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Original article

Cationic surfactants from lysine: Synthesis, micellization and biological evaluation

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ABSTRACT

Biocompatible cationic surfactants from the amino acid lysine (hydrochloride salts of N^{ε}-lauroyl lysine methyl ester, N^{ε}-myristoyl lysine methyl ester and N^{ε}-palmitoyl lysine methyl ester) have been prepared in high yields by lysine acylation in ε position with three natural saturated fatty acids. The micellization process of these surfactants has been studied using the PGSE-NMR technique. The compounds were tested as antimicrobial agents against Gram-positive and Gram-negative bacteria. The surfactants show moderate antimicrobial activity against the Gram-positive bacteria but Gram-negative bacteria are resistant to these surfactants in the concentration range tested. The haemolytic activity is considerably lower than those reported for other cationic N^{α}-acyl amino acid analogues. The acute toxicity against Daphnia magna and biodegradability was studied. The toxicity is clearly lower than that reported for conventional cationic surfactants from quaternary ammonium and the three surfactants from lysine can be classified as ready biodegradable surfactants.

1. Introduction

Although surfactants are widely used in both consumer and industrial applications, they can adversely affect the environment. Therefore, there is a growing demand for mild, biodegradable and nontoxic products that are made from natural raw materials. Moreover, there is also a demand for multifunctional compounds in order to reduce cost and to reduce the amount of chemicals added to wastewater. Accordingly, preparation of surfactants which mimic the structure of natural compounds such as lipoaminoacids, phospholipids and glycerolipids has assumed a new importance because of their unique physicochemical and biological properties [1]. Amino acid based surfactants with one or two hydrocarbon tails have been described in the literature [2,3] particularly salts of long chain N^{α}-acyl amino acids that are currently used as detergents, foaming agents and shampoos because they are mild, non-irritating to the human skin and highly biodegradable [4].

In the last two decades, our group has published a number of papers addressing the synthesis and properties of biocompatible cationic amino acid based surfactants of different structures [5–11]. These surfactants show a low toxicity profile and an antimicrobial activity similar to those of conventional cationic surfactants. From the health and the environmental points of view, cationic amino acid surfactants have aroused considerable interest. Nowadays, cationic surfactants are being tested in new biomedical applications such as drug delivery systems in cationic vesicles. The use of cationic amphiphiles for mediating DNA transfection has become widespread ever since it was shown that cationic lipids, known as cytofectins, are capable of delivering functional genes [12]. Cationic amino acid based surfactants are good candidates to act as transfection agents given their low toxicity, high biodegradability and their structural characteristics suitable for DNA transfection. Some surfactants based on dibasic amino acids have been considered for interaction with DNA [13,14] and the most promising results have been obtained with lysine and arginine [15]. In this field it is necessary to strike a balance between antimicrobial activity on the one hand and low toxicity and efficient biodegradability on the other. For these uses, it would be suitable the use of cationic surfactants with low antimicrobial activity because typically they present high biodegradability and less toxicity. For all these reasons, the knowledge of the aggregation behaviour as well as the biological properties (in particular biodegradation and cytotoxicity) is essential to establish structure/activity relationships.

In this paper, we describe the chemical synthesis and characterization of the hydrochloride salts of N^{ε}-acyl lysine methyl ester with alkyl chain lengths of 12 (LKM), 14 (MKM) and 16 (PKM) carbon atoms (Fig. 1). These amino acid based cationic surfactants have a hydrophobic chain attached to the ε-amino group of the lysine through an amide bond. We focus on the systematic study of

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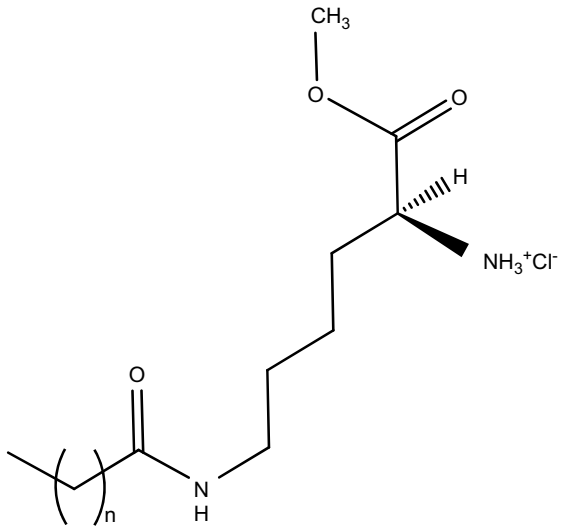

Fig. 1. Molecular structure of the N^{ε}-acyl lysine methyl ester surfactants: $n = 10$ LKM, $n = 12$ MKM and $n = 14$ PKM.

biological and surface properties of these lysine derivatives in order to determine their possible use in new biomedical applications.

In the relevant literature we can find several works concerning the synthesis and properties of N^{α}-acyl lysine [16] derivatives but the studies referring to N^{ε}-acyl lysine compounds [17–19] are scarce and none of these works reported a systematic study on the biocompatibility and micellization process of these compounds.

2. Chemistry

With the aim to systematically study the influence of the alkyl chain length on the properties of long chain N^{ε}-acyl lysine methyl ester salts we synthesized at multigram scale three pure homologues with alkyl chain of 12 (LKM), 14 (MKM) and 16 (PKM) carbon atoms (Fig. 1). The procedure used for the synthesis of these compounds was easy and very efficient. It consisted of three steps (Fig. 2) using the N^{α}-Cbz-lysine·HCl as starting material. (1) Preparation of the N^{α}-Cbz-lysine methyl ester HCl salt (α-carboxylic group protection). (2) Synthesis of N^{ε}-acyl-N^{α}-Cbz-lysine methyl ester by acylation of ε-amino group of the N^{α}-Cbz-lysine methyl ester HCl salt with the corresponding long chain acid chloride. The reaction progress was monitored by HPLC, after 4 h a 92% conversion of the starting reagent was obtained. The isolation of pure compounds was carried out by silica gel chromatography and satisfactory HPLC analyses were obtained for these materials yielding FABMS and NMR spectra, which were consistent with the target compounds. (3) Deprotection of the Cbz group by catalytic hydrogenation of the corresponding N^{ε}-acyl-N^{α}-Cbz-lysine methyl ester using Pd over charcoal. The reaction was carried out by controlling the pH to prevent the hydrolysis of the ester linkage present in these compounds. Pure compounds were obtained after several crystallisations in methanol/acetonitrile. The chemical structure of these compounds was checked by NMR. The proton and carbon NMR spectra were in concordance with the proposed structure. Elemental analyses for the derivatives were in good agreement with the calculated ones.

3. Pharmacology

3.1. Antimicrobial activity

Antimicrobial tests were performed using bacteria and fungi which are stored in our laboratory. Microorganisms were *Bacillus cereus var. mycoides*, *Enterococcus hirae*, *Micrococcus luteus*, *Staphylococcus aureus*, *Bacillus subtilis*, *Staphylococcus epidermis*, *Mycobacterium phlei*, *Candida albicans*, *Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Bordetella bronchiseptica*, *Serratia marcescens*, *Enterobacter aerogenes*.

3.2. Haemolytic activity

The determination of the haemolytic activity was made on human red blood cells obtained from healthy donors by venipuncture (Blood Bank of Hospital Vall d'Hebron, Barcelona, Spain), following the ethical guidelines of the Hospital, and collected in citrated tubes.

4. Results and discussion

4.1. PGSE-NMR results

PGSE-NMR was used to determine self-diffusion coefficients [20] in aqueous solutions of LKM and MKM at 25 °C. The PKM has been studied at 50 °C owing to the very low solubility of this surfactant at 25 °C.

The micellization process for each surfactant was followed by measuring samples with a surfactant concentration below CMC and above the CMC (Fig. 3). For all the systems considered, the intradiffusion coefficient of the surfactant ion, D, plotted as a function of

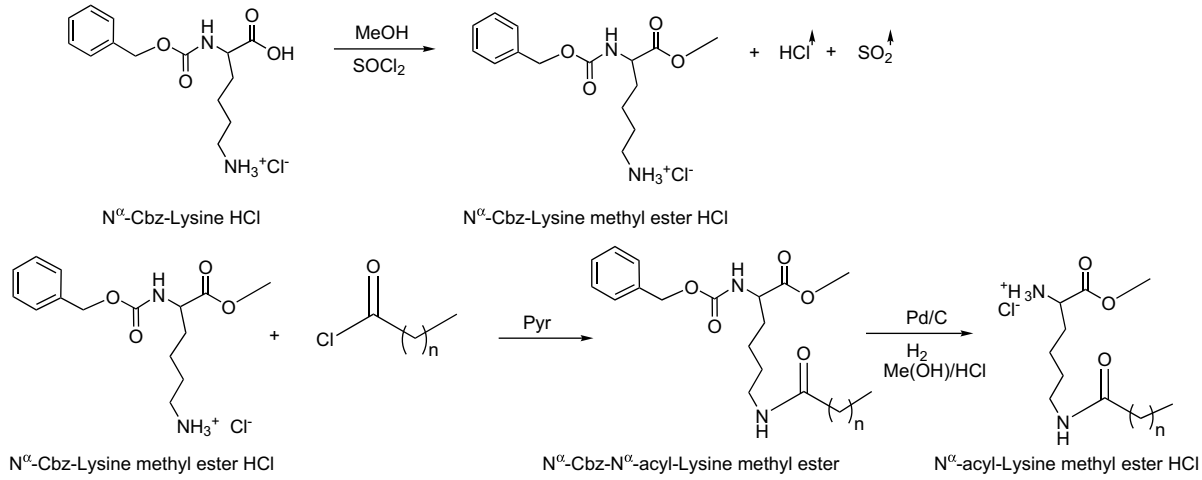

Fig. 2. Synthetic procedure used to obtain the lysine based surfactants.

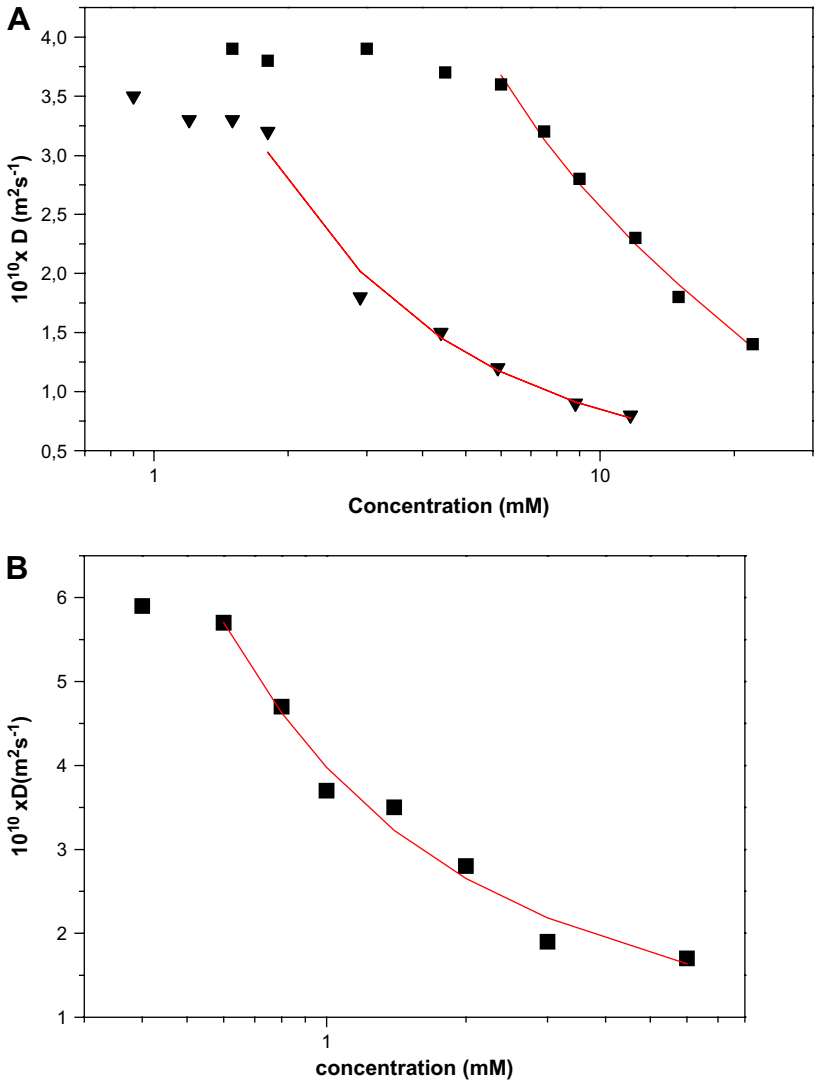

Fig. 3. Diffusion coefficients versus concentration. (A) LKM (■) and MKM (▼) at 25 °C, (B) PKM at 50 °C.

the surfactant molarity shows a change of slope at the onset of micellization, allowing for the determination of CMC. The monitoring of the association process is based on the difference in the self-diffusion coefficient for the surfactant monomers and micelles. The association process of these surfactants is reflected in a sensitive manner by the decrease of their self-diffusion coefficients. The effective translational mobility of the surfactant is considerably reduced because they diffuse in the form of an aggregate. Moreover, the proton spectra at high concentrations show typical high-resolution features with Lorentzian bandshapes, which indicates the presence of spherical micelles. Large aggregates give rise to proton spectra with little structure and broadened signals [21]. From the chemical structures of these surfactants, the formation of spherical micelles is expected. The aggregate type formed by the surfactants depends on the surfactant packing parameter $P = v/al$ were v and l are the volume and the length of the surfactant alkyl chain and a is the surface area occupied by the head group [22]. Surfactants with $P < 1/3$ give rise to spherical micelles. The N^{ε}-lysine derivatives have one polar head and one hydrophobic chain, these compounds usually present conical shape with $P < 1/3$, therefore they form spherical micelles. Studies carried out with one cationic surfactant from arginine, the N^{α}-lauroyl-arginine methyl ester, LAM (Fig. 4), surfactant with one cationic head group and the same alkyl chain, demonstrate that this compound forms spherical micelles [23,24].

Given the rapid exchange between free and micellized surfactant molecules, in the micellar composition range the experimental surfactant intradiffusion coefficient is a mean value between that of free monomers, D_{free}, and that of the micellized molecules, D_{mic}. This can be described using the two-site exchange model [23].

$$D_{obs} = p_{mic}D_{mic} + (1 - p_{mic})D_{free} \quad (1)$$

where $p_{mic} = (c - CMC)/c$ is the fraction of micellized surfactant and D_{obs}, D_{mic} and D_{mon} are the observed mean diffusion coefficient, the self-diffusion coefficient of the micelles and the monomers, respectively. Table 1 shows the D_{mic}, the D_{mon} and the CMC for the

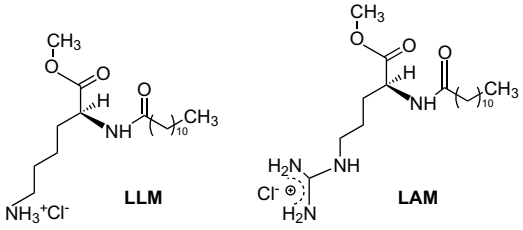

Fig. 4. Molecular structure of the N^{α}-lauroyl lysine methyl ester (LLM) and N^{α}-lauroyl-arginine methyl ester (LAM).

Table 1
D_{mic}, D_{mon} and the CMC for the N^{ε}-acyl lysine surfactants.

Surfactant	CMC (mM)	$D_{mon} \times 10^{10}$ ($m^2 s^{-1}$)	$D_{mic} \times 10^{10}$ ($m^2 s^{-1}$)	R_h (nm)
LKM	5.5	3.9	1.03	1.9
MKM	1.6	3.3	0.37	5.4
PKM[a]	0.6	5.8	1.4	2.6

[a] Diffusion coefficients determined at 50 °C.

two surfactants obtained by fitting Eq. (1) to the descending parts of the experimental curves (Fig. 3). This table also shows the micellar parameters obtained for PKM at 50 °C.

Micellization (or CMC value) of these surfactants from lysine takes place at 5.5 mM for the LKM, and 1.6 mM for MKM. As expected, the CMC decreases when the alkyl chain increases as a consequence of the higher hydrophobic content of the molecule. The CMC of the PKM at 50 °C is 0.60 mM.

At constant temperature, for homologous straight-chain ionic surfactants in aqueous medium, the relation between the CMC and the number of carbon atoms N in the hydrophobic chain takes the form [25]

$$\log \mathrm{CMC} = A - BN \quad (2)$$

where A and B are constants that depend on the molecular structure of the surfactant.

From the CMC values of the LKM and MKM it is possible to estimate the $\log \mathrm{CMC} = A - BN$ relation corresponding to these analogues at 25 °C (Fig. 5). Considering this relation the expected CMC of the PKM at 25 °C would be 0.48 mM. This value is slightly lower than those obtained at 50 °C (0.6 mM). This fact indicates that the temperature induces only small changes in the CMC of this compound. Usually, in cationic surfactants the CMC slightly increases with the temperature.

From the self-diffusion coefficient of the micellized surfactant, the hydrodynamic radius (R_h) (Table 1) can be calculated using the Stokes–Einstein equation.

$$D_{mic} = K_B T / 6 R_h \pi \eta \quad (3)$$

where K_B is the Boltzmann constant, T is the temperature, η is the viscosity of the solvent and R_h is the hydrodynamic radius of the aggregates.

Similar micellar sizes have been obtained for LKM and PKM but the big value of hydrodynamic radius obtained for MKM suggests that this compound forms large micelles. For this surfactant, post-micellar concentrations are near the two phases region boundary (micellar solution and hydrated solid), because of that, higher interaction between micelles and micelle growth is expected, some micelles can diffuse together giving rise to lower micellar diffusion coefficients and consequently the calculated hydrodynamic radius is bigger than expected.

The micellar parameters obtained for LKM (CMC, D_{mic}, R_h) are similar to those obtained for LAM (arginine derivative with the same number of carbon atoms in the hydrophobic part [23]). The results indicate that the cationic charge type in these ionic amino acid surfactants does not affect the micellization process; this process is mainly controlled by the hydrophobic part.

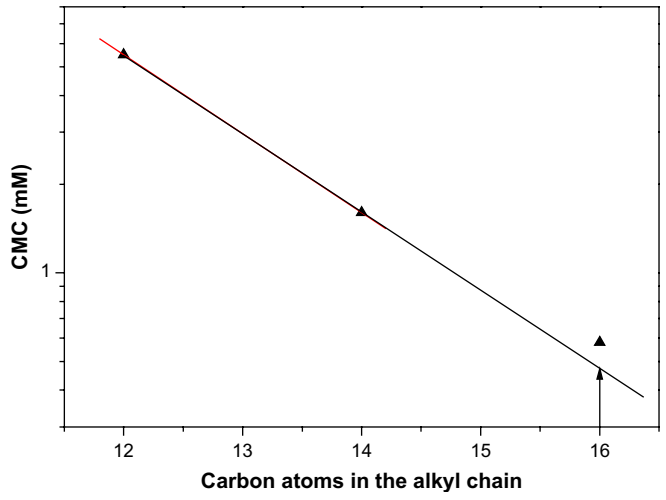

Fig. 5. CMC versus carbon atoms in the hydrophobic chain for the N^{ε}-acyl lysine surfactants.

4.2. Antimicrobial activity

Minimum inhibitory concentration (MIC) values for LKM, MKM and PKM compounds are summarised in Table 2. Due to the low solubility of MKM and PKM, they were dissolved in the minimum possible amount of dimethylformamide (DMF) and then diluted in the medium to the testing concentration [26].

To ensure that the solvent had not effect on the microorganism growth, a control test was performed with the test medium supplemented with DMF as the same concentration used in the experiments. For the sake of comparison, the MICs of two amino acid cationic surfactants, one from arginine, LAM (N^{α}-lauroyl-arginine methyl ester hydrochloride) and the other from lysine LLM [9] (Fig. 4) (N^{α}-lauroyl lysine methyl ester hydrochloride) have been assessed. In these surfactants (LAM, LLM), the alkyl chain is linked to the α-amino group of the corresponding amino acid and the positive charge lies in the ε-amino group (Fig. 4, Table 2). Table 2 also includes the MIC values corresponding to the hexadecyl trimethyl ammonium bromide [27], a classical antimicrobial surfactant agent. The three N^{ε}-acyl lysine methyl ester hydrochlorides show antimicrobial activity against a wide range of Gram-positive bacteria. The activity against Gram-positive bacteria is similar to that shown by the LLM and LAM. Compared with the classical quaternary ammonium surfactants (QAS) [27,28] these substances show a moderate activity level against bacteria with MIC values of 150–350 μM. However the use of QAS in some fields is limited due to the developed microbial resistance against QAS [29], their high acute toxicity and low biodegradability [30]. For medical applications, the use of cationic surfactants with low antimicrobial activity would be necessary because they use to present high biodegradability and less toxicity.

In general, the antimicrobial activity of the surfactants depends on the alkyl chain length, however the correlation between the alkyl chain length and the microbial activity is not linear. For the long chain N^{ε}-acyl lysine methyl ester series under study the chain length does not affect the antimicrobial properties. The only difference is that the LKM presents activity against *C. albicans* and *K. pneumoniae* whereas the MKM and PKM are not active against these bacteria at the highest concentration tested.

On the other hand, Gram-negative bacteria (except *E. coli*) are resistant to these surfactants in the concentration range tested. By contrast, LAM and LLM surfactants present a similar activity against both Gram-positive and Gram-negative bacteria. Other cationic lysine derivatives also show a similar activity against Gram-positive and Gram-negative bacteria [31,32]. Gram-negative bacteria are generally more resistant to antimicrobial agents than are Gram-positive bacteria. This can be explained by the different cell membrane structure of the two bacterial types. The external layer of the outer membrane of the Gram-negative bacteria is almost entirely composed of lipopolysaccharides and proteins that restrict the entrance of biocides and amphiphilic compounds [33]. The perturbation of this outer membrane requires a fine tuning of the

Table 2
MIC values (μM) for compounds LKM, MKM, PKM, LLM (N^{α}-lauroyl lysine methyl ester), LAM (N^{α}-lauroyl-arginine methyl ester) and hexadecyl trimethyl ammonium bromide (HTAB).

Microorganism		MIC μM (mg/L)					
		LKM	MKM	PKM	LLM	LAM	HTAB
Gram positives	*Bacillus cereus var. mycoides*	169 (64)	157 (64)	148 (64)	–	39 (16)	–
	Enterococcus hirae	338 (128)	157 (64)	74 (32)	–	39 (16)	–
	Micrococcus luteus	338 (128)	157 (64)	74 (32)	11 (4)	39 (16)	44 (16)
	Staphylococcus aureus	338 (128)	157 (64)	148 (64)	85 (32)	39 (16)	–
	Bacillus subtilis	338 (128)	157 (64)	148 (64)	42 (16)	157 (64)	44 (16)
	Staphylococcus epidermis	169 (64)	157 (64)	148 (64)	42 (16)	20 (8)	10 (4)
	Mycobacterium phlei	169 (64)	78 (32)	148 (64)	–	39 (16)	–
	Candida albicans	169 (64)	R	R	–	157 (64)	–
Gram negatives	*Klebsiella pneumoniae*	338 (128)	R	R	85 (32)	79 (32)	44 (16)
	Escherichia coli	169 (64)	157 (64)	R	42 (16)	157 (64)	44 (16)
	Salmonella typhimurium	R	R	R	85 (32)	157 (64)	–
	Pseudomonas aeruginosa	R	R	R	169 (64)	157 (64)	–
	Bordetella bronchiseptica	R	R	R	42 (16)	157 (64)	20 (8)
	Serratia marcescens	R	R	R	338 (128)	315 (128)	–
	Enterobacter aerogenes	R	R	R	–	157 (64)	–

R: resistant microorganism at the highest concentration tested (128 mg/mL); –: not available result.

hydrophobic–hydrophilic balance of the surfactants. The difference between LKM, LLM and LAM lies in the polar group. The polar group of LKM consists of the lysine amino acid with a positive charge in the α-amino group (p*K*a = 8.9). The polar group of the LLM is based on the lysine amino acid with a positive charge in the ε-amino group (p*K*a = 10.5) and the polar head in the LAM consists of the arginine amino acid with a positive group in the guanidine function (p*K*a = 12.4). The main difference between these compounds is the p*K*a associated to the protonated amino group on the polar heads. The p*K*a of the protonated guanidine group of the arginine and protonated ε-amino group of the lysine is considerably higher than that of the protonated α-amino group of the lysine. Because the p*K*a can be affected by aggregation, we have calculated the p*K*a of the LKM and MKM at concentration values that show antimicrobial activity [34] from pH measurements.

The apparent p*K*a values for these surfactants at these concentrations are similar to the p*K*a reported in the literature for the α-amino group of the lysine (p*K*a = 8.2 for LKM and p*K*a = 7.8 for MKM). At pH equal to the p*K*a, the amino acid will be 50% protonated. This means that the average charge will be 0.5. As the pH is decreased more than 2 units from the point of pH = p*K*a it can be considered that the amino acid is 100% protonated. This would mean that the average charge is 1. Given that the p*K*a of the N^{ε}-lysine derivatives is around 8 the average charge of the compound could be lower than 1 at the pH of the test medium (pH = 6), and hence not suitable for disrupting the membrane of the Gram-negative bacteria. These results allow us to state that the antimicrobial properties of pH-sensitive amino acid surfactants correlate with the pH of the medium.

As for the effect of the compound net charge on its bactericidal activity, numerous studies show that the electrostatic interactions play a key role in the action of cationic systems, and that a decrease in the charge density of the cationic compound results in a reduction in adsorption and bactericidal effects [35,36]. Accordingly, the antibacterial activity varies with the pH in systems in which the net charge depends on the pH. For example, a decrease of pH increases the antimicrobial activity of chitosan and some peptides [37].

4.3. Aquatic toxicity assessment

It has been shown that the toxicity of surfactants against the aquatic species is caused by the ability of the monomers to disrupt the integral membrane by a hydrophobic/ionic adsorption phenomenon at the cell membrane/water interface in a way similar to that of the antimicrobial mode of action. The results of *Daphnia magna* 24-h immobilisation tests [38] (IC_{50}) of the investigated surfactants are given in Table 3. The lower the IC_{50} value, the higher the toxicity of the compound. PKM has not been tested owing to the very low solubility of this surfactant in the medium.

The acute toxicity of the cationic surfactants from lysine was clearly lower than the toxicity reported for conventional monoquats [30], and even lower than that reported for other environmentally friendly cationic surfactant molecules such as arginine based Gemini cationic surfactants [39] and single chain arginine surfactants [40] with two cationic charges in the head group. Toxic activity is comparable to that shown by LAM (Table 3). Our data indicated that LKM exhibited lower aquatic toxicity than the MKM. Concerning the effect of the alkyl chain length, higher toxicity would be expected with greater hydrophobicity in the molecule, as reported for anionic surfactants on *D. magna* [41] and for alcohol ethoxylated surfactants on *Photobacterium phosphoreum* [42] and on *D. magna* [43]. This could explain the increase in toxicity between these two compounds.

4.4. Biodegradability assessment

Biodegradation is the most important mechanism for the irreversible removal of chemicals from the aquatic and terrestrial environments. It may be defined as the destruction of chemical compounds by the biological action of living organisms. The biodegradability of these surfactants was evaluated by applying the Modified Screening Test [44]. 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. In the course of the biodegradation test, the DOC (dissolved organic carbon) concentrations were determined at the beginning and at regular time intervals for a 28-day period. Biodegradation was stated as the percentage of DOC removal within 28 days (Fig. 6).

Table 3
Aquatic toxicity values on *Daphnia magna* for the lysine derivative surfactants and for the LAM.

	IC_{50} (μM)	
	Mean value	95% Confidence range
LAM	36	(27–54)
LKM	32	26–53
MKM	7.3	2–29

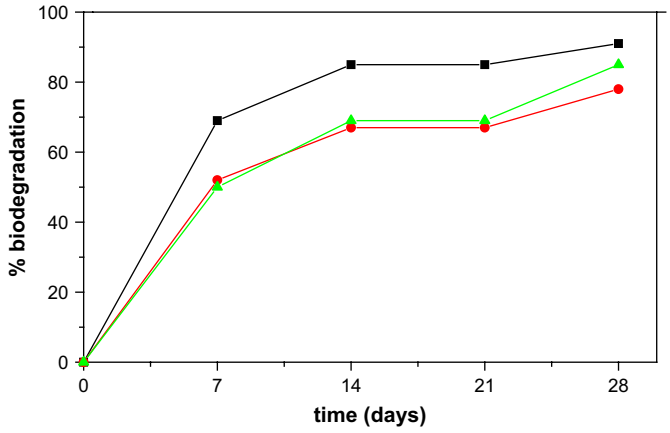

Fig. 6. Biodegradation level obtained for the LKM (◆), MKM (▲) and sodium dodecyl sulphate (■).

The biodegradation curve obtained for PKM was removed from Fig. 6 because inconsistent results were obtained probably due to the low solubility of this compound in the test medium. Biodegradation percentages of approximately 75% at day 28, clearly exceed the specified biodegradation pass level in this test (70%), allowing to classify LKM and MKM as readily biodegradable compounds. 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 [45] and are similar to the findings on cationic surfactants from arginine amino acids [46].

It should also be noted that these compounds reach the biodegradation level in half the time proposed in the test. In fact, this chemical structure has been designed to obtain ready biodegradable compounds. One of the strategies used by the bacteria to access the carbon in surfactants is the initial separation of the hydrophile from the hydrophobe (hydrophile attack). The amide linkage between the alkyl chain and the lysine is readily attacked by the microorganisms and then the fatty acids follow the pathway of chain-shortening through fatty acid β-oxidation [47] and the microorganisms completely degrade the naturally occurring lysine. On the other hand, the resistance of Gram-negative bacteria to these surfactants could facilitate the biodegradability process of these molecules. The alkyl chain length does not affect the biodegradation level, even the surfactant with 14 carbon atoms in the molecule (which has a high hydrophobic character) exceeds the biodegradation level in 15 days.

4.5. Haemolytic assessments

Haemolysis by surfactants is a process of great fundamental and practical importance. The human erythrocyte lacks internal organelles and since it is the simplest cellular model obtainable, it is the most popular cell membrane system to study the surfactant–membrane interaction [48]. For all these reasons it is adopted as a convenient model system. In addition, the potential uses of surfactants as drug delivery systems make of great importance the evaluation of haemolysis.

Evaluation of the concentration that induces the haemolysis of 50% of the erythrocytes (HC50) was determined and quantified from plots of percentage haemolysis as a function of amphiphile concentration (Table 4). Interestingly, the long chain N^{ε}-acyl lysine methyl ester series showed significantly lower haemolytic activity than the N^{α}-acyl lysine and N^{α}-acyl arginine compounds. The positive charge nature could affect the capability of these surfactants to disrupt the fragile cellular surface of the erythrocytes. Surfactant intercalation into membrane leads to changes in the membrane molecular organization and increase of membrane permeability that concludes with cell lyses [49]. However, haemolysis depends upon the adsorption of the surfactant to components of the membrane surface which is influenced by electrostatic attraction between the surfactant molecules and membrane components, among other factors. In this sense Shalel et al. [49], demonstrated that the ionic strength of the solution diminishes the effects of the erythrocyte negative surface charge at physiological pH and thus decreases the rate and amount of surfactant incorporated into the cell membrane, and consequently reduces the membrane permeability. The decrease of the haemolytic activity could be associated with the decrease of the antimicrobial activity of these surfactants. The change of the positive charge from the ε-amino group to the α-amino group in the amino acid belongs to compounds with moderate antimicrobial activity and lower cellular toxicity.

Table 4
Haemolytic assessment of the N^{ε}-acyl lysine surfactants and N^{α}-acyl arginine methyl ester (LAM).

Compound	HC_{50} (μM)
LAM	150
LLM	199
LKM	391
MKM	1232
PKM	1454

The haemolytic activity of these lysine surfactants decreases with increasing length of the hydrophobic tail. This behaviour contrasts with those described for the cationic surfactants in witch the haemolytic activity typically increases with increasing chain length [50]. The decrease of the haemolytic activity with increasing of the alkyl chain has been reported for partially fluorinated pyridinium bromides [51]. On the other hand the haemolytic activity showed for these lysine derivatives is clearly lower than those found for classical cationic quaternary ammonium surfactants as alkyltrimethylammonium salts [50], CTAB (hexadecyltrimethylammonium bromide) and DODAB (dioctadecyldimethyl ammonium bromide) and for new Gemini QACs [52] (benzalkonium chloride) and partially fluorinated pyridinium bromides [53]. These results showed that the erythrocytes-disrupting ability of these surfactants is correlated to the cationic charge type and to the alkyl chain length.

For these lysine surfactants the HC50 decreases linearly as the CMC increases. For LKM haemolysis occurs at monomer concentration, whereas MKM induces erythrocyte lysis at concentrations closer to its CMC and PKM at micellar concentrations. Different studies were performed to demonstrate a positive correlation between haemolytic action and the CMC of surfactants; however, no clear conclusions were reported in the literature. In the case of non-ionic surfactants, it has been pointed out that hydrophobic interaction and micellization are the main driving forces in the induction of haemolysis [54]. In a similar way, Gemini surfactants [55] or antipsychotic drugs [56] presented good correlation between haemolytic effects and CMC. In contrast, this good relationship cannot be established for different chemicals with amphiphile properties [56,57].

5. Conclusions

This work combines the study of the biological and physicochemical properties of the N^{ε}-acyl lysine surfactants. The results obtained have been compared with those reported for N^{α}-acyl amino acid surfactants with the aim of knowing how the cationic

charge type affects the behaviour of these kinds of compounds. Satisfactory synthesis for the preparation of long chain N^{ε} acyl lysine methyl ester hydrochloride salts as surfactants was carried out in a three-step procedure. These compounds act as moderate antimicrobial agents against Gram-positive bacteria. The haemolytic activity is considerably lower than those reported for other cationic surfactants, N^{α}-amino acid or ammonium quaternary type. As regards the aquatic toxicity, they present a good behaviour, moderate toxicity and high biodegradation level. The surfactants form micelles at millimolar concentrations. The results clearly show that the positive charge nature affects the antimicrobial properties as well as the haemolysis activity but not the biodegradability and micellization parameters. Taking into account the high biodegradation level and the low haemolytic activity of these compounds could be considered safe surfactants in relation to the cell of the human body. These properties make the compounds suitable candidates for biological applications such as the construction of cationic synthetic delivery systems.

6. Experimental protocols

6.1. Chemistry

All solvents were reagent grade and were used without further purification. The acyl chlorides with 12, 14 and 16 carbon atoms were from Fluka. The progress of the reactions was monitored by HPLC, model Merck-Hitachi D-2500 using UV–VIS detector L-4250 at 215 nm. A Lichrospher 100 CN (propylcyano) 5-μm, 250 × 4 mm column was used. A gradient elution profile was employed from the initial solvent composition of A/B 75/25 (by volume), changing during 24 min to a final composition of 5/95 where solvent A is 0.1% (vol/vol) trifluoro-acetic acid (TFA) in H_2O and solvent B is 0.085% of TFA in H_2O/CH_3CN 1:4. The flow-rate through the column was 1.0 mL min^{-1}.

The structures of the pure compounds were checked by ^1H and ^{13}C nuclear magnetic resonance (NMR) analyses which were recorded on a Varian spectrometer at 499.803 (^1H) and 125.233 (^{13}C) MHz, respectively, using the deuterium signal of the solvent as the lock. Chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane (TMS). All measurements were carried out on 0.6 mL samples in 5 mm tubes using a 5 mm indirect broadband probe. ^{13}C NMR spectra were recorded under composite decoupling to eliminate ^{13}C–^1H coupling. The distortionless enhancement by polarization transfer spectra (Dept) were run in a standard way to separate the CH/CH_3 and CH_2 lines phased up and down, respectively.

Mass spectroscopy (MS) spectra with fast atom bombardment (FAB) or electrospray techniques were carried out with a VG-QUATTRO from Fisons Instruments. Elemental analysis of the final compounds was also achieved.

6.2. General procedure synthesis of long chain N^{ε}-acyl lysine methyl ester hydrochloride salts

The synthesis of LKM, MKM, and PKM has been carried out following a three-step procedure starting from the commercial N^{α}-Cbz-LysOH·HCl (N^{α}-bencyloxycarbonyl-LysOH·HCl) (Fig. 2).

6.2.1. Synthesis of N^{α}-Cbz-lysine methyl ester·HCl (α-carboxylic group protection)

Methanol (400 mL) was cooled to −80 °C using acetone/solid carbon dioxide, then, N^{α}-Cbz-LysOH·HCl (25 g, 0.089 mol) was added. Thereafter, 19.5 mL of thionyl chloride (0.027 mol) was dropwise added. The mixture was stirred at room temperature for 20 h. The end of the reaction was checked by HPLC with the decrease in the peak area corresponding to N^{α}-Cbz-LysOH·HCl.

Methanol, excess thionyl chloride and chloride acid formed during the reaction were eliminated by successive vacuum evaporations. After lyophilisation a white solid corresponding to the title product was obtained.

Analytical data and spectral assignments for N^{α}-Cbz-lysine methyl ester·HCl.

Yield: 97%. HPLC, $t_r = 5.88$ min. MW 330.5. Elem. Analy. Found: C, 51.13; H, 6.89; N, 8.11; Calcd. for $C_{15}H_{23}O_4N_2Cl \cdot 1H_2O$: C, 51.65; H, 7.17; N, 8.03. ^1H NMR: δ_H (CD_3OD): 1.42–1.89 [m, 6H, $3CH_2$ side chain of lysine group], 2.85 [t, 2H (–CH_2–NH_2), 3.74 [s, 3H (–COO–CH_3)], 4.21 [m, 1H (–NH–CH–COO–)], 5.17 [s, 2H (Cbz–CH_2–)], 7.25–7.31 [m, 5H of the Cbz group (Z)].

^{13}C NMR: δ_C (CD_3OD): 23.83, 28.60, 32.05 [–CH_2– side chain of lysine], 40.49 [–CH_2–NH_2], 52.75 [–COO–CH_3], 55.12 [–NH–CH–COO–], 67.67 [Cbz–CH_2–], 128.81–129.48 [6C of the Z group (Cbz)], 158.69 [COO–NH of the Z group (Cbz)], 174.38 [–COO–CH_3].

6.2.2. Synthesis of N^{ε}-acyl-N^{α}-Cbz-lysine methyl ester

A solution of pyridine (30 mL) containing 0.015 mol (5 g) of N^{α}-Cbz-lysine methyl ester·HCl was placed in a round-bottomed flask. To this solution, 0.015 mol of the corresponding acyl chloride was dropwise added and stirring continued at room temperature for 4 h. After completion of the reaction, pyridine was removed by vacuum pump. The resulting solid was dissolved in methanol (25 mL) and extracted three times with petroleum ether to remove the corresponding fatty acid excess. The solvent was evaporated and the solid was dissolved in chloroform (25 mL). Thereafter, this solution was shaken with water to eliminate water-soluble impurities. Silica acid flash chromatography: 100 mL of silica (Chromagel 60A CC, 70–230) was packed into a flash chromatography column. Then 3 g of the N^{ε}-acyl-N^{α}-Cbz-lysine methyl ester dissolved in chloroform was loaded into the column and was eluted from chloroform to chloroform/methanol. The fractions containing the pure target products were pooled. The identification of the products was carried out by HPLC, Elemental analysis, ^{1} HNMR and ^{13} CNMR.

6.2.2.1. Analytical data and spectral assignments for N^{ε}-acyl-N^{α}-Cbz-lysine methyl ester

6.2.2.1.1. N^{ε}-lauroyl-N^{α}-Cbz-lysine methyl ester. Yield: 91%. HPLC, $t_r = 19.55$ min. MW 476.29. ESI-MS, $m/z = 477.34$ corresponding to $(M + H)^+$.

6.2.2.1.2. N^{ε}-myristoyl-N^{α}-Cbz-lysine methyl ester. Yield: 93%. HPLC, $t_r = 20.34$ min. MW 504.29. ESI-MS, $m/z = 505.36$ corresponding to $(M + H)^+$.

6.2.2.1.3. N^{ε}-palmitoyl-N^{α}-Cbz-lysine methyl ester. Yield: 90%. HPLC, $t_r = 20.72$ min. MW 532.29. ESI-MS, $m/z = 533.39$ corresponding to $(M + H)^+$.

6.2.2.1.4. ^1H NMR and ^{13}C NMR resonances of the N^{ε}-acyl-N^{α}-Cbz-lysine methyl ester compounds. ^1H NMR: δ_H (DMSO-d_6), 0.84 [t, 3H, CH_3 alkyl chain], 1.22–1.63 [m, CH_2 alkyl chain, $2CH_2$ side chain of lysine group], 2.00 [t, 2H (–CH_2–CO–NH– alkyl chain)], 2.98 [m, 2H (–CO–NH–CH_2– side chain of lysine group)], 3.61 [s, 3H (–CH_3 lysine group)], 3.99 [m, 1H (–NH–CH–COO–)], 5.02 [s, 2H (Cbz–CH_2–O–)], 7.29–7.36 [m, 5H (Z group)].

^{13}C NMR: δ_H (DMSO-d_6), 13.7 [CH_3– alkyl chain], 21.9, 22.7, 25.1, 28.5, 28.6, 28.7, 28.8, 28.8, 30.2, 31.1 [–CH_2– alkyl chain and side chain of lysine group], 35.3 [–CO–CH_2–CH_2– alkyl chain], 37.9 [–CH_2–NH–CO lysine group], 51.6 [–CH–COO–], 53.8 [–COO–CH_3], 65.4 [Cbz–CH_2–], 127.5, 127.6, 128.2 [–CH– Z group], 171.8 [–CONH–], 172.7 [–COO–].

6.2.3. Synthesis of long chain N^{ε} acyl lysine methyl ester hydrochloride salt

The target compounds were obtained by hydrogenation of the corresponding pure N^{ε}-acyl-N^{α}-Cbz-lysine methyl ester

(0.002 mol) in 30 mL methanol/HCl (HCl moles/lysine derivative moles = 1.3) using Pd in activated charcoal (10% Pd) as catalyst. The reaction was carried out at atmospheric pressure. The mixture was stirred for 30 min. Given that HCL is present in the reaction medium, the final surfactants were obtained as HCl salts. At the end of the reaction, the catalyst was filtered off on celite. The solvent was evaporated under reduced pressure. Pure compounds were obtained by several crystallizations from methanol/acetone.

6.2.3.1. Analytical data and spectral assignments for N^{ε}-acyl lysine methyl ester HCl salts

6.2.3.1.1. N^{ε}-lauroyl lysine methyl ester hydrochloride salt. Yield 70%. HPLC, $t_r = 14.8$ min. MW 378.45: ESI-MS, *m*/*z*: 343.2 (M^+ without the Cl^-). Elem. Analy. Found: C, 57.02; H, 11.20; N, 7.27; Calcd. for $C_{19}H_{39}O_3N_2Cl \cdot 1.2H_2O$: C, 56.99; H, 10.35; N, 6.99. ^{1}H NMR: δ_H (CD_3OD) 0.89 [t, 3H, (CH_3 alkyl chain)], 1.29 [s, 16H, $8CH_2$ alkyl chain], 1.50–1.60 [m, 6H, (CH_2–CH_2–CO–NH–), (–CH_2–CH_2–CH_2–CH_2–NH–)], 1.92 [m, 2H, (–CH_2–CH_2–CH_2–CH_2–NH–)], 2.18 [t, 2H (–CH_2–CO–NH–)], 3.20 [t, 2H (–CH_2–CH_2–CH_2–CH_2–NH–)], 3.8 [s, 3H, (–COO–CH_3)], 4.0 [m, 1H (–NH–CH–$COOCH_3$)].

^{13}C NMR: δ_C (CD_3OD), 14.4 [CH_3–, alkyl chain], 23.3, 23.7, 27.0, 29.9, 30.3, 30.4, 30.6, 30.7, 31.1, 33.0, [–CH_2– alkyl chain and side chain of lysine group] 37.1, [–CO–CH_2–CH_2– alkyl chain], 39.6 [–CH_2–NH–CO– lysine group] 53.6 (–CH–COO–), 53.8 (–COO–CH_3), 171.0 (–COO–), 176.4(–COHN–).

6.2.3.1.2. N^{ε}-myristoyl lysine methyl ester hydrochloride salt.- Yield 75%. HPLC, $t_r = 15.4$ min. MW 406.66: ESI-MS, *m*/*z*: 371.5 (M^+ without the Cl^-). Elem. Analy. Found: C, 61.77; H, 10.78; N, 6.73; Calcd. for $C_{21}H_{43}O_3N_2Cl$: C, 62.02; H, 10.56; N, 6.88. ^{1}H NMR: δ_H (CD_3OD), 0.84 [t, 3H, (CH_3 alkyl chain)], 1.26 [s, 20H, $10CH_2$ alkyl chain], 1.36–1.48 [m, 6H, (CH_2–CH_2–CO–NH–), (–CH_2–CH_2–CH_2–CH_2–NH–)], 1.73 [m, 2H, (–CH_2–CH_2–CH_2–CH_2–NH–)], 2.03 [t, 2H (–CH_2–CO–NH–)], 3.17 [t, (–CH_2–CH_2–CH_2–CH_2–NH–)], 3.8 [s, 3H, (–COO–CH_3)], 4.0 [m, 1H (–NH–CH–COO)].

^{13}C NMR: δ_C (CD_3OD), 14.4 [CH_3–, alkyl chain], 23.2, 23.7, 27.0, 29.9, 30.3, 30.4, 30.5, 30.6, 30.7, 30.8, 31.1, 33.1 [–CH_2– alkyl chain and side chain of lysine group] 37.1, [–CO–CH_2–CH_2– alkyl chain], 39.6 [–CH_2–NH–CO– lysine group] 53.6 (–CH–COO–), 53.8 (–COO–CH_3), 171.04 (–COO–), 176.4(–COHN–).

6.2.3.1.3. N^{ε}-palmitoyl lysine methyl ester hydrochloride salt.- Yield 82%. HPLC, $t_r = 18.07$ min. MW 434.66. ESI-MS, *m*/*z*: 399.35 (M^+ without the Cl^-). Elem. Analy. Found: C, 63.61; H, 11.20; N, 6.37; Cl, 8.27; Calcd. for $C_{23}H_{47}O_3N_2Cl$: C, 63.49; H, 10.81; N, 6.44; Cl, 8.05. ^{1}H NMR: δ_H (CD_3OD), 0.83 [t, 3H, CH_3 alkyl chain], 1.214 [s, 22H, $12CH_2$ alkyl chain], 1.34–1.43 [m, 6H, (–CH_2–CH_2–CH_2–CO–NH), (–CH_2–CH_2–CH_2–CH_2–NH–)], 1.70 [m, 2H (–CH_2–CH_2–CH_2–CH_2–NH–)], 2.00 [t, 2H (–CH_2–CO–NH–)], 2.98 [m, 2H (–CH_2–CH_2–CH_2–CH_2–NH–)], 3.69 [s, 3H, (–COO–CH_3)], 3.80 [m, 1H (–NH–CH–COO)].

^{13}C NMR: δ_H (CD_3OD), 14.46 [CH_3– alkyl chain], 23.2, 23.7, 27.1, 29.9, 30.3, 30.4, 30.5, 30.6, 30.7, 30.8, 31.1, 33.1 [–CH_2– alkyl chain and side chain of lysine group], 37.0 [–CO–CH_2–CH_2– alkyl chain], 39.8 [–CH_2–NH–CO– lysine group] 53.6 [–CH–COO–], 53.8 [–COO–CH_3–], 170.9 [–COO–], 176.6 [–CONH–].

6.3. PGSE-NMR

The pulsed gradient stimulated echo experiment was used to determine the self-diffusion coefficients of the surfactants at 25 (±0.5) °C. The ^{1}H NMR measurements were obtained on a Varian Inova 500-MHz spectrometer equipped with a standard 5-mm indirect detection, pulse field gradient (PFG) probe. The combination provides a *z* gradient strength up to 0.33 T m^{-1} (33 G cm^{-1}).

All the NMR signals give rise to single exponential decays in the diffusion experiment, and the diffusion coefficient, *D*, is obtained by fitting the following equation to the NMR data.

$$I = I_0 e^{\left[(-\gamma G\delta)^2 D(\Delta - \delta/3)\right]} \quad (4)$$

In Eq. (4), *I* is the measured signal intensity, I_0 is the eco intensity in the absence of field gradient pulses, Δ is the time between the two gradients in the pulse sequence, γ is the magnetogyric ratio, *G* is the field gradient strength and δ is the duration of the gradient pulse. The experiment has been carried out by varying the gradient strength *G*. Typically, a value of 50–250 ms is used for Δ, 5–10 ms is used for δ and *G* is varied from 0.03 to 0.33 T m^{-1} in 15–20 steps. The combination of *G*, δ and Δ was chosen to generally obtain 90–95% total signal attenuation throughout the experiment. The calibration of the gradient strength was performed using heavy water with trace amounts of light water (D_{HDO}) [58] 18.72×10^{-10} m^2 s^{-1}. The intradiffusion coefficients were calculated by following the signal intensities of the CH_2 groups in the alkyl chain.

6.4. Antimicrobial activity

The compounds tested were dissolved in nutrient agar medium in the concentration range of 0.1–256 μg/mL. Then 10 μL of a nutrient broth starter culture of the each bacterial strain was added to achieve final inoculums of ca. 5×10^{-4}–5×10^{-5} colony forming units per mL. The cultures were incubated overnight at 37 °C. Nutrient broth medium without the compound served as control. The growth of the microorganisms was determined visually after incubation for 24 h at 37 °C. The development of turbidity in an inoculated medium is a function of growth. A rise in turbidity reflects the increase in both mass and cell number. Changes in turbidity were correlated with changes in cell numbers. The lowest concentration of antimicrobial agent at which no visible turbidity was observed was taken as the minimum inhibitory concentration.

6.5. Aquatic toxicity tests

To determine aquatic toxicity, *D. magna* acute toxicity tests [38] were carried out. *D. magna*, laboratory bred, not more than 24 h old were used in this test, where the swimming incapability is the end point. The pH of the medium was 8.0 and the total hardness was 250 mg/L (as CaCO3), with a Ca/Mg ratio of 4/1. Tests were performed in the dark at 20 °C. 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 h 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.

6.6. Biodegradation test

The Modified Screening Test [44] described in the Organization for the Economic Cooperation and Development (OECD) Guidelines was applied to determine the biodegradation. A mixture of equal volumes of secondary effluent from a wastewater treatment plant and garden soil aqueous suspension was used as inoculums for the biodegradation test.

6.7. Haemolytic activity

Erythrocytes were washed three times in phosphate buffer isotonic saline (PBS), containing 22.2 mmol/L Na_2HPO_4, 5.6 mmol/L KH_2PO_4, 123.3 mmol/L NaCl, Glucose 10.0 mmol/L in distilled water (pH = 7.4). The erythrocytes were then suspended in PBS at a cell density of 8 × 109 cell/mL.

6.7.1. Haemolysis assay (HC50)

A series of different volumes of surfactant solution (1 mg/mL), ranging from 10 to 80 μL, were placed in polystyrene tubes and an aliquot of 25 μL of erythrocyte suspension was added to each tube. The tubes were incubated at room temperature for 10 min under shaking conditions. Following incubation, the tubes were centrifuged (5 min at 5000 rpm). The haemolysis degree was determined by comparing the absorbance (540 nm) of the supernatant with that of the control samples totally haemolysed with distilled water.

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