pH = 2. Comparing plots in Figure 4a and Figure 4b for the DPPC/LLM mixture, we see that they correspond to the same behavior within experimental errors. Therefore, cationic charges do not play any fundamental role in monolayer behavior which responds to the sum of hydrophobic interactions and hydrogen bonding.

The monolayer behavior of the DPPC/LALM mixture is similar to the behavior shown by DPPC alone (Figure 2b) but slightly shifted toward larger molecular areas. Gas-expanded liquid and expanded liquid-condensed liquid are clearly identified at about 75 and 50 $Å^2$ per molecule. As illustrated in Scheme 1, LALM features are an alkyl chain and a polar head with two cationic amino acids. Repulsion forces place the amino acids in the subphase as far as possible from each other. In this scenario, polar heads can be oriented in space as illustrated in Figure 5 making plausible an electrostatic attraction between the positive charge of one amino acid and the negative charge on the DPPC dipole. Electrostatic, hydrophobic and hydrogen bond interactions properly combined would favor arrangements of DPPC and LALM molecules according to a perpendicular orientation with respect to the monolayer plane causing a minimal distortion to the monolayer of DPPC in water. Therefore, the presence of LALM results in molecular areas slightly greater while transitions are still basically those of DPPC.

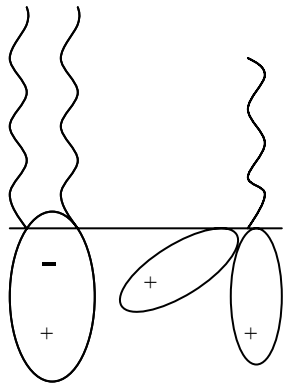
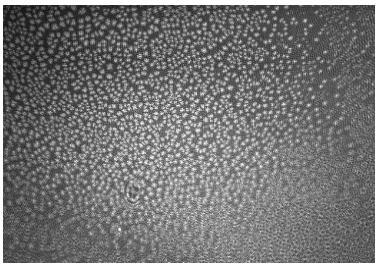


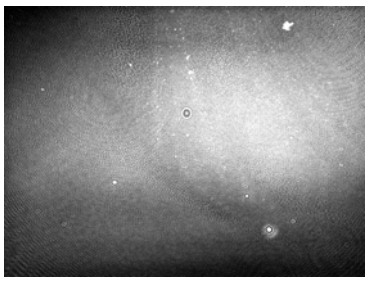
Figure 5. Schematic representation of DPPC-LALM electrostatic interactions.

Finally, the monolayer of DPPC/$C_6(LL)_2$ mixture at pH = 2 (Figure 4b) is the same as the one at pH = 12 (Figure 4a) within experimental error. Therefore, the pH of the subphase has no noticeable effect on the monolayer behavior which can be attributed to the lack of strong water-surfactant interactions. Overall, the pH seems to play a minor role in the π-A compression isotherms, while the presence of gemini surfactants strongly influences both the isotherm´s shape and the range of molecular areas spanned.

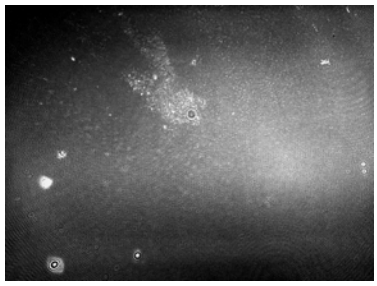
BAM microscopy was used to validate π-A compression isotherms. Images of the actual monolayers formed by the mixtures studied were captured using BAM microscopy at pH = 12 and pH = 2. Surface pressure was always 30 mNm^{-1} (See Figure 6).



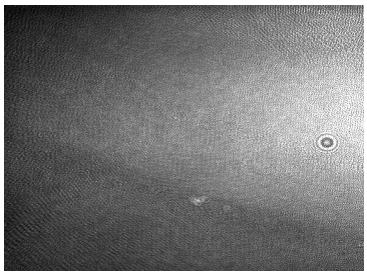
DPPC



DPPC/LLM pH =12

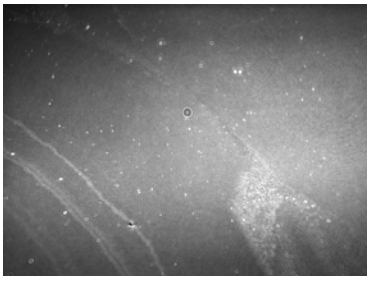


DPPC/LLM pH = 2

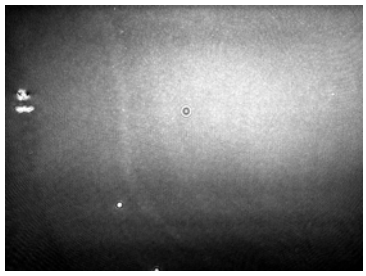


DPPC/LALM pH = 12

DPPC/LALM pH = 2



DPPC/$C_6(LL)_2$ pH = 12



DPPC/ $C_6(LL)_2$ pH = 2

Figure 6. BAM images at 30 mNm^{-1}surface pressure.

The BAM image of pure DPPC evidences the existence of typical bright domains suspended in a darker phase. [38] For DPPC/lysine mixtures BAM observations clearly indicate that, at pH = 12, uncharged lysine surfactants have tendency to form three-dimensional structures that under compression form stripes. At pH = 2, the film homogeneity for the three mixtures

studied increases. The presence of lysine surfactants in the mixture causes the characteristic DPPC domains to be indistinguishable in the film. For the investigated mixtures, there is no evidence of phase separation, thus the miscibility of DPPC and lysine surfactants can be assured.

Polar groups are one of the structural factors which affect molecular packing in mixed monolayers. Their nature, orientation, mutual interactions and degree of hydration are crucial for molecular packing.[39] On the one hand, results yielded by the mixtures studied in this work show that pH plays a minor role and that monolayer behavior is mainly governed by hydrophobic interactions. On the other hand, considering that the three surfactants studied have the same alkyl chain, if hydrophobic interactions were the major factor affecting the monolayer behavior, one would expect that the monolayer for the three mixtures would be almost the same. However, this is not the case and we can attribute the differences in monolayer behavior to the different polar heads featured by each surfactant which adopt different stereo chemical configurations allowing for ionic interactions with DPPC.

The π-A compression isotherms for DPPC/Lysine surfactants in aqueous NaCl 0.5 M are plotted in Figure 7. Comparing π-A isotherms in Figure 7 with those in Figure 4, the only noticeable difference is that now the liquid condensate phase of DPPC/LALM mixture is shifted towards molecular areas smaller than in Figure 4. This indicates that part of the surfactant and the DPPC are expelled from the monolayer, dissolving into the subphase probably by forming mixed micelles.[40]

π-A compression isotherms evaluated at pH = 2, pH = 12 and in aqueous solutions of NaCl (pH of about 6.7) for each surfactant studied do not show significant differences and therefore the behaviour of mixture monolayers is mainly governed by hydrophobic interactions between mixture components. However, the size of the polar heads also has some effect on the perturbation of the DPPC monolayer. In fact, the gemini surfactant which has the bigger polar head (two amino acids plus one spacer chain) shows the greater DPPC monolayer perturbation.

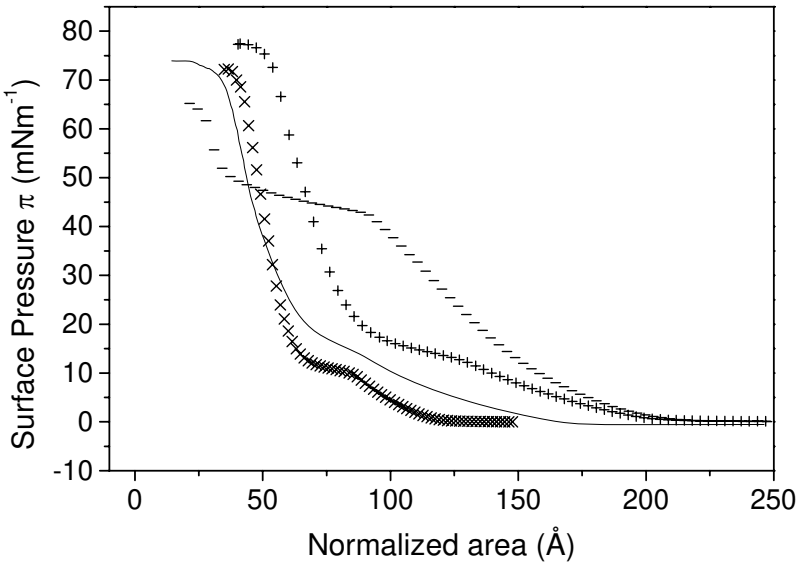


Figure 7. π-A compression isotherms for binary mixtures (50/50) of DPPC/lysine surfactants. DPPC (x), DPPC/LLM (+), DPPC/LALM (Straight line), DPPC/C_6 $(LL)_2$ (Bar -).

Antimicrobial Activity

Biological membranes contain a variety of phospholipids whose composition depends on the species and on their functionality.[41] The membrane of Gram-negative bacteria has a complex structure consisting of two bilayers, the outer membrane and the inner or cytoplasmatic membrane. Gram-positive bacteria only have a cytoplasmatic membrane that consists to a large extent of phosphatidyl glycerol which gives a global negative character to the membrane. For a surfactant to be active against bacteria, it must be able to distort the structure of the outer membrane in order to reach and to permeabilize the inner phospholipid membrane, whose integrity is essential for biological viability.[42] In this context, the antimicrobial activity of LLM, LALM and $C_6(LL)_2$ lysine-based surfactants was tested by determining the MIC values against the Gram-positive S. *aureus,* and the Gram-negative *E. coli*. Measured values are collected in Table 2. MIC values correspond to surfactants with a moderate antimicrobial activity against Gram-positive bacteria, whereas they present low activity against the Gram negative.

Table 2. MIC values of lysine based surfactants

	MIC/μg mL^{-1}(μM)		
Surfactant	LLM	LALM	$C_6(LL)_2$
S. aureus	63	31	125
	(166)	(54)	(154)
E. coli	125	125	250
	(331)	(218)	(308)

MIC values of LLM, LALM and $C_6(LL)_2$ are smaller than those of CMC[19,20]. This suggests that their antimicrobial activity can be attributed to the interaction between compound monomers and the bacterial membrane.

In general, the antimicrobial activity of cationic surfactants depends on two factors. One is the amount of surfactant that diffuses from the bulk solution toward the bacterial membrane, which is governed by ionic interactions between opposite charges present on the surfactant and the bacterial outer membrane. The other factor we need to consider is the usually hydrophobic interactions between the surfactants adsorbed on the membrane wall and some membrane wall components.

Let us correlate surfactant behavior with respect to the DPPC simple membrane model with the antimicrobial activity measured by MIC values. First, recall that in the Binary Systems section we have discussed how interactions between DPPC and LLM, LALM and $C_6(LL)_2$ surfactants are essentially governed by hydrophobic interactions.

For LALM and LLM surfactants, the higher the ability to interact with the DPPC monolayer the higher their antimicrobial activity is, or equivalently, the smaller the MIC values.

Consider now the gemini surfactant $C_6(LL)_2$. We observed three facts. First, the π-A compression isotherms of DPPC/$C_6(LL)_2$ mixtures show that the gemini surfactant destroys the mixture monolayer, therefore we expected the surfactant $C_6(LL)_2$ to exhibit the highest antimicrobial activity. Second, the DPPC/$C_6(LL)_2$ BAM images showed fibrous structures forming multilayer assemblies, associated with fracture into multilayer structures that coexist with a bare interface resulting from the monolayer destruction. Measured MIC values where greater than those measured for LALM and LLM. This third fact clearly contradicts the first two. We will give a rationale for this behavior later on after considering the results yielded by

observing with Transmission Electron Microscopy the actual effect of $C_6(LL)_2$ gemini surfactant on the two tested bacteria.

Bacterial Viability

The effect of the lysine based surfactants on bacterial population was studied. Two surfactant concentrations were selected on a MIC basis: one was 50% greater than the corresponding MIC (3/2 MIC), the other was equal to the MIC. These concentrations were always lower than the corresponding CMC. Figure 8 shows the reduction of viable cells versus exposure time. No differences can be observed neither for the different surfactants nor for the different concentrations employed. In all assays, a complete inhibition of cell growth occurred consistently after approximately 30 minutes of contact, indicating that surfactants interact with bacteria right from the beginning of contact.

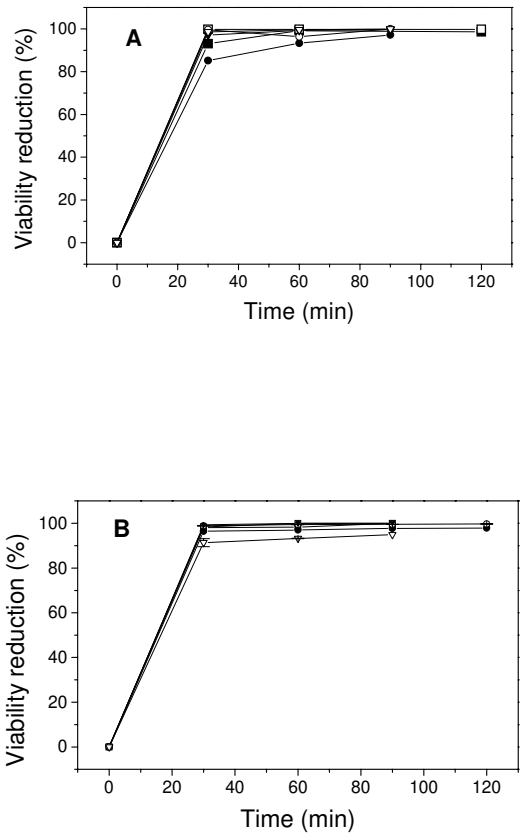


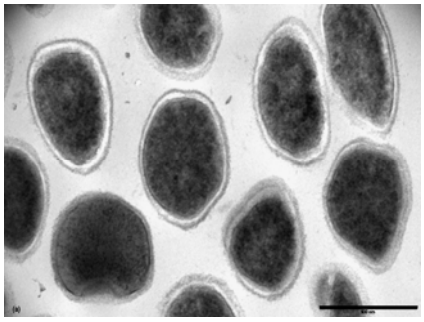
Figure 8. Percentage of viable bacterial cells versus time. *S. aureus* (A), *E. coli* (B). LLM MIC ■; LLM 3/2 MIC □, LALM MIC ●, LALM 3/2 MIC ○; $C_6(LA)_2$ MIC ▼; $C_6(LA)_2$ 3/2MIC ∇.

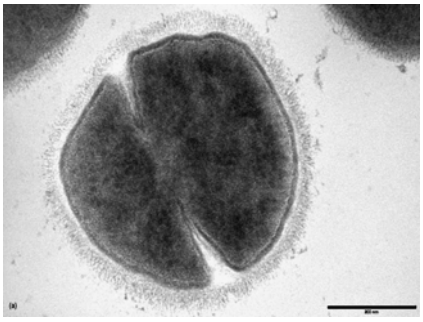
Transmission Electron Microscopy

Surfactants can interact with bacterial cells in three possible regions: the cell wall, the cytoplasmic membrane and the cytoplasm. It is well known that, in general, cationic surfactants interact with the cell wall.[43, 44] Surfactants begin their action covering the surface of the cell membrane by means of electrostatic forces, then interact with the hydrophobic part of the membrane causing distortions in the packing of the lipid membrane bilayer. These distortions involve the formation of pores and the consequent loss of ions and molecules, essential for the cell, after which death occurs. The effect of lysine based surfactants on bacterial membranes was evaluated on two bacteria, the gram-positive *S. aureus* and the gram-negative *E. Coli.*

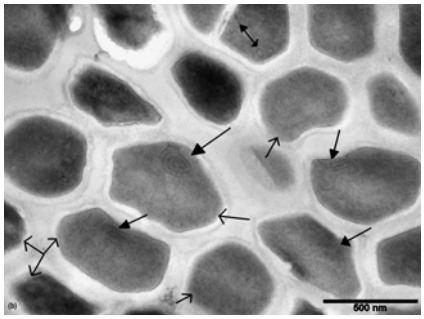
The Figure 9a shows electron micrographs of *S. aureus* alone which is used as control. The rest of electron micrographs in Figure 9 show the *S. aureus* after exposure to LLM, LALM and $C_6(LL)_2$ respectively.

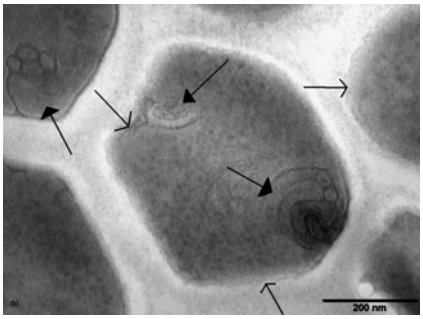
(a)



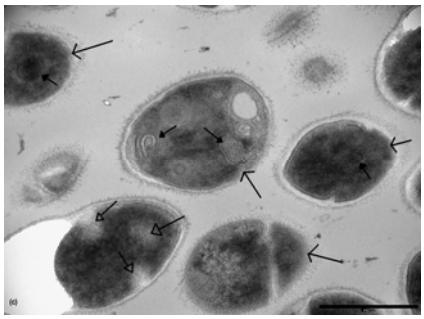


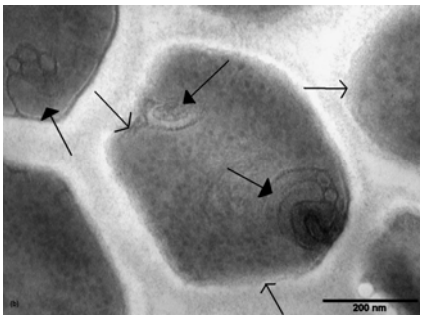
(b)



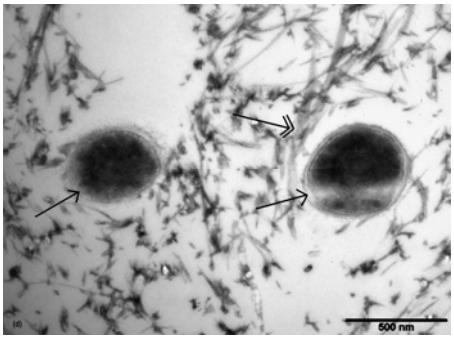


(c)





(d)



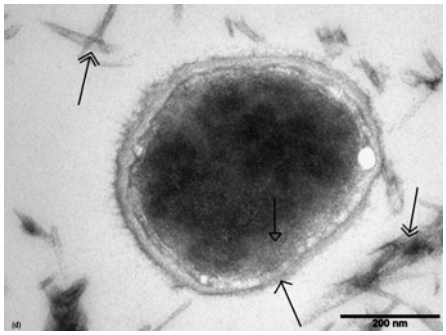


Figure 9. Effect of lysine based surfactants on *S. aureus* bacterial structure. (a) control, (b) LLM (c) LALM and (d) $C_6(LL)_2$. Time of contact was always 30 min and the surfactant concentration was the MIC.

The surfactant LLM (Figure 9b) caused morphological deformation yielding polygonal shapes. In Figure 7b, invaginations in the membrane and the typical deformations in the form of mesosomes near the cell membrane (black arrows) are clearly visible. The presence of fewer dark zones within the cell (open arrows) may be attributed to cytoplasmic dissolution. Similar results were obtained for a cationic arginine based surfactant[45] which, although it did not produce cell disruption, caused disturbance in the membrane potential as well as structural changes. The cellular stress caused by LALM is higher than the one caused by LLM, this observation is consistent with the MIC values. As illustrated in Figure 9c, LALM values for the number of mesosomes and folds, cytoplasmatic material dissolution (black and triangular open arrows) and cell wall disruption (open arrows) were higher than those shown by LLM.

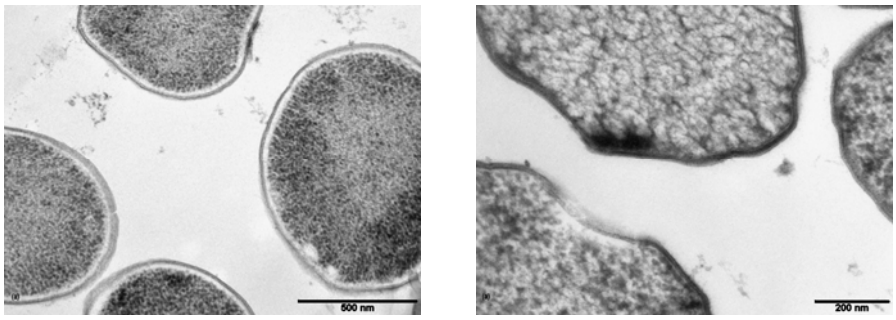
The lethal action of the $C_6(LL)_2$ gemini surfactant is apparent. Figure 9d shows low cellular density, a very high loss of cell integrity (open arrows) and completely lysed cells (double arrow), all of them indicate that severe cell damage has been produced.

Now we describe the effects of the LLM, LALM and $C_6(LL)_2$ surfactants on *E. coli*. As before, for control purposes, the first row of Figure 10 shows electron micrographs of *E. coli*. The rest of electron micrographs in Figure 10 show the *E. coli* after exposure to LLM, LALM and $C_6(LL)_2$ respectively.

Control cells of *E. coli* in the first row of Figure 10a show the cell wall and membrane that characterize this type of bacteria. As shown in Figure 10b, the presence of the surfactant LLM induces the formation of vesicles (double arrows). Moreover, within the cell appeared mesosomes and invaginations (open arrows) of the cell membrane along with dark zones within the cytoplasm (open arrows). Exposure of the bacteria to LALM (Figure 10c) resulted in severe damage in portions of the cell wall and cell membrane suggesting leakage of intracellular material (open arrows). In addition, cytoplasmic disintegration was evidenced by the presence of fewer dark areas in the cells and blebs (triangular arrows) which are visible on the outer membrane. The bacteria resulted severely damaged when exposed to the gemini

surfactant $C_6(LL)_2$ (See Figure 10d). The action of this surfactant was lethal exemplified by a massive loss of cell integrity.

(a)



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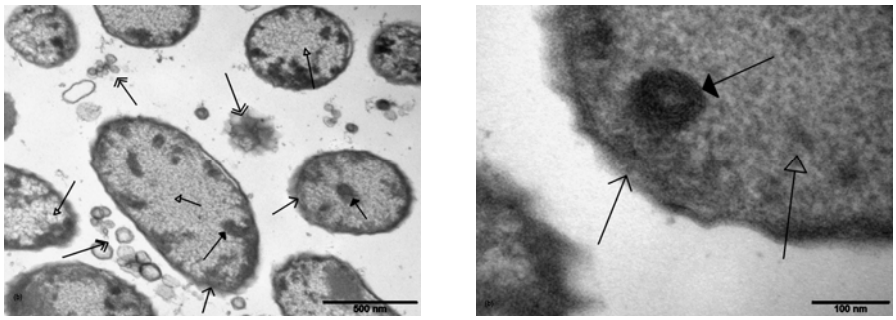
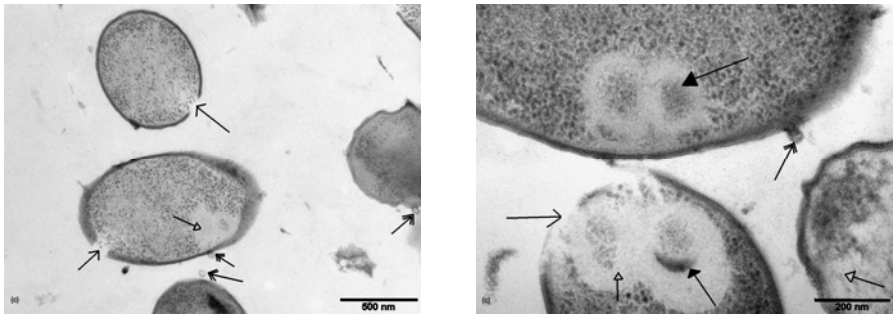


(c)



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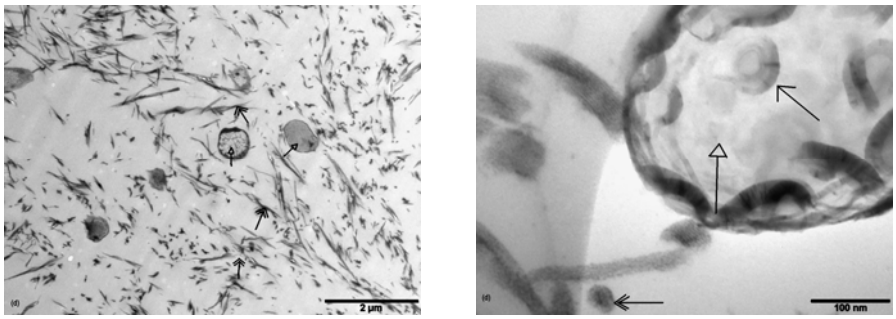

Figure 10. Effect of lysine based surfactants on *E. coli* bacterial structure. (a) control (b) LLM (c) LALM. (d) $C_6(LL)_2$. Time of contact was always 30 min and surfactant concentration was equal to MIC.

Electron micrographs of bacterial $C_6(LL)_2$ systems clearly show that the gemini surfactant causes a drastic damage to the membrane of the bacteria tested. Once this fact has been confirmed, we can give a rationale for the question raised in the Antimicrobial Activity section concerning the correlation between MIC values and antimicrobial activity of the gemini surfactant $C_6(LL)_2$.. High antimicrobial activity of $C_6(LL)_2$ was anticipated by its π-A compression isotherms resulting from measurements taken in the DPPC membrane model. However, MIC values suggested that the gemini surfactant´s antimicrobial activity should be rather weak. The unexpected high MIC value can now be attributed to the fact that the gemini surfactant´s solubility in the bacterial culture medium is low. Thus measured MIC values take into account the extra surfactant needed to allow it to reach and interact with the bacterial membrane.

CONCLUSIONS

We have studied the antimicrobial properties of a set of lysine-based surfactants: i) a monocatenary surfactant (LLM) with one alkyl chain and the lysine amino acid as polar group, ii) a monocatenary surfactant (LALM) which has one alkyl chain and the arginine and lysine polar groups and iii) a gemini surfactant ($C_6(LL)_2$) with two alkyl chains and two lysine amino acids in the polar group.

π-A compression isotherms of studied surfactants in NaCl 0.5 M solutions were evaluated at pH = 2, at pH = 12 and in pH = 6.7 (0.5M NaCl). Isotherms of LLM and LALM did not show significant differences and therefore ionic interactions play a secondary role. The monolayer

behaviour of mixtures should be governed by the sum of hydrophobic interactions and hydrogen bonding. Isotherms of $C_6(LL)_2$ changed with the pH, and for mixtures with DPPC ionic interactions besides hydrophobic and hydrogen bonding interactions in the monolayer must be taken into account.

The compression isotherms behavior for binary mixtures with DPPC depends on the structure of the lysine based surfactants. Gemini surfactants cause the highest expansion in the pure DPPC monolayer.

The surfactants studied show a moderate antimicrobial activity which depends up to a large extent on the surfactant structure. The antimicrobial activity weakens with the decrease of the polar head size and the number of alkyl chains, the gemini surfactant $C_6(LL)_2$ being the one with the strongest activity and LLM the weakest one.

The membrane model used based on the DPPC monolayer gives significant partial information about the interaction of surfactants with biological membranes but can not explain all the effects observed. This scenario agrees with previously reported results for other cationic surfactants.[46,47]

ACKNOWLEDGEMENTS

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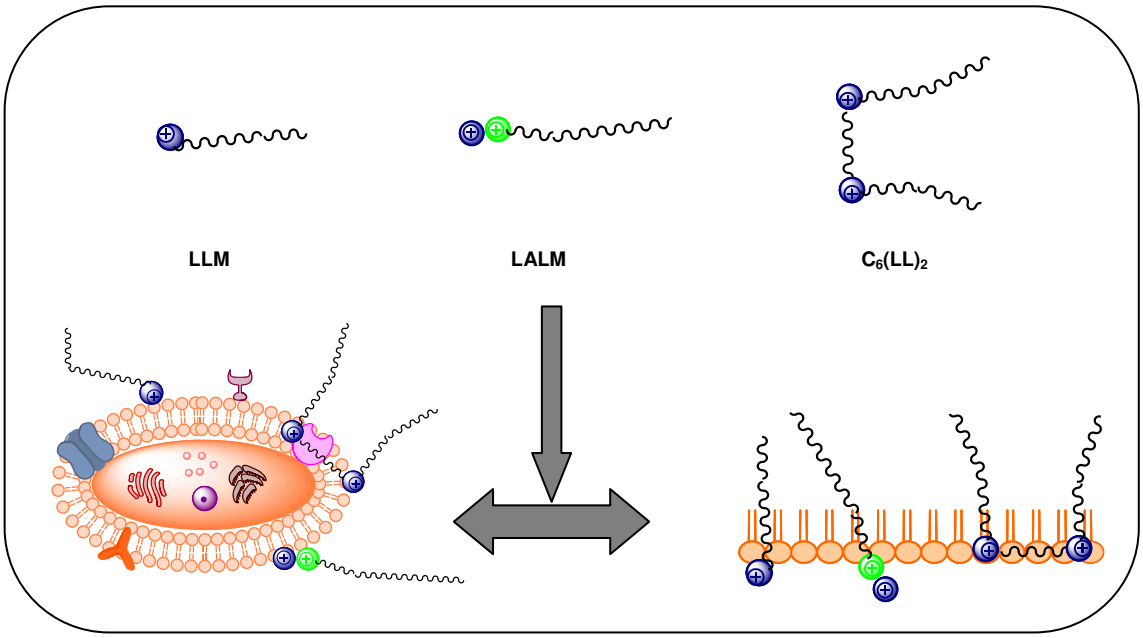

Schematic representation of the antimicrobial mechanism of three cationic lysine surfactants, LLM, LALM and C_6 $(LL)_2$