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pH SENSITIVE SURFACTANTS FROM LYSINE: ASSESSMENT OF THEIR CYTOTOXICITY AND ENVIRONMENTAL BEHAVIOR

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ABSTRACT

The toxicity and environmental behaviour of new pH sensitive surfactants from lysine are presented. Three different chemical structures are studied: surfactants with one amino acid and one alkyl chain, surfactants with two amino acids on the polar head and one alkyl chain and Gemini surfactants. The pH-sensitivity of these compounds can be tuned by modifying their chemical structures. Cytotoxicity has been evaluated using erythrocytes and fibroblast cells. The toxic effects against these cells depend on the hydrophobicity of the molecules as well as their cationic charge density. The effect of hydrophobicity and cationic charge density on toxicity is different for each type of cells. For erythrocytes the toxicity increases as hydrophobicity and charge density increases. Nevertheless, for fibroblast cationic charge density affects cytotocicity in a opposite way, the higher charge density the lower toxicity. The effect of the pH on hemolysis has been evaluated in detail. The aquatic toxicity was established using *Daphnia magna*. All surfactants yielded EC_{50} values considerably higher than that reported for cationic surfactants based on quaternary ammonium groups. Finally, their biodegradability was evaluated using the CO_2 headspace test (ISO 14593). These lysine derivatives showed high levels of biodegradation under aerobic conditions and can be classified as "readily biodegradable compounds".

INTRODUCTION

Surfactant intercalation into the cell membrane leads to changes in the membrane's molecular organization and increases membrane permeability that concludes with cell lysis. The potential use of surfactants in pharmaceutical and biological applications[1,2,3,4,5,6] makes the evaluation of cytotoxicity and environmental behaviour of great importance.

The toxicity of cationic surfactants is still an obstacle for the use of these compounds in biological applications. It is necessary to have a good understanding of the toxicity of these lipids before employing them in biological systems. Usually, cationic surfactants are composed of three basic domains: a positive polar head, a hydrophobic fraction and a linker between these two regions. Some of these surfactants have in the polar head functional groups capable of being protonated in acidic media while others, such as quaternary ammonium salts, exhibit a permanent positive charge.[7] Changes in the polar or hydrophobic domains can have dramatic effects on the toxicity of these molecules. In general the toxicity has been found to follow certain trends. For aliphatic single-chain cationic surfactants, the toxicity increases as the alkyl chain length increases[8,9] while for double-chain cationic surfactants the toxicity decreases as the alkyl chain length increases.[10] The double tail surfactants are less toxic than the single chain counterparts.[11] Nevertheless, several published results do not follow this general trend, for example, the single chain 6-lauroxyhexyl ornithinate presented lower cytotoxicity than the DOTAP (1,2-dioleoyl-3-trimethylammonium propane), a double tail cationic surfactant widely used in gene therapy.[12] Concerning the polar head, the toxicity of cationic surfactants is mainly governed by the cationic charge. The cationic charge can be situated on a quaternary ammonium group, on an amine group (primary, secondary or ternary amine) or delocalized in a heterocyclic ring or guanidine group. In general, quaternary ammonium based surfactants[13] are the most toxic. Surfactants that can spread the cationic charge in a heterocyclic or guanidine group[14] are the least toxic.

On the other hand, cationic lipids were generally found to have acute toxicity and poor biodegradability. Insufficient biodegradation of the chemical compounds increases the toxicity of chemicals given that aquatic micro-organisms are more time in contact with them. At present, the European Community demands greener products to avoid these undesirable effects on the environment.

The publishing results to date indicate that the effects of both the hydrophobic group and the polar head group on toxicity have not been adequately addressed. It is essential to generate significant data on structure-activity relationships.

Over the last years, our group has synthesized cationic surfactants from different amino acids. Amino acid based surfactants can be prepared from renewable raw materials and are characterized by their high biodegradability and low toxicity against aquatic micro organisms.[15,16,17,18] Regarding the toxicity against humans cells, these compounds show moderate toxic levels. This toxicity depends on the alkyl chain length and structure of the molecule.[19,20,21]

Recently, we have reported the synthesis and biological properties of different cationic lysine derivatives where the type of polar head group, the character of the spacer and the type and number of cationic charges on the head group region were systematically varied.[22] The alkyl chain of these surfactants has always been twelve carbon atoms except for one of the monocatenary surfactant that contains fourteen carbon atoms. Figure 1 shows the chemical structure of the prepared compounds.

In this work we have studied the influence of pH on the cationic charge of these molecules and the toxicity of these compounds against two types of eukaryotic cells: fibroblasts and erythrocytes. We have also determined the effect of pH on the hemolysis of these compounds. Moreover, the aquatic toxicity as well as the biodegradation of these compounds have been evaluated. The purpose of these studies was to approach to an explanation of how can affect the

different structural parameters on the toxicity and environmental behaviour of these surfactants. Determining how each of the structural components affects toxicity is of fundamental interest to rationalize the design of environmentally friendly cationic lipids with low cytotoxicity.

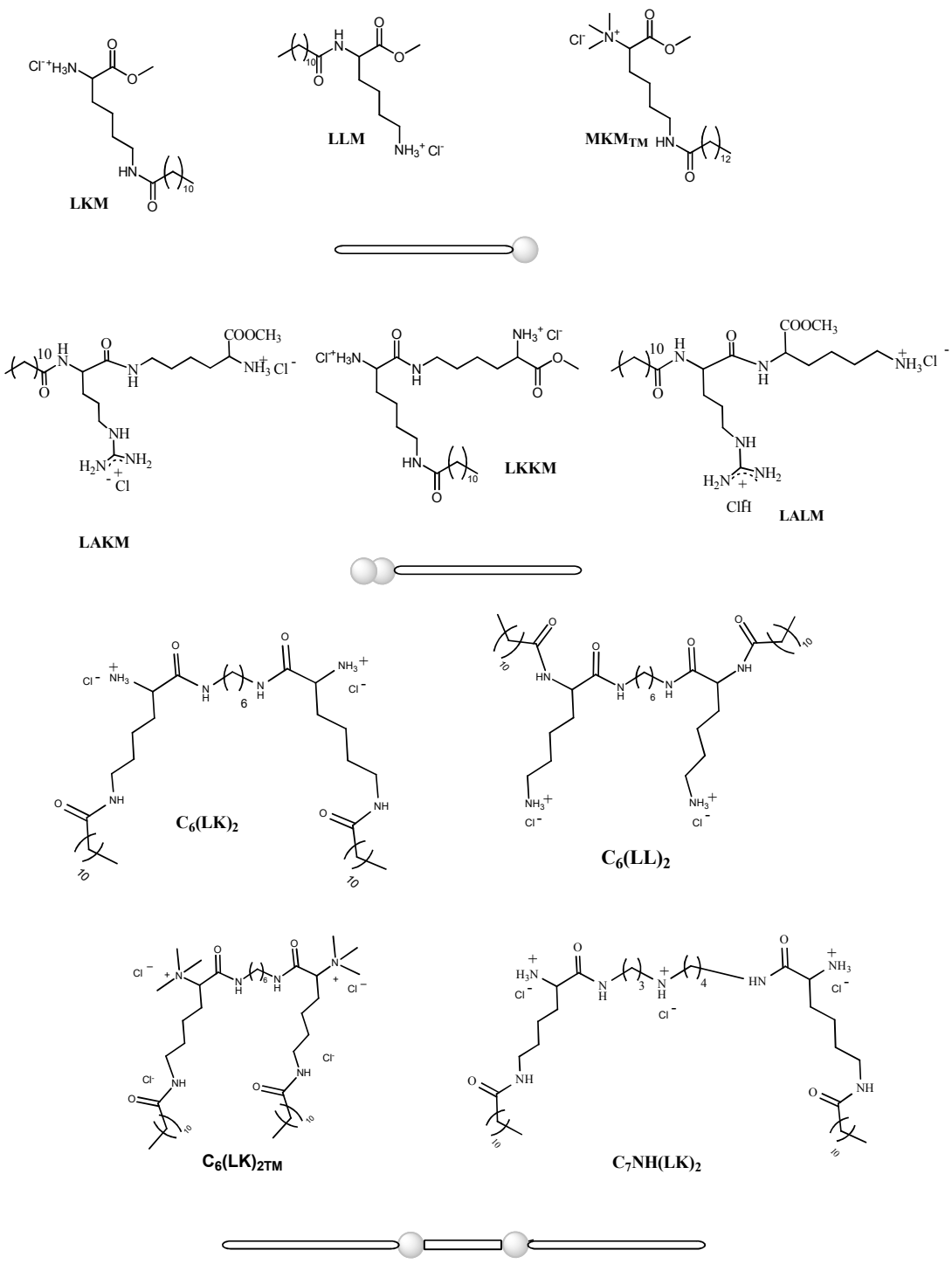


Figure 1. Chemical structure of cationic lysine surfactants.

RESULTS AND DISCUSSION

Physicochemical studies

All the cationic surfactants studied in this work, except the MKM_{TM} and $C_6(LL)_{2\ TM}$, have the cationic charges situated in amino protonated groups (Figure S1 in supplementary information). Depending on the pH value, in aqueous solution these surfactants can dissociate, losing the cationic charge. Therefore, knowledge of the pKa of these compounds is essential to explain their behaviour in aqueous solution. The pKa values of these surfactants have been determined by titration of aqueous solutions of surfactants with Na(OH) water solutions. Table 1 contains the pKa values that were obtained at the half-equivalence point in the titration curves (Figure S2 in supplementary information). In the case of the LALM, LAKM and the Gemini $C_6(LL)_2$, the titration curve showed two inflection points, this indicates that the two amino groups do not have identical acidic character.

Table 1. CMC and pKa values for the lysine derivative surfactants.

Surfactant	Cationic Charges on the polar head	Number of alkyl chains	CMC mM (pH)	CMC mM (Conductivity)	pKa
LALM	2	1	17 ± 5	25 ± 6	9.1 ± 0.1 /10.4 ± 0.1
LAKM	2	1	15 ± 4	26 ± 5	7 ± 0.1 / 9 ± 0.1
LKKM	2	1	15 ± 5	22 ± 5	6.8 ± 0.1
LLM	1	1	1.6 ± 0.8	7.2 ± 3.0	8.8 ± 0.1
LKM	1	1	2.3 ± 0.9	5.5 ± 2.1	6.1 ± 0.1
MKM_{TM}	1	1		2.2 ± 1.3	-
$C_6(LK)_2$	2	2	0.2 ± 0.08	0.50 ± 0.2	4.8 ± 0.1
$C_6(LL)_2$	2	2	0.7 ± 0.3	0.74 ± 0.4	7.4 ± 0.1 / 8.2 ± 0.1
$C_7NH(LK)_2$	3	2	2 ± 0.9	1.9 ± 0.7	6.9 ± 0.1
$C_6(LK)_{2TM}$	2	2		0.75 ± 0.3	-

Considering the pKa values of the **α** and **ε** amino groups of the lysine amino acid, 8.8 and 10.7 respectively[23] it can be stated that the introduction of one hydrophobic group in the amino acids

drastically reduces the pKa of their amino groups. The pKa of the **ε** amino group of the lysine in the LLM was 8.8 and the pKa of this same group in the Gemini surfactant counterpart $C_6(LL)_2$ was 7.4 and 8.2, that is, for both surfactants the pKa of the **ε** amino group has been reduced by 2/3 units. The same behavior was observed for the LKM and its Gemini counterpart $C_6(LK)_2$, the pKa of the **α** amino group in both cases has change in 3/4 units. Surfactants with two different amino acids on their polar head showed two pKa values, one corresponding to **α** or **ε** amino group of the lysine and the other to the guanidine group of the arginine amino acid.

The obtained pKa values for these surfactants went from 10.4 to 4.8, this means that they have weak acidic properties; consequently the cationic character depends on the pH. This behavior is common in amino acid based surfactants[24] as well as in other cationic surfactants in which the cationic charge is situated on a protonated amine group.[25,26] Surfactants with lower pKa values are those in which the positive charge is located on the alpha amino group.

Regardless of the position of the cationic charge, the acid character of these compounds increases with increasing hydrophobicity in the molecule. With increasing hydrophobicity the hydrophobic interactions are greater and the fatty chains become more tightly packed, consequently to avoid unfavorable repulsion forces of the cationic polar groups the surfactants release more protons, showing lower pKa values. These results agree with those reported by Spelios et al.[10], these authors attribute the decrease of the pKa value of the amino group to the reduced hydration of the cationic lipids compared to the free amine as well as the tight packing of the hydrophobic chains. The $C_7NH(LK)_2$ had only one pKa value despite having another amino protonated group in the spacer chain.

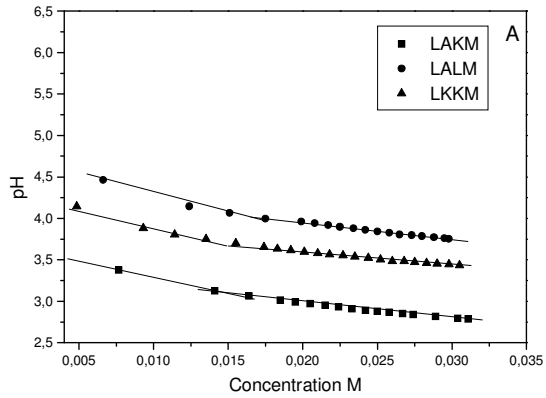
In view of the acidic properties observed it can be predicted that at high pH (the pH values depend on the surfactant alkyl chain length) the compounds lose the cationic charge, becoming nonionic surfactants, at low pH values they act as cationic surfactants while at intermediate pH values the aqueous solutions will contain cationic and non ionic species. It is noteworthy the

low pKa of the Gemini $C_6(LK)_2$ which indicates that this surfactant has only cationic character at low pH values.

These behaviors could be of interest in medicinal chemistry. Variations in the protonation state of pH-titratable head groups would lead to changes in the head group area, and as a result in their aggregation state. These kinds of surfactants have been shown to be efficient vectors for gene therapy because they improve the release of DNA into the cells and consequently the level of gene transfection may be augmented.[27,28,29]

Determination of Critical Micellar Concentration

Given the pKa of these compounds, the pH of the aqueous solutions of these surfactants is acidic, showing the loss of the cationic charge (Figure 2).



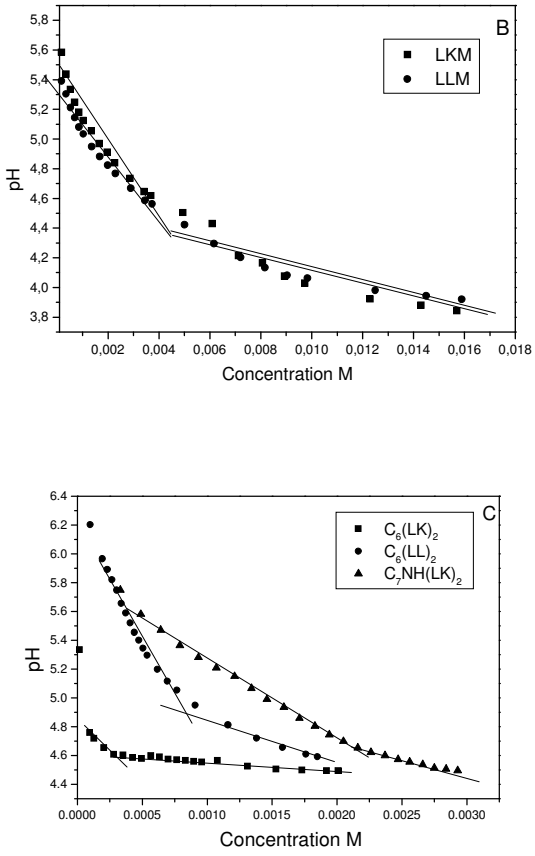


Figure 2: pH values against surfactant concentration for the lysine based surfactants. a) diamino acid surfactants, b) single chain amino acid surfactants and c) Gemini surfactants.

For these pH sensitive surfactants, pH measurements also provided a simple method for the determination of CMC. Table 1 shows the CMC obtained for each pH sensitive surfactant. At premicellar concentrations, the pH decreased as the concentration increased, after the CMC the pH continued decreasing but with lower intensity. The formation of micelles shifts the acid-base equilibrium toward the acid species that corresponds to the cationic charged surfactant. The electrostatic attraction between Cl and cationic micelles causes the neutralization of the molecule and reduces the capability of releasing the proton. Because that, table 1 also contains the CMC values obtained by conductivity measurements that have been carried out by a previously reported method. [22] For the trimethylated surfactants $(MKM)_{TM}$ and $C_6(LL)_{2TM}$, the determination of CMC was not possible by pH measurements given that the pH of these systems

does not depend on surfactant concentration.By conductivity it is possible to calculate the CMC of all cationic surfactants. It can be observed that CMC values obtained by pH measurements were in concordance with those obtained using conductivity.

As expected, because of higher hydrophilicity, diamino acid surfactants (LAKM, LALM, LKKM) showed the highest CMC values. The single tail surfactants have CMC values similar to those reported for monocatenary arginine cationic surfactants with the same hydrocarbon chain length[30] and lower than the CMC values corresponding to the commercial 12-carbon-straight-chain cationic surfactant such as dodecyltrimethylammonium bromide (DTAB).[31] Gemini surfactants had very low CMCs, the dimeric structure increases hydrophobic interactions giving rise to compounds with very low CMC and high efficiency by reducing the surface tension of water.[32] The results indicate that the micellization process is governed by the hydrophobic/hydrophilic balance and not by the type or density of cationic charge. For instance, LKM and LLM have very different pKa values which indicates that the protonation and consequently the density of cationic charge will also be different, even though the CMC of the two compounds is comparable.

Cytotoxicity

Despite the potential use of cationic surfactants in a wide range of pharmaceutical and biotechnological applications, structure-membrane toxicity relationships are poorly understood nowadays. In this work we have evaluated the influence of several structural parameters on the toxicity of cationic lipids against eukaryotic cells.

The interaction of these compounds with human cells has been evaluated using erythrocytes, which lacks internal organelles and is the most widely used cell membrane system to study surfactant–membrane interactions.[33]

Evaluation of the concentration that induces the hemolysis of 50% of the erythrocytes (HC_{50}) was determined and quantified from plots of percentage of hemolysis as a function of amphiphile concentration. Table 2 shows the HC_{50} values obtained for lysine derivatives and the

HC_{50} value obtained for the hexadecyltrimethylammonium bromide (HTAB), a widely used commercial cationic surfactant.

Table 2. Hemolytic activity (HC_{50}) and cytotoxic effect (IC_{50}) of lysine based-surfactants

	HC_{50}		IC_{50}	
	μg/mL	μM	μg/mL	μM
LALM	550 ± 60	962 ± 105	67.1 ± 6.6	117 ± 11
LAKM	452 ± 20	791 ± 35	97.9 ± 15.6	171 ± 27
LKKM	157 ± 33	290 ± 60	13.4 ±1.4	24.6 ± 2.5
LLM	75.4 ± 8	199 ± 21	5.4 ±0.1	14.2 ± 0.2
LKM	148 ± 26	391 ± 68	2.4 ± 0.1	6.3 ± 0.2
MKM_{TM}	23.4 ± 3	52 ± 7	22.2 ± 0.7	49.4 ± 1.5
$C_6(LK)_2$	93.9 ± 9	116 ± 7	3.4 ± 0.2	4.2 ± 0.2
$C_6(LL)_2$	384 ± 24	475 ± 29	4.1 ± 0.5	5.1 ± 0.6
$C_7NH(LK)_2$	10.5 ± 2	12 ± 2	63.6 ±7.0	72 ± 8
$C_6(LK)_{2TM}$	9.8 ± 1	11 ± 1	11.8 ±0.6	13.2 ± 0.7
HTAB	8.7 ± 1.2	14.5 ± 2	4.7 ± 0.9	8.7 ± 1.5

Results are expressed as Mean ± SEM of three independent experiments . Hemolys assays were carried out at pH =7.4 using phosphate buffer saline (PBS) and toxicity against 3T3 cells were determined at pH=7.4 in DMEM media.

It can be observed that the less active compounds were the single chain diamino acid surfactants while the most hydrophobic surfactants, the Gemini ones, presented the highest hemolytic activity. These findings indicate that the hydrophobicity and the capacity of forming micelles affect the toxicity levels of these cationic surfactants. The pattern observed here for lysine based-surfactants has also been described for bis(Quats) Gemini surfactants and their corresponding monoQuats.[34] However, different behaviour has been observed for quaternary ammonium surfactants with one and two alkyl chains. In this case the HC_{50} of the single chain derivative hexadecyltrimethylammonium bromide (HC_{50}=0.7 μM) is lower than that of vesicles formed by the double chain compound dioctadecyldimethylammonium bromide (HC_{50}= 4.0 μM).[35]

Among the diamino acid compounds, the LALM and LAKM presented lower hemolytic activity than the LKKM. The LKKM surfactant has two protonable amino groups with the same pKa (about 6.6) while the LAKM and LALM have unlike pKa values for every amino protonable group (7 and 9 for the LAKM and 9 and 10.4 for the LALM). At the physiological pH (7.4) it is expected that LALM has the guanidine group fully protonated and the ε lysine amino group almost fully protonated, consequently the molecule would display more or less two positive charges. The LAKM also has one positive charge on the guanidine group, but in this case the amino group of the lysine is not protonated at all, so that this compound contains more than 1 and less than 2 positive charges. Finally, at this pH the LKKM does not have any of the two amino groups fully protonated, then the density of cationic charge will be probably less than 1. This means that the hydrophobicity of the compounds shows the following trend: LKKM > LAKM > LALM. Considering all these observations, the results suggest that for this subgroup of surfactants the hemolytic activity increases as the hydrophobicity rises.

Within the group of monocatenary surfactants with only one amino acid, the hemolytic activity seems to be related with the cationic charge density exhibited at this physiological pH. Indeed, LKM, which correspond to the compound with the lower pKa value and consequently with the minor density of cationic charge, is the less hemolytic while MKM_{TM}, that always is fully protonated, shows the highest hemolytic activity. For these monocatenary surfactants toxicity seems to increase when the cationic charge density increases.

Gemini surfactants are very hydrophobic compounds with low CMCs and show, in general, higher hemolytic activity. It is well known that the Gemini structure enhances hydrophobic interactions, giving rise to surfactants that can aggregate at very low concentrations. The increase in the hemolytic character of Gemini surfactants could be due to the increase of these interactions. In our case, for the Gemini compounds, the most hemolytic surfactants are also those with higher charge density $C_6(LK)_{2TM}$ and $C_7NH(LK)_2$.

The activity of these surfactants against bacteria is different. Indeed, the antimicrobial activity of Gemini surfactants is lower to that of their corresponding Gemini counterpart.[22] Moreover, in general, except for C7NH(LK)2 and C6(LK)2, the lysine derivative surfactants possess antibacterial activity to Gram-positive bacteria at lower concentrations than those in which they show hemolytic activity.

The results obtained confirm that it is difficult to predict the hemolytic activity of surfactants, based only on their structure. The toxicity against red blood cells increases as the hydrophobicity increases. Morover, for molecules with similar hydrophobic character it has been observed that the number of cationic charges, the density of charge and the position of the cationic charges play an important role in the hemolytic activity of these compounds. But the influence of structural parameters is different for every type of chemical architecture. For monocatenary compounds with two amino acid on the polar head the most hemolytic was the LKKM and the less one was the LALM, this means that charge density does not affect and hemolysis increases as the hydrophobicity rises. Nevertheless, for the single chain amino acid surfactant with one lysine on the polar head and for Gemini surfactants the charge density affects hemolysis significantly; the MKM_{TM} and the $C6(LL)_{2TM}$ are by far the most hemolytic compounds.

Compared with other cationic lipids it is noticeable that these surfactants showed in general significantly lower hemolytic activity than the HTAB, a known biocide product (Table 2), and also lower than those reported for other cationic surfactants based on quaternary ammonium.[36-38]

Despite the potential use of cationic pH sensitive lipids in biological systems, structure-toxicity relationships of these compounds are poorly studied, especially the influence of pH on the toxicity of these compounds. For this type of surfactants the variation of pH promotes a variation in the protonation state that would lead to changes in their aggregation shape. These

changes can modify the interaction of the surfactants with the cells and consequently their toxic effects.

In this work we have analyzed the influence of the pH on the hemolytic activity of the lysine derivatives with two amino acids on the polar head as well as for Gemini surfactants. The hemolysis test has been carried out at four different pH values: 5.4, 6.4, 7.4 and 8.0. For these surfactants, the intrinsic ionization constant and the local pH environment will determine the percentage ionized. The ratio of protonated / non protonated species can be estimated using the mass action law:

$$pH = pK_a + \log \frac{[A^-]}{[HA]}$$

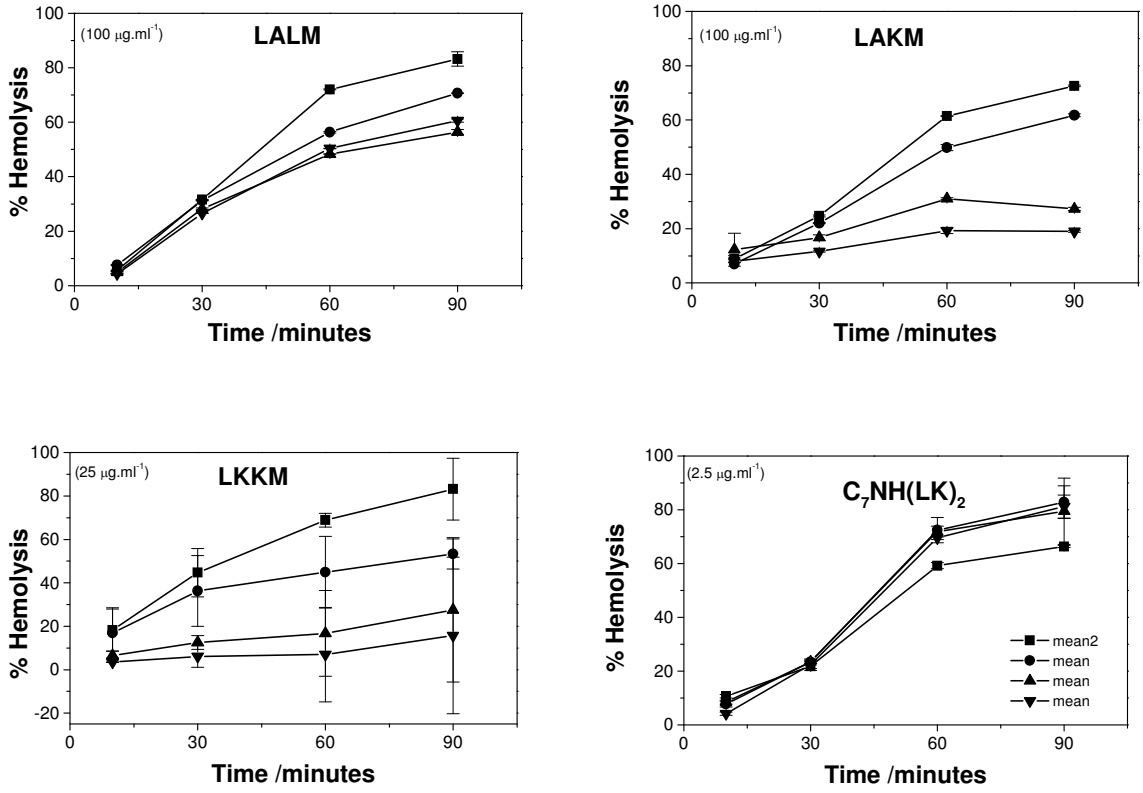
Table 3 shows the percentage of protonated molecules at the four pHs used to determine hemolysis. Surfactants with two different titratable groups such as LAKM or LALM show two pKa values and these amphiphiles can be double charged, mono-charged and less than mono-charged depending on the pH of the medium.

Table 3. Percentage of protonated surfactants at different pH values

Surfactant	pk_a	pH=5,4	pH=6,4	pH=7,4	pH=8,0
LKKM	6.8	96.1	71.5	20.0	5.9
LAKM	7/9	97.5 / 99.9	79.9 / 99.7	28.5 / 97.5	9.1 / 90.9
LALM	9.1/10.4	99.9 / 100	99.8 / 99.9	98.0 / 99.9	92.6 /99.6
$C_6(LL)_2$	7.4/ 8.4	99.0 / 99.9	90.9 / 99.0	50.0 / 90.9	20.0 / 71.5
$C_6(LK)_2$	4.8	20.0	2.45	0.25	0.06
$C_7NH(LK)_2$	6.7/ 6,9	95.2 / 96.9	66.61 / 75.9	16.6 / 20.4	4.8 / 7.3

It can be observed that the surfactants with the positive charge on the α amino group of the lysine have a low percentage of protonated molecules at pH higher than 6. In the case of $C_6(LK)_2$ the percentage of protonated molecules at the lowest pH studied is only 20%. For the

surfactants with two amino groups and different pKas for every group table 3 shows the percentage of protonation for every group. Notice that the pKa values of these surfactants have been calculated by titration of water solution of surfactants. The pKa is significantly affected by the ionic strength of the medium. Then the obtained values can deviate from those of the surfactants in the biological mediums used in the test. Usually the pKa increases as the ionic strength increases, in the case of Vectamidine, a pH sensitive surfactant with two amino groups the pKa varies from 4.6 and 8.8 (30 mM NaCl) to 5.2 and 9.4 (130 mM NaCl) respectively.[39] Thus the acid dissociation constants are approximate and table 3 gives a rough calculation of the different ratio of protonated and non protonated species that can be presented in the biological solution. It is expected that in the biological medium the pKa and consequently the percentage of protonated amino groups would be higher than that showed in table 3.



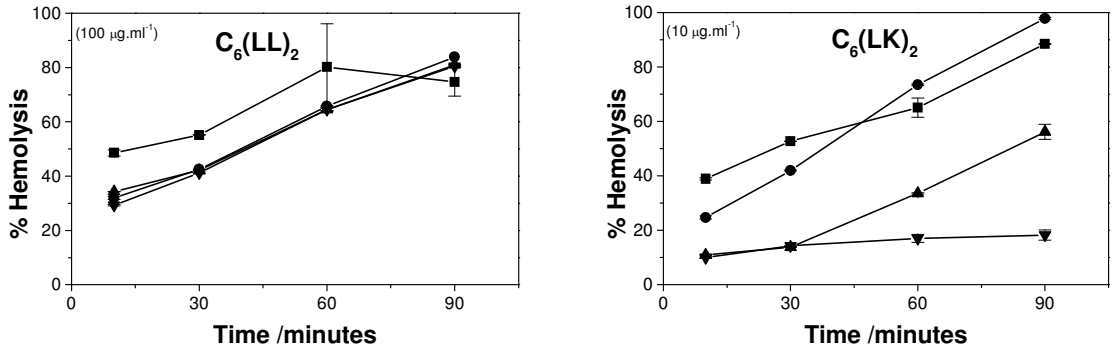


Figure 6: *Evaluation of the pH-sensitive hemolysis activity at different incubation times (-■- pH= 5,4; -●- pH=6,4;-▲- pH=7,4 y -▼- pH=8,0.)*

Figure 3: Evaluation of percentage of hemolysis at different pH´s after 90 minutes of incubation.

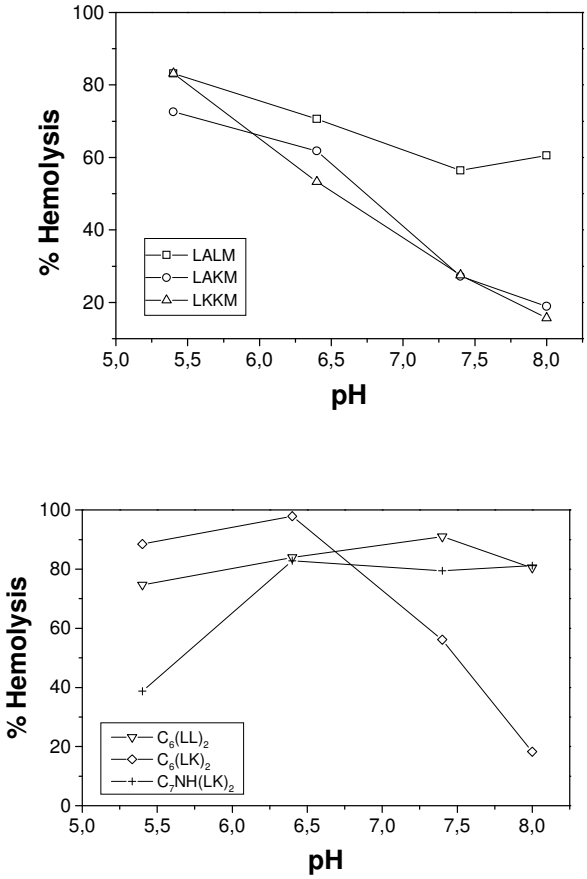


Figure 4: Evaluation of percentage of hemolysis at different pH´s after 90 minutes of incubation.

Figure 3 contains the percentage of hemolysis at different times for the four pH values and figure 4 shows the percentage of hemolysis at 90 minutes for the different pH values.

For surfactants with two amino acids on the polar head the percentage of hemolysis increased with time but the performance was different for every compound. The activity of LAKM and LKKM at pH 7.4 and 8 was very low compared with the activity at 5.4 and 6.4. Moreover the hemolysis of these compounds at high pHs was nearly constant with time. Gemini surfactants showed analogous behaviour, the $C_6(LK)_2$ had very different activity at the different pHs while for the $C_6(LL)_2$ it was obtained more or less the same activity at the four pH values. This behaviour can be discussed in terms of surfactant cationic charge and surfactant packing. Surfactants with high pKa values such as LALM and $C_6(LL)_2$ are greatly protonated at all the pHs tested, then hemolysis does not vary a lot with the pH. On the other hand, for surfactants with lower pKa values (LKKM or C6(LK)2) the percentage of protonated amino groups increases by a long way at reduced pH. When the pH-value decreases more amino groups are protonated, the surfactant becomes more hydrophilic due the cationic charge and the aggregation of the amphiphilic compound can vary. As a result of these changes the compounds have greater hemolysis. In a similar way, our group has described recently that the antimicrobial activity of these lysine derivatives with the positive charge in the ε amino group also increases at reduced pH.[22]

It is also interesting that polymerizable surfactants with pH-sensitive amphiphilicity are prepared based on the hypothesis that pH-sensitive compounds can be more effective in gene therapy. In these compounds the hemolytic activity also increases with decreasing the pH due to the higher number of protonated amino groups on the molecule.[40] This observation open the possibility of studying these lysine based-surfactants for this potential uses.

The cytotoxic effects of the cationic surfactants were evaluated with the 3T3 cells, a murine fibroblast cell line, with a colorimetric assay which measures the capacity of living cells to

metabolize a tetrazolium colorless salt to a blue formazan (MTT assay) as indirect measurements of cell viability. Cytotoxicity studies offer relevant information about the mechanism of toxic action and the suitability of 3T3 and MTT as endpoint to assess the basal cytotoxicity has been recently demonstrated.[41]

The results relative to the MTT assay are reported in Figure 5. All the cationic surfactants induced a clear dose-response relationship, which allow us to determine the half maximal inhibitory concentrations or IC_{50} (Table 2).

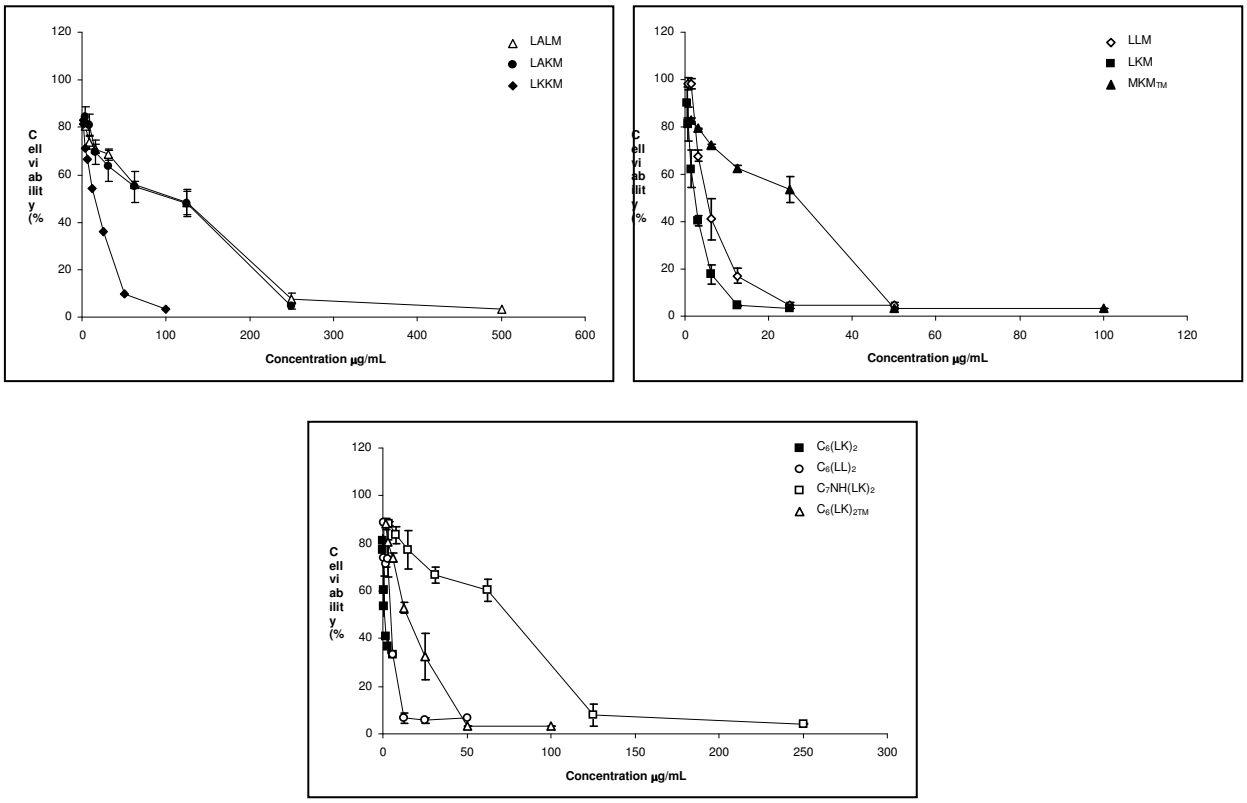


Figure 5: Concentration–response curves from 24-h exposure of the 3T3 fibroblasts to new lysine derivative surfactants. Data are expressed as mean ± S.E.M. of three independent experiments, performed in triplicate.

The IC_{50} values listed in Table 2 reveal that the surfactants derived from lysine present different cytotoxic levels. The types of cationic charge as well as the architecture of the molecules strongly affect the toxicity to mammalian cells. It has been established that ionic surfactants exhibit higher cytotoxicity than the non ionic ones, and that cationic surfactants are more potent than their anionic counterparts.[42] This behavior has been observed using both *in vivo* and *in vitro* assays.[43]

The way in which hydrophobicity and cationic charge density affects toxicity against 3T3 cells and hemolytic activity is very different, because different mechanisms of action are evaluated. It can be observed that the less toxic compounds against both cells are those with two amino acids on the polar head. Nevertheless, monocatenary compounds with only one lysine on the polar head and Gemini surfactants show similar cytotoxicity against 3T3 but very different hemolytic behavior. Moreover, the most noticeable is that trimethylated surfactants, MKM_{TM} and $C6(LL)_{2TM}$ are the most hemolytic compounds but are the less cytotoxic as expressed by the higher IC_{50} values. This means that cationic charge density affects cytotoxicity in an opposite way as described for hemolysis, the higher charge density the lower toxicity. Moreover, in this case, the activity of surfactants can not be related to an increase in surfactant's hydrophobicity as observed in the case of hemolytic behavior. These differences should be attributed to the cellular toxic mechanisms evaluated by the two biological assays employed in this study. Hemolysis is centered in the interactions of surfactants with plasmatic membranes. The MTT assay determines the metabolic activity that occurs in the mitochondrial compartment but also the reduction by oxido-reductase-type enzymes in microsomal and cytosolic fractions.[44] Thus, the MTT assay appears to be more sensitive in detecting cell damage and our results suggest that the mechanism of toxicity exerted by these surfactants involves an early effect on the metabolic activity of the cells, while plasma membrane could be affected at a later stage.

In general the toxicity of these single tail lysine surfactants is similar than that reported for single chain cationic alkyl trimethyl ammonium bromide, with IC_{50} values from 4.8 $\mu g.mL^{-1}$ for the C_{10} derivative to 1.6 $\mu g.mL^{-1}$ for the C_{16} derivative.[45] In a similar way, we have reported in a previous work that cytotoxicity of monocatenary surfactants from arginine also rise as the alkyl chain increased.[46]

The molecular weight of the Gemini surfactants is considerably higher than that of the single alkyl chain derivatives. ~~K.P~~. Wilhelm et al, [47] reported that for compounds with high molecular

weights the toxicity can be overestimated using *in vitro* tests. These authors compared the toxicity of different compounds using an *in vitro* method and an *in vivo* assay and they found a good correlation between *in vitro* and *in vivo* citotoxicity for numerous compounds except for those with molecular weights ≥1000 $g.mol^{-1}$.

These lysine Gemini compounds show comparable toxicity against mammalian cells than cholic-acid-based amphiphiles that also contain in their molecules one or more protonable amino groups,[48] 1,6,hexanediyl bis(dimethyldodecylammonium) bromide (IC_{50} 12 μM) and Gemini surfactants of pyridinium with C_{12} alkyl chains (IC_{50} 15.7 μM).[49]

Notice that except the MKM_{TM}, all the other lysine derivatives studied in this work have alkyl chains of 12 carbons atoms. In the case of single chain surfactants it is expected that the toxicity increases with longer alkyl chains. Nevertheless, in the case of Gemini surfactants it would be probable that the toxic levels decrease with higher alkyl chains. In fact, the toxicity of pure dialkyldimethyl ammonium bromides[50], pure diacyl glycerol arginine and pure Gemini pyridinium surfactants[49] decreases as the alkyl chain length increases. In the case of pH sensitive surfactants with two alkyl chains the lipids with two 12 alkyl chains were poorly tolerated while the homologues with hydrophobic tails from 14 to 18 showed cell viability greater than 60%.[10] In fact, the toxicity against fibroblast of vesicles prepared with pure quaternary ammonium compounds with two alkyl chains of 18 carbon atoms, dioctadecyl-dimethyl-ammonium bromide, is lower than that of these lysine surfactants. The dioctadecyl-dimethyl-ammonium bromide cationic vesicles are non toxic below 1 mM.[51]

It is clear that Gemini cationic surfactants have toxic effects at low concentrations but the toxic effects occur at concentrations above the ones normally used in biological and pharmacological applications. On the other hand it has been reported that factors present in human serum reduce the cytotoxicity of active compounds in some human cells.[52]

The toxicity of cationic lipids can be reduced using vesicular systems; in fact vesicles are commonly used in cosmetics and pharmacy as vehicles for active agents. Cationic lipid vesicles formed by pure cationic lipids can be very toxic but the toxicity can be reduced adding another compound to the vesicular systems. For example the CTAB is a cytotoxic surfactant (IC_{50} lower than 10 $\mu g.mL^{-1}$) for different cell lines, but when cationic vesicles were formed by CTAB-SDS, cell survival was higher than 60% for a concentration of 75 μM.[53] Moreover, for some cationic lipids such as Dioctadecyl-dimethyl-ammonium bromide cytotoxicity towards human cells is drastically reduced when this surfactant is included in cationic vesicles formed by cholesterol and phosphatidylcholine.[54]

Biodegradability assessment

Biodegradation of surfactants is an important criterion to evaluate these products. The quick and complete biodegradation of chemicals after use is needed in terms of the establishment of green and sustainable chemistry. Biodegradation is the most important mechanism for the irreversible removal of chemicals from aquatic and terrestrial environments. It may be defined as the destruction of chemical compounds by the biological action of living organisms. The aerobic ultimate biodegradability of these lysine derivatives was evaluated by applying the ISO 14593-CO_2 headspace test. In this test, the ultimate biodegradation or mineralization of the surfactants (i.e., the microbial transformation of the parent chemical into inorganic final products of the degradation process, such as carbon dioxide, water, and assimilated biomass) was evaluated. A surfactant is considered as readily biodegradable if the biodegradation level exceeds 60% within 28 days in the test.

Biodegradation percentages of all cationic surfactants studied in this work exceed the specified biodegradation pass level in this test (60%), classifying them as readily biodegradable compounds (Figure 8). The conditions in this test are so stringent (relatively low density of not preadapted bacteria, relatively short duration, and absence of other sources of organic carbon)

that chemicals exceeding the specified biodegradation level will rapidly and completely biodegrade in an aquatic environment under aerobic conditions. These good results contrast with the low biodegradation level that usually reaches the cationic surfactants from quaternary ammonium[55] and are similar to the findings on cationic surfactants from arginine amino acids.[56] In all cases, degradation started immediately and no induction period was detected. The single chain derivatives LLM and LKM rapidly degraded and reached 60% in 14 days and about 70% after 28 days. Surfactants with two amino acids on the polar head reached the threshold value of 60% in 20 days but the biodegradation rate was the same after 28 days. Finally, the Gemini surfactants can also be considered as readily biodegradable compounds but they needed more time to reach the 60% of degradation.

These good biodegradation levels could be due to the amide linkage between the polar head and the hydrophobic part of the molecules as well as to the low toxicity of initial compounds and intermediates. Usually, the introduction of an ester or amide linkage between the alkyl chain and the hydrophilic moiety improves the biodegradation of surfactants.[57] One of the strategies used by bacteria to access the carbon in surfactants is the initial separation of the hydrophilic group from the hydrophobic part (hydrophile attack). The amide linkage between the alkyl chain and the lysine is readily attacked by microorganisms and then the fatty acids follow the pathway of chain-shortening through fatty-acid β-oxidation[58] and microorganisms completely degrade the natural occurring lysine. This estimated biodegradation pathway indicates a safe biodegradation process given that neither the initial compounds nor the intermediates are toxic products. Cationic ester surfactants based on quaternary ammonium are biodegradable compounds but the Gemini homologues do not reach the 60% of biodegradation after 28 days despite having ester linkages between the polar head and the alkyl chain.[59] The low biodegradation of these bisQuats was attributed to the low biodegradability of the degradation intermediates having two ammonium groups. Gemini amphoteric surfactants with ammonium groups on the polar head[60]

also presented biodegradation levels lower than 60% and ionic liquid containing ammonium groups also showed poor biodegradation.[61] In the Gemini surfactants presented in this work the polar head is linked to the spacer chain through amide linkage and the cationic hydrophilic moiety does not contain ammonium groups. These structural features improve biodegradation levels.

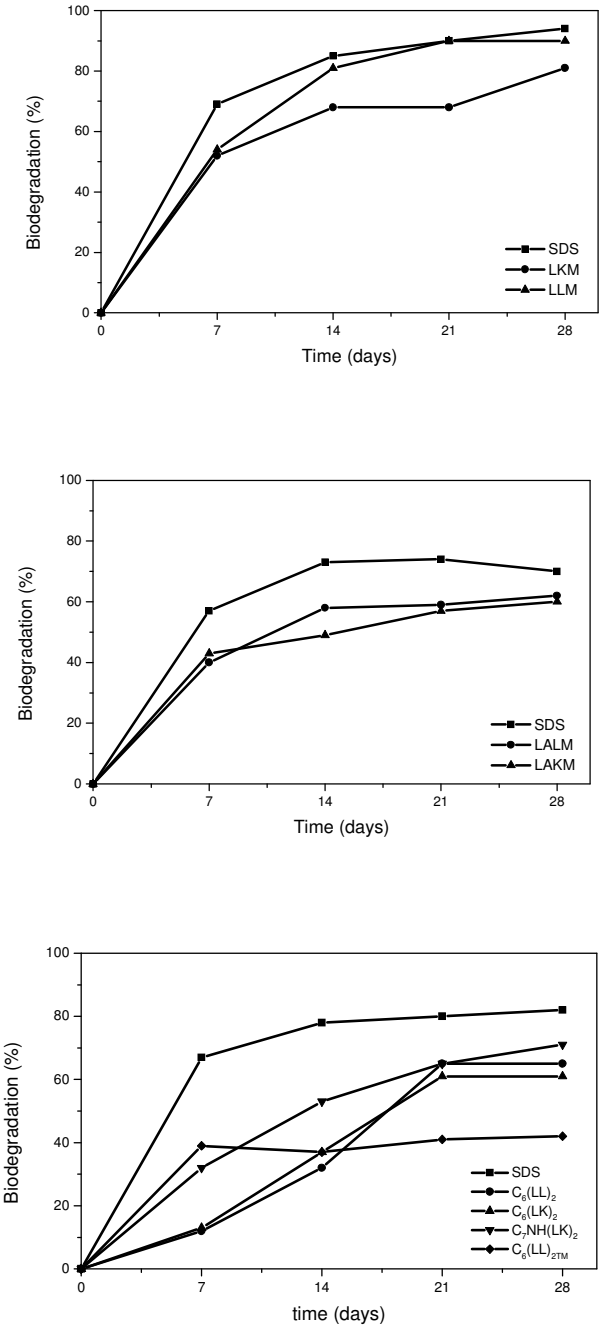


Figure 8. *Biodegradation curves of lysine based surfactants*

From the results obtained it can be stated that the pKa and density of cationic charge does not affect the biodegradation process of these surfactants. This process is mainly governed by the structure type. The single chain structures with only one amino acid are the compounds that degrade more rapidly. They consist of one alkyl chain and one amino acid, the microorganisms separate these two parts and can degrade quickly the fatty acid and the amino acid. The Gemini surfactants are the most complicated structures, micro-organisms can separate the alkyl chains but the polar head still has two amino acids and one spacer. For this reason, Gemini surfactants need more time to reach the pass level needed to be considered readily biodegradable compounds.

Aquatic toxicity

Cationic surfactants have in general higher aquatic toxicity than other surfactants. It has been shown that the toxicity of surfactants against aquatic species is caused by the ability of the monomers to interact strongly with negatively charged surfaces including the lipid membranes of cells. The results of *Daphnia magna* 24-h immobilisation tests (IC_{50}) of the investigated surfactants are given in Table 4. The lower the IC_{50} value, the higher the toxicity of the compound.

The acute toxicity of the cationic surfactants from lysine was clearly lower than the toxicity reported for conventional monoquats.[17] The results in table 4 indicate that it is not easy to establish trends in the comparative toxicity of these surfactants. Single chain compounds with two amino acids are in general less toxic than the other lysine derivatives. The introduction of two amino acids on the polar head reduces lipophilicity and as a result the toxicity decreases. The toxicity of the single chain compounds with one lysine on the polar head depends on the type of cationic charge. MKM_{TM} showed the lowest EC_{50}, this higher toxicity could be due to the quaternary ammonium group in the polar head. It is noticeable that LLM was clearly less

toxic than LKM. LLM was not toxic at 50 mg/L, the highest concentration tested, while LKM was toxic at lower concentrations. Nevertheless, LLM was clearly more toxic than LKM against erythrocytes and fibroblasts. The density of cationic charge affects the toxicity against these aquatic microorganisms in different way.

Table 4. IC_{50} values against *Daphnia magna*

Surfactant	IC_{50} (mg/L) (48 hours)	
	mean value	CI 95%
LKM	7,4	6,9-8,0
LLM	>50	-
MKM_{TM}	4,1	3,1-5,5
LALM	>40	>40
LAKM	26	22-32
LKKM	40	35-50
$C_6(LL)_2$	---	
$C_6(LK)_2$	---	
$C_7NH(LK)_2$	4,0	3,5-4,5
$C_6(LL)_{2TM}$	2,9	1,8-3,8

More interesting was the behaviour of Gemini surfactants. Single chain surfactants presented a clear dose response relationship (Figure 9a) while for $C_6(LL)_2$ and $C_6(LK)_2$ the toxicity did not increase with increasing the concentration (Figure 9b). This behavior could be related with the aggregates formed by these surfactants. The CMC of the single chain derivatives is lower than their corresponding IC_{50} values, and then the toxicity is exerted by the monomers in solution. Gemini surfactants have CMCs one order of magnitude lower than the corresponding monomeric compounds. This means that the highest concentration of monomers in solution is much lower. Gemini monomers are more toxic than their corresponding single chain homologues due to the higher hydrophobic character. Nevertheless, the concentration of Gemini monomer is always very low given their low CMCs. Because of that, at low concentrations,

Gemini present toxic effects but these remain in the same order when the concentration increases because the concentration of monomers remains constant. On the other hand, Gemini surfactants tend to form higher aggregates that usually have more difficulties to interact with biological membranes.[32]

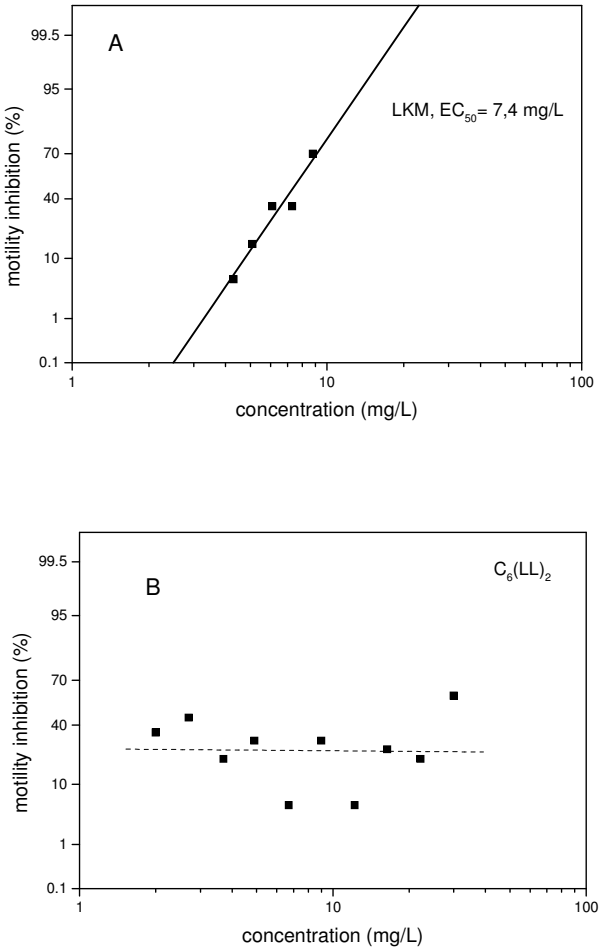


Figure 9. Mobility inhibition of Daphnia magna at different concentrations of LKM(a) and $C_6(LL)_2$ (b).

MATERIALS AND METHODS

Materials: DMSO was purchased from Fluka. Sodium dodecyl sulphate (SDS), NaOH, HCl, NaCl, Na_2HPO_4 and KH_2PO_4 were purchased from Merck (Darmstadt, Germany). TEGO Betaine T-50 (TGB) was obtained from Goldschmidt Ltd. (Essen, Germany). Dulbecco´s Modified Eagel´s Medium (DMEM), L-Glutamine (200 mM), trypsin/EDTA solution (170.000

U.L^{-1} of trypsin and 0.2 g.L^{-1} of EDTA), sterile phosphate buffer solution (PBS), antibiotic solution penicillin (10,000 U.mL^{-1})-streptomycin (10,000 µg.mL^{-1}) and fetal bovine serum (FBS) were obtained from Lonza (Verviers, Belgium). The tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and the HTAB was from Sigma – Aldrich (St. Louis, MO, USA). The 75 cm^2 flasks and 96-well plates were purchased from TPP (Trasadingen, Switzerland).

All the cationic surfactants from lysine studied in this work have been synthesised using a previously reported method. [22] The purity of all compounds has been determined using HPLC and combustion analysis; the two techniques confirm that the purity of surfactants is higher than 95%.

Analytical data for the lysine derived surfactants:

LALM: HPLC, t_r= 11.4 min. **Elem. Analy.** Found: C 47.2, H 9.28, N 14.0, Cl 11.7 Calc. for $C_{25}H_{52}Cl_2N_6O_4{\cdot}3H_2O$, C 48.0, H 9.3, N 13.5. **LAKM: HPLC**, t_r= 11.4 min. **Elem. Analy**. Found: C 49.0, H 9.1, N 13.9, Cl 12.3 Calc. for $C_{25}H_{52}Cl_2N_6O_4{\cdot}2H_2O$, C 49.5, H 9.2, N 13.8, Cl 11.7. **LKKM: HPLC**, $t_{r=}$ 12.8 min. **Elem. Analy**. Found: C 47.2, H 9.28, N 14.0, Cl 11.7 Calc. For $C_{25}H_{52}Cl_2N_6O_4$ ·3H2O, C 48.0, H 9.3, N 13.5, CL 11.2. **LKM: HPLC**, t_r=14.8 min. **Elem. Analy.** Found: C, 57.02; H, 11.20; N, 7.27; Cal. for $C_{19}H_{39}O_3N_2Cl$ ·1.2 H_2O, C, 56.99; H,10.35; N, 6.99. **LLM: HPLC**, t_r=14.8 min.**Elem. Analy.** Found: C, 59.30; H, 10.40; N, 7.25; Cal. for $C_{19}H_{39}O_3N_2Cl$, C, 60.2; H,10.37; N, 7.39. **$C_6(LL)_2$: HPLC**, t_r= 17.6 min; **Elem.Analy. Found**: C 60.8, H 10.6, N 10.0, Cl 8.3 Calc. for $C_{42}H_{86}N_6O_4Cl_2{\cdot}1.5H_2O$, C 60.2, H 10.6, N 10.1, Cl 8.4. **$C_6(LK)_2$: HPLC**, t_r= 17.8 min; **Elem.Analy. Found**: C 56.6, H 10.4, N 9.6, Cl 9.0 Calc. for $C_{42}H_{86}N_6O_4Cl_2{\cdot}4H_2O$, C 57.1, H 10.6, N 9.5, Cl 8.0. **$C_7NH(LK)_2$: HPLC**, t_r=18.7min; **Elem.Analy. Found**: C 55.5, H 10.3, N 10.3, Cl 12.0 Calc. for $C_{42}H_{86}N_6O_4Cl_2{\cdot}2.5H_2O$, C 56.0, H 10.3, N 10.6, Cl 11.5. **$C_6(LK)_{2TM}$: HPLC**, t_r= 20.5 min. **Elem.Analy. Found**: C 58.2, H 10.9, N 8.3, Cl 7.2 Calc. For $C_{48}H_{98}N_6O_4Cl_2{\cdot}5H_2O$, C 58.5, H 10.9, N 8.5, Cl 7.2.

Acid-base titration. pKa values were determined with a pH electrode (Model 8102 ROSS Thermo Orion, Beverly, USA) at 25 ºC under nitrogen gas atmosphere and magnetic stirring. Aqueous surfactant solutions (1.5 mL) of 5 mM were titrated with aqueous Na(OH) solution of the same concentration. The experiment was twice repeated.

CMC by pH measurements. The pH of different concentrations of surfactant water solutions under nitrogen gas was measured using a pH electrode (Model 8102 ROSS Thermo Orion, Beverly, USA). Measurements were made at increasing concentrations of surfactant to minimize errors from possible contamination from the electrode.

Cytotoxicity evaluation on 3T3 cells.

Culture of 3T3 cell line. Murine Swiss albino 3T3 (ATCC CCL92) fibroblast cell line was grown in DMEM culture media containing 10% (v/v) of FBS, 2 mM glutamine and antibiotics (penicillin 100 $U.mL^{-1}$ and streptomycin 100 $\mu g.mL^{-1}$). The cells were harvested when 80% confluent with trypsin/EDTA and seeded 100 µL per well from a suspension of a density of 8.5 $x10^4$ $cell.mL^{-1}$, into the central 60 wells of a 96 well-plate and allowed to adhere overnight at 37 ºC in the CO_2 incubator.

Chemical exposure. Stocks solutions of surfactants were prepared in DMSO and sterile PBS (pH 7.4). Serial dilutions were subsequently prepared in DMEM medium supplemented with 5% FBS, 2 mM L-glutamine, and 1% antibiotic solution (final concentration of DMSO in culture medium < 1%)**.** After removal of the medium culture, 3T3 cells were exposed to the surfactants in multiple plates. Controls containing only culture medium were included in each plate and they were independent for each of the different surfactants tested. Plates were incubated at 37 ºC, 5% CO_2 for 24 hours.

MTT assay: the cytotoxicity of surfactants against 3T3 fibroblast cells was determined using a tetrazolium-based assay.[62] MTT was dissolved in PBS (5 $mg.mL^{-1}$) and added to the cells in a 1:10 dilution in medium without phenol red and serum. After the treatment with surfactants,

medium was removed and replaced by MTT solution (100 µL per well) and the plates were incubated for 3 hours at 37 ºC and 5% CO_2. Thereafter, cultures were washed with PBS, and dimethylsulphoxide (100 µL per well) was added to dissolve the purple formazan product while shaking for 10 minutes at room temperature. The absorbance of the resulting solutions was read at 550 nm wavelength in a Bio-Rad 550 microplate reader. Results are expressed as percentage of control.

Hemolysis assay. We followed an adaptation of the method described by Pape et al. (1987).[63] A series of different volumes of surfactant solution (1-10 $mg.mL^{-1}$), ranging from 10 to 80 µL, were placed in polystyrene tubes, 25 µL of erythrocyte suspension and phosphate buffered saline were added to each tube, and PBS solution was finally added to a total volume of 1 mL. Samples were incubated at room temperature while shaking for 10 minutes. Following incubation, the tubes were centrifuged (5 min at 5000 rpm). The percentage of hemolysis was determined by comparing the absorbance (540 nm) of the supernatant of the samples with that of the control totally haemolysed with distilled water. Concentration-response curves were determined from hemolysis results and the concentrations inducing 50% hemolisys (HC_{50}) were calculated.

Preparation of erythrocyte suspension. Erythrocytes were obtained from the blood of healthy volunteers with previous consent. They were washed three times in a phosphate buffer isotonic saline (PBS), containing 5.6 $mmol.L^{-1}$ KH_2PO_4, 22.2 $mmol.L^{-1}$ Na_2HPO_4 and 123.3 $mmol.L^{-1}$ NaCl, in distilled water (pH = 7.4). The erythrocytes were then suspended in PBS at a cell density of 8 $x10^9$ $cell.mL^1$.

pH – Sensitive hemolysis. Surfactants were dissolved in PBS with pH adjusted to 5,4; 6,4; 7.4 and 8.0 with HCl or Na(OH) solutions. Sample solutions (10 mL) were incubated at room temperature while shaking for 90 minutes. Following incubation times of 10, 30, 60 and 90 minutes, aliquots of 1 mL were taken and centrifuged 5 minutes at 5000 rpm. The absorbance of

the supernatant was measured at 540 nm in a spectrophotometer. The percentage of hemolysis was determined by comparing the different absorbance of the samples with that of the positive control totally haemolysed with distilled water. Hemolysis percentage – time curves at different pHs were calculated from hemolysis results.

Biodegradation. ISO 14593 - CO_2 Headspace test. To evaluate the biodegradability of new surfactants, the "CO_2 Headspace" test (ISO 14593, OECD 310) was applied. This method allows the evaluation of the ultimate aerobic biodegradability of an organic compound in an aqueous medium at a given concentration of microorganisms by analysis of inorganic carbon. The tested surfactant, as the sole source of carbon and energy, was added at a concentration of 40 $mg.L^{-1}$ to a mineral salt medium. These solutions were inoculated with activated sludge collected from an activated sludge treatment plant, washed and aerated prior to use and incubated in sealed vessels with a headspace of air. Biodegradation (mineralisation to carbon dioxide) was determined by measuring the net increase in the total organic carbon (TOC) levels over time compared with unamended blanks. Sodium *n*-dodecyl sulfate (SDS) was used as reference substance. The test ran for 28 days. The extent of biodegradation was expressed as a percentage of the theoretical amount of inorganic carbon (ThIC) based on the amount of test compound added initially.

***Daphnia magna* test**. To determine aquatic toxicity, the *Daphnia magna* acute toxicity assay [64] was carried out. Laboratory bred *Daphnia magna*, less than 24 hours old, were used in this test, where the swimming incapability is considered the end point. The pH of the medium was 8.0 and the total hardness was 250 $mg.L^{-1}$ (as $CaCO_3$), with a Ca/Mg ratio of 4/1. Tests were performed in the dark at 20 ºC. Twenty Daphnia, divided into four groups of five animals each, were used at each test concentration. For each surfactant, ten concentrations in a geometric series were tested in the concentration range first established in a preliminary test. The percentage immobility at 24 hours was plotted against concentration on logarithmic-probability

paper. Normal statistical procedures were then employed to calculate the IC_{50} and to determine the 95% confidence ranges for the calculated IC_{50} values.

CONCLUSIONS

It has been shown that the pKa and pH-sensitivity of cationic lipids from basic amino acids can be tuned by modifying the type of amino acid present on the polar head, the free amino group of the amino acid, the alkyl chain length and the structure type of the surfactants.

For these lysine derivative surfactants, the hydrophobicity, number of cationic charges as well as the density of charge play an important role in the toxicity of these compounds. The influence of these parameters is different for each kind of chemical architecture and also changes for the type of cell used for evaluating the toxicity.

The toxic effects of cationic lipids from lysine against erythrocytes are lower than those reported for cationic lipids based on quaternary ammonium groups. The most hemolytic compounds are the more hydrophobic ones and, for surfactants with similar hydrophobicity the hemolytic activity increases as the cationic charge density does. Different trends have been observed in the case of fibroblast cells, where the hydrophobicity is not the parameter that mostly affects cytotoxicity. Indeed, Gemini and monocatenary surfactants have similar toxic effects, whereas trimethylated surfactants, compounds with the highest cationic charge density, are the less toxic ones.

All surfactants studied in this work can be classified as ready biodegradable compounds and in general they present low aquatic toxicity.

These results confirm that it is very difficult to predict the toxicity of cationic surfactants. Because of that, a long way is still ahead in order to establish meaningful structure-activity relationships.

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Supporting Information Available: dissociation states in the lysine based surfactants as well as titration curves of aqueous solutions of cationic lysine surfactants. This information is available free of charge via the Internet at http://pubs.acs.org.

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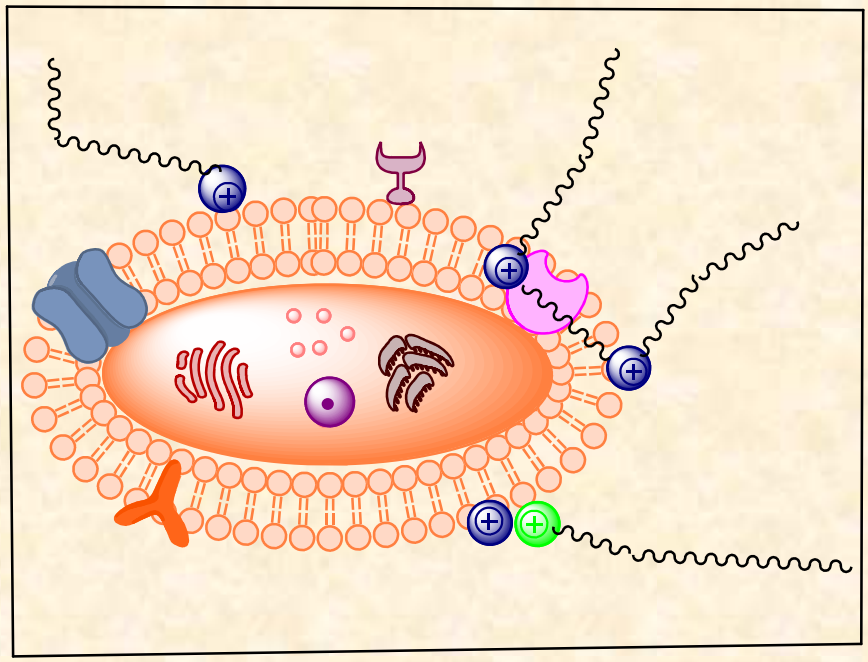
TABLE OF CONTENTS GRAPHIC

pH Sensitive Surfactants from Lysine: Assessment of their Cytotoxicity and Environmental Behavior



The influence of the acidic properties on the toxicity and biodegradability of cationic surfactants with different chemical structure and cationic charge density is reported. This study leads to the better understanding of the influence of structural parameters on the biological properties of cationic lipids.