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ORIGINAL ARTICLE

Self-Assembly of Lipoaminoacids-DNA Based on Thermodynamic and Aggregation Properties

Célia Faustino[1] · Tiago Martins[1] · Noélia Duarte[1] · Maria H. Ribeiro[1]

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Abstract Lipoamino acids (LAA) are biocompatible and biodegradable biosurfactants, a promising alternative to viral vectors in gene delivery. LAA are constituted by a polar head, the amino acid, and a hydrocarbon (alkyl) chain usually from a fatty acid or fatty acid derivative, such as a fatty amine or a fatty alcohol. In this work, dodecyl LAA was produced from dodecylamine and natural L-amino acids cystine (Cys), lysine (Lys), and phenylalanine (Phe) using an enzyme-based approach with porcine pancreatic lipase. The self-assembly behavior of LAA solutions, in the absence or presence of DNA, was studied by conductivity and fluorescence regarding the application as transfection agents. Conductivity measurements yielded important system parameters, including critical micelle concentration (CMC) and standard Gibbs energy of micellization (ΔG°_{mic}) for pure LAA systems, and apparent critical aggregation concentration (CAC_{app}) and apparent standard Gibbs energy of aggregation (ΔG°_{agg}) for the mixed LAA-DNA systems. The CMC increased in the order of decreasing lipophilicity: $(C_{12}Cys)_2 < C_{12}Phe < C_{12}Lys$, CMC values were higher in the presence of DNA, suggesting the formation of a LAA-DNA complex responsible for hindering the micellization process. Binding of the LAA with DNA was confirmed from fluorescence measurements for the ethidium bromide exclusion assay. Results suggest a weak interaction of the LAA with DNA which can be attributed to their relatively short dodecyl chains and/or the ionic strength of the buffer solution, supporting the role of hydrophobic interactions in complex formation between DNA and the oppositely charged surfactant in combination with electrostatic interactions. The CAC_{app} values decreased with increasing LAA hydrophobicity, reflecting the relevance of hydrophobic interactions in complex coacervation.

Keywords Lipoamino acid · DNA · Cationic surfactant · Lipase · Gene delivery vectors

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Introduction

Gene therapy has emerged as a promising approach for the treatment or prevention of both genetic and acquired diseases (Ilarduya et al., 2010; Wettig et al., 2008). However, there are several hurdles to overcome in developing effective gene-based therapeutics, namely cellular uptake, endosomal escape avoiding DNA degradation, and nuclear localization (Ilarduya et al., 2010; Singh et al., 2011; Singh et al., 2012; Wettig et al., 2008). Successful gene therapy depends crucially on the development of effective vectors, especially the introduction of the selected gene into living cells toward the target site. Some drawbacks may be associated with viral vectors, namely residual infectivity and immunogenic and inflammatory responses (Ilarduya et al., 2010; Wettig et al., 2008). Cationic lipids or cationic surfactants that bind and condense the negatively charged phosphate backbone of DNA into nanosized cationic lipid-DNA complexes (lipoplexes) are attractive synthetic alternatives to viral vectors due to easy preparation, higher loading capacity, lower cytotoxicity, and safer immunogenic profile (Ilarduya et al., 2010; Wettig et al., 2008).

Lipoamino acids (LAA), which are condensation products of amino acids with fatty acids or their derivatives, usually have good biocompatibility and biodegradability (Bordes and Holmberg, 2015; Pérez et al., 2014). The polar headgroup of

✉ *Maria H.L. Ribeiro
mhribeiro@ff.ul.pt

[1] Faculty of Pharmacy, Research Institute for Medicines (iMed. ULisboa), Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisbon, Portugal

LAA, which contains both donor and acceptor hydrogen bonding groups capable of intra- and intermolecular interactions, adds further complexity to their self-assembly behavior. LAA have been employed as drug delivery agents for hydrophobic drugs (Ménard et al., 2012; Serafim et al., 2016), enhancing drug solubilization and bioavailability. Moreover, LAA are obtained from naturally renewable sources and can be produced by green-chemistry approaches that include biotechnological procedures, such as fermentation or enzymatic catalysis (Ménard et al., 2012; Pérez et al., 2014; Pinazo et al., 2011; Serafim et al., 2016). Cationic LAA are thus promising transfection agents that meet the requirements of both physiological and ecological compatibility.

Dodecyl LAA varying in the nature of the headgroup and number of dodecyl chains has been obtained from renewable raw materials by enzyme-catalyzed coupling of dodecylamine (DDA) with lysine, phenylalanine, and cystine (Fig. 1).

The LAA from cystine (the dimer of cysteine) with its dimeric structure comprising two cysteine residues linked by a disulphide bond, is expected to promote the release of the complexed DNA and enhance transfection efficiency (Ilarduya et al., 2010; Wettig et al., 2008), through reductive cleavage of the disulphide spacer by endogenous glutathione. Furthermore, dimeric or gemini LAA are versatile molecules able to interact with biological interfaces and biomacromolecules, including membrane phospholipids (Faustino et al., 2011), serum albumin proteins (Branco et al., 2015) and DNA (Faustino et al., 2015).

The presence of pH-sensitive amino acid headgroups in the LAA structure allow for changes in the aggregation morphology with variations in the protonation state of the amino acid residues that facilitate membrane fusion and DNA escape from the endosome often avoiding the need for addition of helper lipids (Ilarduya et al., 2010; Wettig et al., 2008). In this work, the supramolecular behavior of the dodecyl LAA obtained and their interactions with DNA were studied regarding further application as transfection agents. Conductivity measurements yielded important system parameters, including critical micelle concentration (CMC) and standard Gibbs energy of micellization (ΔG°_{mic}) for pure LAA systems as well as apparent critical aggregation concentration (CAC_{app}) and apparent standard Gibbs energy of aggregation (ΔG°_{agg}) for the mixed LAA-DNA systems.

Materials and Methods

Materials

Double-stranded deoxyribonucleic acid (dsDNA) in the form of sodium salt from salmon testes was supplied by

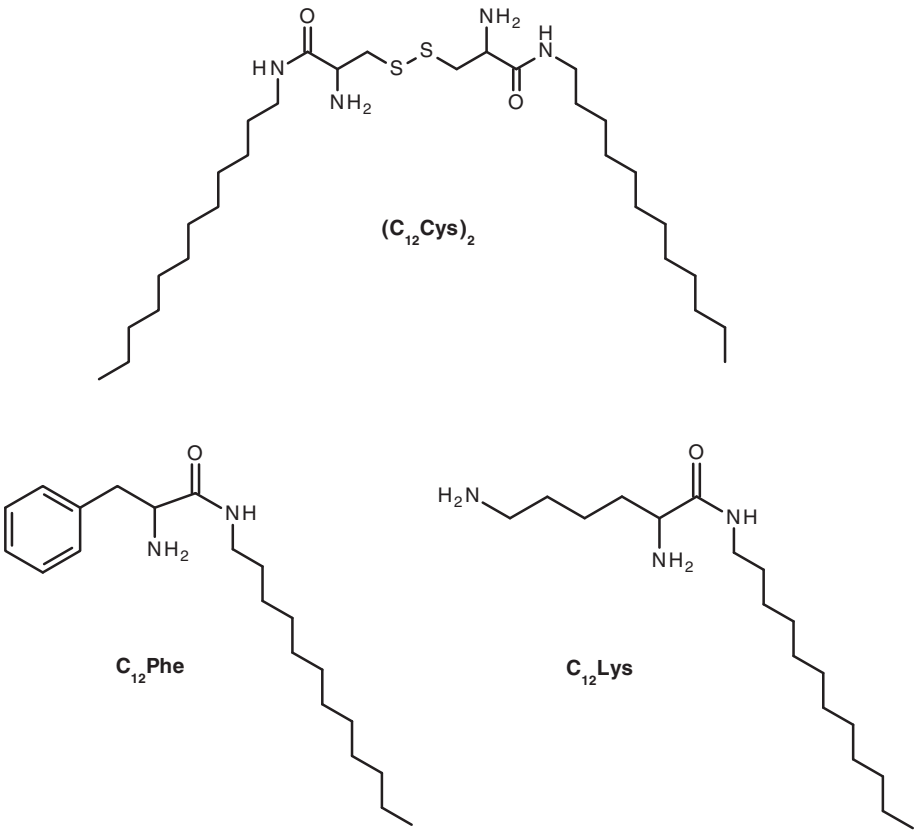

Fig. 1 Lipoamino acids produced by biocatalysis

Sigma-Aldrich (St. Louis, MO, USA) and used as received. Ethidium bromide (EB; 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide) solution (0.5 mg mL^{-1} in water), tris(hydroxymethyl) aminomethane (Tris), DDA L-cystine (Cys), L-lysine (Lys), L-phenylalanine (Phe) and porcine pancreatic lipase (PPL), type II crude, were also obtained from Sigma-Aldrich (St. Louis, MO, USA).

Biosynthesis

The dodecylLAAs were prepared from natural L-amino acids cystine, lysine, and phenylalanine by condensation with DDA using the biocatalyst, lipase PPL (EC number 232–619-9) encapsulated in sol–gel, in a reaction media of eutectic mixture, Dowtherm©A (Dow Chemicals, Midland, MI, USA).

PPL was immobilized using a developed in-house process. The immobilization of PPL was carried out in sol–gel, following the method described by Vila-Real et al. (2010). Briefly, in an eppendorf were added 96 mg of glycerol (98%), 70 μL of distilled water, 15 μL of HCl (80 mM), and 300 μL of TMOS. The mixture was sonicated for 20 min at a temperature from 0 to 4 °C. To the formation of the hydrogel a ratio of 1:1 was used, adding to 25 μL of sol solution, 25 μL PPL 2.5 mg mL^{-1} in 10 mM Tris buffer pH 8. The lens shape was created by using a 96 rounded well microplate as a recipient for the sol–gel with the PPL. Gentle tapping on the microplate was used to unify the mixture.

The lenses (126 mg) were used in the bioconversion assays at 45 °C, with the substrate, DDA, which is liquid at this temperature. One gram of DDA was weighted into each reaction flask, which was stabilized at 45 °C, to complete melting of the DDA. After, 300 mg of cystine, or 300 mg lysine, or 300 mg phenylalanine was added to each flask, along with 500 μL of the eutectic mixture Dowtherm®A. The bioconversion assays were performed for 96 h. All the assays were carried out in triplicate.

The products were obtained in a solid state form, precipitated and separated from the reaction media. A yield of approximately 85% was attained.

The final products obtained in a solid form were identified by high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) and characterized by nuclear magnetic resonance (NMR).

The samples analyzed were prepared by collecting 2 mg of the reaction and adding 2 mL of dimethylsulfoxide for a final concentration of 1 mg mL^{-1}.

The HPLC analyses were performed on a Waters Alliance 2695 (Waters®, Dublin, Ireland) equipped with a quaternary pump, solvent degasser, auto sampler, and column oven, coupled to a Photodiode Array Detector Waters 996 PDA (Waters®, Dublin, Ireland). The tandem mass spectrometer (MS/MS) used was a MicroMass Quattromicro® API (Waters®, Dublin, Ireland), triple quadrupole type. Compound ionization was performed by an electrospray source in positive mode (ESI+). For acquisition and processing of HPLC-MS/MS data MassLynx® version 4.1 was used.

The separation was performed on a normal-phase column (Luna HILIC 1000 × 3.00 mm) at 35 °C using an injection volume of 10 mL. Photodiode Array Detector was used to scan wavelength absorption from 210 to 600 nm. The mobile phase consisted of Milli-Q (Millipore, Bedford, MA, USA) water containing 0.5 formic acid (i): acetonitrile (ii) at a flow rate of 0.30 mL min^{-1} and the following eluting conditions were used: 0 min—5% (A) and 95% (B); 5.0 min—100.0% (A) and 0.0% (B); 7.0 min—100.0% (A) and 0.0% (B); 10.0 min—5.0% (A) and 95.0% (B); 15.0 min—5.0% (A) and 95.0% (B).

NMR spectra were recorded on a Fourier 300 spectrometer (300 MHz) from Bruker (Billerica, MA, USA) using $CDCl_3$ as solvent, based on the studies of Faustino et al., (2010). ^1H chemical shift is expressed in parts per million, δ (ppm), referenced to the solvent used.

$(C_{12}Cys)_2$. ^1H NMR ($CDCl_3$): δ (ppm) = 0.88 (t, 6H, 2 × C<u>H</u>$_3$), 1.25 (m, 36H, 2 × (C<u>H</u>$_2$)$_9$CH$_3$), 1.53 (m, 4H, 2 × C<u>H</u>$_2$CH$_2$NHCO), 2.78 (m, 4H, 2 × C<u>H</u>$_2$NHCO), 3.15 (m, 4H, 2 × C<u>H</u>$_2$S), 3.56 (d, 2H, 2 × C<u>H</u>). M^+ (*m*/*z*): 575.

C_{12}Lys. ^1H NMR ($CDCl_3$): δ (ppm) = 0.88 (t, 3H, C<u>H</u>$_3$), 1.25 (m, 18H, (C<u>H</u>$_2$)$_9$CH$_3$), 1.45 (m, 4H, C<u>H</u>$_2$CH$_2$NHCO + H$_2$N(CH$_2$)$_2$C<u>H</u>$_2$), 2.10 (m, 4H, H$_2$NCH$_2$C<u>H</u>$_2$CH$_2$C<u>H</u>$_2$), 2.69 (m, 2H, C<u>H</u>$_2$NHCO), 3.71 (m, 1H, C<u>H</u>). M^+ (*m*/*z*): 314.

C_{12}Phe. ^1H NMR ($CDCl_3$): δ (ppm) = 0.88 (t, 3H, C<u>H</u>$_3$), 1.26 (m, 18H, (C<u>H</u>$_2$)$_9$CH$_3$), 1.45 (m, 2H, C<u>H</u>$_2$CH$_2$NHCO), 2.21 (br s, 2H, C<u>H</u>$_2$Ph), 2.70 (m, 2H, C<u>H</u>$_2$NHCO), 3.75 (d, 1H, C<u>H</u>), 7.10–7.60 (m, 5H, C$_6$<u>H</u>$_5$). M^+ (*m*/*z*): 333.

Sample Preparation

DNA stock solutions at a concentration of 0.01 g L^{-1} were prepared by accurately weighing the appropriate amount of lyophilized material and dissolving in 0.09 mol L^{-1} Tris–HCl aqueous buffer solution (pH 8.0) under gentle magnetic stirring for at least 12 h prior to use. Surfactant solutions, both in the absence and in the presence of 0.01 g L^{-1} DNA, were prepared by accurately weighing the desired amount of surfactant and dissolving in Tris–HCl buffer solution (pH 8.0) or DNA stock solution, respectively. Previously, the solubilization of the new surfactants was tried in water but solubility was lower than that in the Tris–HCl buffer solution, presumably due to the presence of the uncharged form of the surfactant, with low water solubility, eventually resulting from partial deprotonation of the LAA in the aqueous media. LAA solutions were prepared in the concentration range 0.1–2 mg mL^{-1} chosen due to the low solubility of LAA at concentrations above 2 mg mL^{-1} and

conductivity masking by the buffer at LAA concentrations below 0.1 mg mL^{-1}.

Conductance Measurements

Conductance of LAA and LAA-DNA solutions was measured at 20 °C with an uncertainty of less than ±0.1% using a digital conductivity meter (XS Instruments, Carpi, Italy) equipped with a conductivity cell with platinum electrodes and a cell constant of 1.11 cm^{-1} and calibrated with standard KCl solutions (Crison Instruments, Allela, Spain) in the appropriate concentration range.

EB Exclusion Assay

To 20 µL of a DNA solution (0.01 mg mL^{-1}) in 0.09 mol L^{-1} Tris–HCl buffer (pH 8.0) was added 10 µL of EB solution (0.5 mg mL^{-1}). Different amounts of each LAA stock solution (2 mg mL^{-1}) in Tris–HCl buffer were added to the DNA/EB solution in a 96-well microplate and diluted with Tris–HCl buffer to a final volume of 100 µL. Fluorescence intensity was measured in a microplate reader (Zenyth 3100 from Anthos, Salzburg, Austria). The excitation and emission wavelengths were set at 480 and 600 nm, respectively.

Results and Discussion

Self-Assembly Behavior of Pure LAA in Aqueous Media

The self-assembly behavior of the biosynthesized LAA in aqueous medium (in the absence and in the presence of DNA) was studied by conductometry. The conductivity profiles for the studied LAA solutions (in the absence of DNA) are shown in Fig. 2.

The CMC of the pure LAA in buffer solution was determined from the breakpoint in the specific conductivity against LAA concentration plots (Fig. 2), which show two linear regions due to the LAA self-assembly process. At concentrations above the CMC, the slope decreases due to lower conductivity of micelles compared to free surfactant monomers since mobility of condensed counterions at the micellar interface is restricted while the larger micellar size leads to increased friction with the solvent and thus decreased diffusion coefficient (Das et al., 2016; Ali et al., 2009; Faustino et al., 2009a, 2009b).

The average degree of micellar ionization (α) was taken as the ratio of the slopes above and below the CMC (Ali et al., 2009; Das et al., 2016; Faustino et al., 2009a, 2009b), which allowed determination of the degree of counterion binding to micelles (β), according to Equation (1):

$$\beta = 1 - \alpha. \tag{1}$$

The standard ΔG°_{mic} for the LAA systems in the absence of DNA was calculated from Equation (2) based on the phase separation model for ionic surfactants with monovalent counterions in the presence of excess electrolyte (Zana, 1996, 2002):

$$\Delta G^{\circ}_{mic} = RT\,(1+\beta)\,\ln CMC \tag{2}$$

where R is the ideal gas law constant and T the absolute temperature. The thermodynamic parameters obtained for the studied pure LAA solutions are summarized in Table 1.

The hydrophobic effect serves as the driving force for micelle formation, which is thermodynamically favored

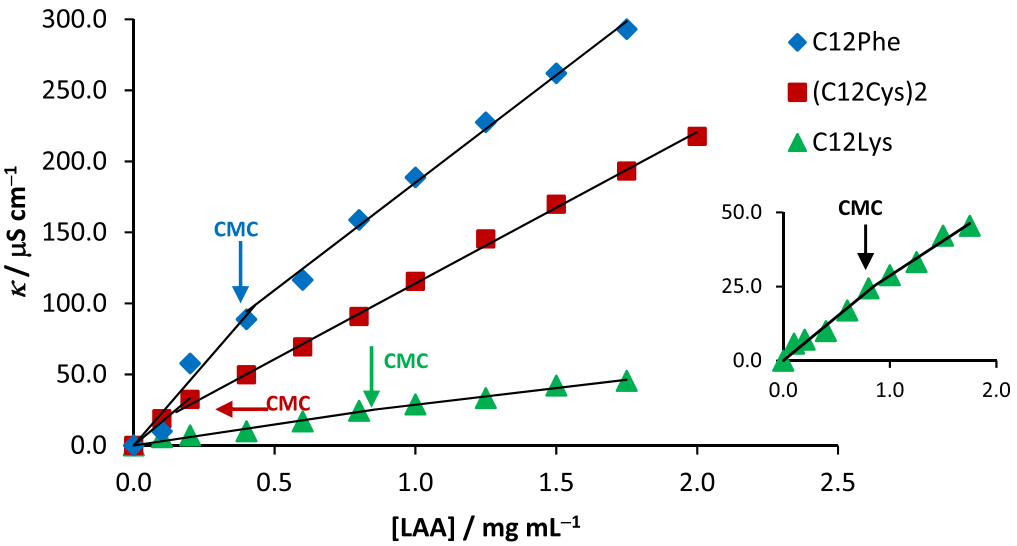

Fig. 2 Conductivity profile for the studied LAA in 0.09 Mol L^{-1} Tris–HCl buffer (pH 8.0) at 293 K. The insert represents the conductivity profile for C12Phe

Table 1 Critical micelle concentration (CMC), degree of micelle ionization (α), degree of counterion binding (β), and standard Gibbs energy of micellization (ΔG°_{mic}) for the lipoamino acids studied in 0.09 Mol L^{-1} Tris buffer (pH 8.0) at 293 K

Surfactant	CMC/mg mL^{-1} (mmol L^{-1})	α	β	ΔG°_{mic}/kJ mol^{-1}
$(C_{12}Cys)_2$	0.15 (0.25)	0.63	0.37	−27.7
$C_{12}Lys$	0.85 (2.72)	0.80	0.20	−17.7
$C_{12}Phe$	0.43 (1.31)	0.66	0.34	−22.1

Note: Values in brackets refer to CMC in Mmol L^{-1}.

(Zana, 1996, 2002; He et al., 2011). Accordingly, the values of ΔG°_{mic} for the self-assembly process of the studied LAA are negative, as shown in Table 1. As expected, the Gemini surfactant, with two hydrocarbon chains, showed higher $|\Delta G^{\circ}_{mic}|$ as a result of increased hydrophobic interactions. The more hydrophobic Gemini surfactant also possessed the highest β while the more hydrophilic $C_{12}Lys$ with a lysine headgroup had the lowest β (Table 1). More hydrophobic surfactants tend to form larger micelles, which reduce the surface area per headgroup, increasing surface charge density and therefore the degree of counterion binding (Faustino et al., 2009a, 2009b, 2010; Pérez et al., 2014; Pinazo et al., 2011). The additional amino group of $C_{12}Lys$ at the side chain of the amino acid residue contributes to increased electrostatic repulsion among the headgroups and thus is more likely to form smaller aggregates with higher surface area per head group, resulting in lower counterion binding (Faustino et al., 2009a, 2009b, 2010; Pérez et al., 2014; Pinazo et al., 2011). A decrease in β has been associated to an increase in the binding ability of surfactants toward DNA (Bhadani and Singh, 2009); thus, the relatively low β values obtained for the studied LAA suggest formation of small micelles with low interface charge density, which can be attributed to the large hydrophilic amino acid headgroup.

The CMC values for the studied LAA increased in the order $(C_{12}Cys)_2 < C_{12}Phe < C_{12}Lys$ due to the corresponding decrease in hydrophobicity of the LAA (Table 1). The micellization process is mainly governed by the number and length of the alkyl chains, and also by the nature of the polar headgroup. Thus, among the studied LAA, the Gemini surfactant $(C_{12}Cys)_2$ produced lower CMC than the other single-tail surfactants of the same alkyl chain length, as expected, due to enhanced hydrophobic interactions that result from the presence of the additional dodecyl chain (Faustino et al., 2010; Pérez et al., 2014; Pinazo et al., 2011). The CMC of the studied Gemini LAA is slightly higher than the ones reported for cationic Gemini surfactants of the same alkyl chain length that also contain a disulphide bond in the spacer, obtained by reaction of dodecyldimethylglycine betaine with cystine or cystamine, 0.065 and 0.120 mmol L^{-1}, respectively (Pinazo et al., 2011). This can be attributed to their quaternary ammonium groups and to the different position of the amide bond in the surfactant structure. A similar effect has been reported for quaternary ammonium ester surfactants (esterquats) of the same alkyl chain length, where the ester bond orientation as $O(C{=}O)(CH_2)_{10}CH_3$ in regular esterquats or as $(C{=}O)O(CH_2)_{11}CH_3$ in betaine esters shifted the CMC from 0.7 to 5 mmol L^{-1}, respectively (Para et al., 2016). Gemini amide quats with dodecyl tails and a tetramethylene spacer, whose structure is very similar to $(C_{12}Cys)_2$, show an almost identical CMC value of 0.23 mmol L^{-1} (Hoque et al., 2012).

On the other hand, the additional amino group at the lysine side chain increases the hydrophilicity of $C_{12}Lys$, which improves water solubility and lowers the tendency of the surfactant to form micelles in aqueous solution, thus contributing to a higher CMC (Colomer et al., 2011; Colomer et al., 2012).

The solubility of the cationic surfactants in 20 mM Tris buffer pH 8 is higher than in (more acidic) water which can be attributed to a decrease in Krafft temperature (T_K) resulting from the increase in ionic strength due to the buffer. Solubility of monomeric ionic surfactants usually increases with temperature up to the critical micelle temperature which for most ionic detergents is identical to T_K (Pérez et al., 2014; Pinazo et al., 2011; Zana, 2002). Electrolyte addition is known to modulate both the CMC and the T_K of ionic surfactants in aqueous solution (Islam et al., 2015). Islam et al., (2015) studied the effect of inorganic sodium salts on the T_K and CMC of anionic surfactant benzyldimethylhexadecylammonium chloride in aqueous media. The authors found that kosmotropic anions such as SO_4^{2-}, F^-, and CO_3^{2-}, which are extensively hydrated in the bulk, lower T_K with increasing concentration of the electrolytes while chaotropic ions like Br^- and I^- tend to form ion pairs with the surfactant, resulting in decreased monomer solubility and increased T_K (Islam et al., 2015). Conductivity measurements and isothermal titration calorimetry for Gemini surfactants also showed that the nature of the counterion strongly influenced CMC and micellar ionization degree, which were correlated with the Hofmeister (lyotropic) series of counteranions (Jiang et al., 2004). The size of the counterion has also been shown to influence micellar growth in *N*-dodecyl alaninate surfactants, which is also promoted in the presence of buffer due to the electrolyte screening effect (Takeuchi et al., 2002).

The nature and size of the amino acid residue can also affect the CMC and T_K of LAA. Haldar and Maji studied *N*-*n*-hexadecanoyl amino acid amphiphiles with hydrophobic C_α-side chains in Tris buffer (pH 9.3) and showed from differential scanning calorimetry measurements that T_K and enthalpy of solution were affected by the type of the amino acid side chain (Haldar and Maji, 2013). The authors eliminated the effect of the counterion on self-assembly, solubility, and ionization behavior of the amphiphiles by using a large excess of Tris buffer compared to the CMC of the surfactants (Haldar and Maji, 2013). Ohta et al., (2003) studied *N*-hexadecanoyl amino acid surfactants and observed that T_K increased with decreasing size of the amino acid residue.

Increasing the concentration of the chloride counterions by addition of Tris–HCl buffer also contributes to decrease the CMC of the studied cationic LAA. The electrostatic repulsion between the charged head groups is reduced due to screening of the surface charge of micelles by the associated counterions resulting in a decrease in CMC and T_K. Similar results have been found by Colomer et al., (2012) for pH-sensitive dodecyl cationic surfactants from lysine and also by our group when comparing the CMC of anionic Gemini surfactants derived from cysteine with octyl chain length in water (CMC = 8.2 mmol $L^{-1;}$ Faustino et al., 2010) and in 0.020 M phosphate buffered-saline at pH 7.4 containing 0.15 mol L^{-1} NaCl (CMC = 0.612 ± 0.014 mmol L^{-1}) (Faustino et al., 2015), both values obtained at 25 °C. Moreover, Colomer et al., (2012) also found that pK_a of the amino group for their cationic lysine-based surfactants usually increased with the increase in ionic strength, which suggests that the studied amino acid–based surfactants are mainly in their protonated form at the pH = 8 of the Tris buffer employed.

Regarding the lysine surfactants, it has been shown that the position of the cationic charge either at the α- or ε-amino group of the side-chain does not significantly affect the CMC. N^{ε}-dodecanoyl lysine methyl ester hydrochloride has a CMC value of 5.5 mmol L^{-1} while N^{α}-dodecanoyl lysine methyl ester hydrochloride has a CMC of 7.2 mmol L^{-1} (Colomer et al., 2011, 2012). The studied lysine dodecyl amide C_{12}Lys has a slightly lower value, close to the CMC value of 1.8 mmol L^{-1} found for arginine dodecyl amide dihydrochloride (Pinazo et al., 2011), which also has an amide bond (instead of ester) at the same position of the studied LAA and unsubstituted nitrogen basic functions.

The phenylalanine-derived surfactant C_{12}Phe has a lower CMC than the corresponding lysine surfactant C_{12}Lys due to the hydrophobic phenyl group at the side chain of the amino acid residue. Thermodynamic studies have revealed that the hydrophobicity of the C_α-substituents in amino acid-based surfactants lower the free energy of micellization and thus the CMC (Chatterjee et al., 2002). Despite the bulky phenyl group in C_{12}Phe, which could hinder micellization, additional micelle stabilization by π-π stacking interactions between the aromatic rings has been described for phenylalanine-derived surfactants (Chatterjee et al., 2002). CMC values of 0.126 and 0.154 mmol L^{-1} (Joondan et al., 2014) have been reported from surface tension and conductivity measurements, respectively, for the dodecyl ester of phenylalanine (hydrochloride salt), which are lower than the one obtained for the corresponding dodecyl amide studied. However, since a similar CMC of 0.212 mmol L^{-1} has been obtained for the hexadecyl amide of phenylalanine quaternized at the α-amino group (Jadhav et al., 2008), a higher value is expected for C_{12}Phe due to the shorter dodecyl chain and thus less hydrophobic character.

The CMC values obtained for the studied LAA are also lower than the one reported for the commercial quaternary ammonium surfactant of the same alkyl chain length, dodecyltrimethylammonium bromide (DTAB), which has a CMC of 16 mmol L^{-1} in aqueous solution (Pérez et al., 2014). Contrary to quaternary ammonium surfactants, whose charge does not depend on the pH of the solution, amino acid-based surfactants are pH-sensitive. Thus, increasing the pH above the pK_a of the surfactant increases the percentage of uncharged species and can lead to decrease in solubility (Chatterjee et al., 2002; Colomer et al., 2012; Pinazo et al., 2011) and/or CMC due to formation of mixed micelles with reduced electrostatic repulsions between the headgroups due to the presence of the uncharged species (Colomer et al., 2012; Mezei et al., 2012).

The α-amino groups of cysteine, phenylalanine, and lysine have pK_a values of 9.1, 9.3, and 10.7, respectively, while the ε-amino group at the lysine side chain has a pK_a value of 10.5 (Colomer et al., 2012, 2013; Pérez et al., 2014; Pinazo et al., 2011). Thus, the studied LAA are expected to be mainly in their protonated form in Tris–HCl buffer (pH 8.0) medium; however, significant pK_a shifts have been reported for amino acid–based surfactants upon micellization as aggregation can induce changes in surfactant protonation (Colomer et al., 2011, 2012; Mezei et al., 2012; Pinazo et al., 2011). Formation of micelles from cationic LAA has been reported to shift the acid-base equilibrium toward the acid species corresponding to the protonated surfactant, since micellar counterion binding stabilizes electrostatic repulsions among the charged headgroups and reduces their proton releasing ability (Colomer et al., 2012, 2013).

Furthermore, pK_a values of weak acids can be influenced by the ionic strength of the medium since increasing the ionic strength stabilizes charge separation and thus increases the acid dissociation constant. For weak acids like

alkylammonium cations, pK_a values have been reported to increase with ionic strength (Colomer et al., 2012). The influence of the ionic strength on the pK_a of lysine- and arginine-derived surfactants has been studied and no significant differences have been observed between apparent pK_a values measured in water and those measured in aqueous saline solutions up to 0.5 mol L^{-1} NaCl added salt (Colomer et al., 2013). Thus, ionic strength is expected to have a minor effect on the apparent pK_a of the studied LAA, suggesting that they remain mainly in their protonated form at pH 8.0 and hence behave like cationic surfactants. On the other hand, high ionic strength of buffer solutions have been reported to lower the CMC due to the electrolyte screening effect, which reduces the electrostatic repulsions between the surfactant headgroups in the micellar aggregates thus promoting micelle formation at lower surfactant concentrations (Faustino et al., 2009a, 2009b; Zhu and Evans, 2006).

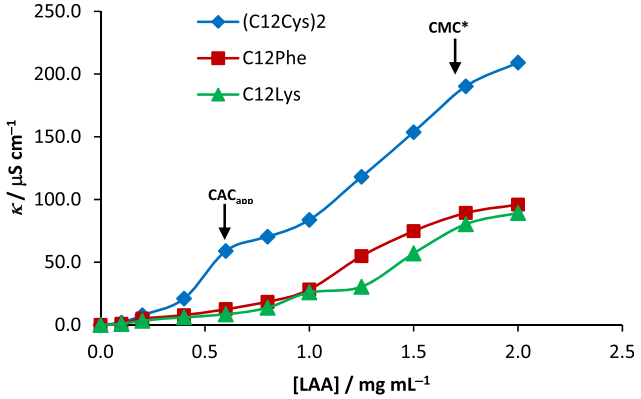

Fig. 3 Conductivity profile for the studied LAA in the presence of 0.01 g L^{-1} DNA in 0.09 Mol L^{-1} Tris–HCl buffer (pH 8.0) at 293 K. curved lines are just guides, not models. Straight lines result from regression analysis in the concentration ranges where linearity between conductivity and surfactant concentration occurred

LAA–DNA Interactions

Self-Assembly Behavior of LAA in the Presence of DNA

Electrical conductivity has been used to provide valuable information on the association process between ionic surfactants and water-soluble polymers or polyelectrolytes, such as polyvinyl pyrrolidone (Ali et al., 2009), inulin (Dan et al., 2009), bovine serum albumin (BSA; Branco et al., 2015; Faustino et al., 2009a, 2009b), lysozyme (Chatterjee et al., 2002), gelatine (Chatterjee et al., 2002; Mitra et al., 2008), sodium hyaluronate (Zhu and Evans, 2006), and sodium carboxymethyl cellulose (NaCMC; Ansari et al., 2013; Das et al., 2016; Wang et al., 2005). Plots of conductivity against surfactant concentration in the presence of polymers and proteins usually show three different linear regions with two characteristic transition points (Ali et al., 2009; Branco et al., 2015; Chatterjee et al., 2002; Faustino et al., 2009a, 2009b). The break point at lowest concentration corresponds to the critical aggregation concentration (CAC), representing the onset of cooperative binding, while the second transition point corresponds to CMC in the presence of the polymer (CMC*) due to bulk micelle formation after polymer saturation has been reached. Although this behavior has also been found in polyelectrolyte-oppositely charged surfactant systems (Bračič et al., 2015; Chatterjee et al., 2002), conductivity dependence on cationic surfactant concentration in the presence of DNA is often more complex due to the tertiary structure of the biopolymer and strong electrostatic interactions. The conductivity profiles for the studied LAA in the presence of 0.01 g L^{-1} DNA are shown in Fig. 3.

All conductivity profiles exhibit two discontinuities, corresponding to apparent CAC (CAC_{app}) and CMC*. Except for the C_{12}Phe-DNA system, deviation from linearity occurred within a narrow LAA concentration range near CAC_{app}, where the increase in conductivity with LAA concentration is interrupted by a slow rise immediately followed by a sharp and linear increase up to the CMC* (Fig. 3). Nonlinearity between CAC and CMC has been observed in polymer-surfactant systems and has been interpreted by assuming that more than one type of aggregate is developing (Chatterjee et al., 2002), as the result of conformational changes (Mitra et al., 2008) or self-assembly of the polymer-surfactant complex is involved (Chatterjee et al., 2002). Transformation of dsDNA from an extended coil state to a compact globule condition has been demonstrated by fluorescence microscopy studies and the onset of DNA compaction are in agreement with the CAC value obtained from conductivity measurements, with a narrow concentration range of coexistence of coils and globules (Dias et al., 2008).

The CAC_{app} and CMC* values obtained from the electrical conductivity profiles for the studied LAA-DNA mixed systems are summarized in Table 2, along with the values found for the standard ΔG°_{agg} for the surfactant-DNA complexation process, which were calculated according to Equation (3):

$$\Delta G^{\circ}_{agg} = RT(1+\beta')\ln(\mathrm{CAC}) \quad (3)$$

where β' is the degree of counterion binding to DNA-surfactant complexes. This value can be obtained from the ratio of the slopes of the conductivity plots against LAA concentration above and below CAC_{app} (while the ratio of the slopes above CMC* and below CAC give the degree of counterion binding to free micelles) whenever there is a decrease in the slopes from the CAC to the CMC* concentration regions. For the studied LAA-DNA systems, the

slope below CAC is lower than the slope above CAC (and also lower than the slope above CMC*); thus, β' could not be determined by this method. Therefore, the β value obtained previously, corresponding to the degree of counterion binding to micelles in the absence of DNA, was used instead of β' in Equation (3). Although lower counterion binding is usually found for polymer-bound micelles due to their smaller size (Ali et al., 2009; Bračič et al., 2015; Faustino et al., 2009a, 2009b), the β values obtained for the free micelles are low and thus are not expected to differ significantly from β'.

For the studied LAA-DNA systems, initial binding of LAA to the polyelectrolyte is expected to take place through electrostatic interactions between the cationic headgroups of the surfactant and the anionic phosphate groups of the DNA backbone along with hydrophobic interactions involving DNA-bound LAA molecules (Zhou et al., 2012). For aromatic LAA, such as C_{12}Phe, additional π-π interactions between the phenyl substituent of the phenylalanine headgroup and the DNA nucleobases can occur (Zhou et al., 2012).

Cooperative binding of cationic surfactants to DNA usually occurs at a critical surfactant concentration (i.e., CAC) much lower than CMC due to the strong electrostatic interactions, similar to other polyelectrolyte-oppositely charged surfactant systems (Ansari et al., 2013; Chatterjee et al., 2002; Das et al., 2016; Wang et al., 2005). The CAC of DDA and DTAB was found to shift to lower values in the presence of dsDNA and synthetic polyribonucleotides in buffer solution (1 mmol L^{-1} Tris–HCl, pH 7.2, 10 mmol L^{-1} NaCl) as compared to CMC of pure DDA and DTAB buffer solutions (Petrov et al., 2002). From Table 2, it is evident that the CAC values (CAC_{app}) obtained are all higher than the CMC for the corresponding LAA (Table 1), which usually means that formation of free micelles is favored over polymer-bound ones, and DNA complexation does not occur. However, comparison of the conductivity profiles for the studied LAA in the absence and in the presence of DNA (Figs. 2 and 3, respectively) show striking differences, suggesting that interaction with DNA does occur. Moreover, surfactant concentration corresponding to formation of free micelles in solution (CMC) was higher in the presence of DNA, suggesting formation of a LAA-DNA complex responsible for hindering the micellization process (Ali et al., 2009; Branco et al., 2015; Chatterjee et al., 2002; Faustino et al., 2009a, 2009b). DNA complexation by the studied LAA was further confirmed by the EB exclusion assay, as will be discussed below.

Table 2 Apparent critical aggregation concentration (CAC_{app}), critical micelle concentration in the presence of biopolymer (CMC*), and standard Gibbs energy of aggregation (ΔG°_{agg}) for the lipoamino acids studied in the presence of 0.01 g L^{-1} DNA in Tris buffer (0.09 Mol L^{-1}, pH 8.0) at 293 K

Surfactant	CAC_{app}/mg mL^{-1} (mmol L^{-1})	CMC*/mg mL^{-1} (mmol L^{-1})	ΔG°_{agg}/ kJ mol^{-1}
$(C_{12}Cys)_2$	0.63 (1.09)	1.76 (3.06)	−24.7
C_{12}Lys	1.12 (3.57)	1.74 (5.56)	−20.4
C_{12}Phe	0.90 (2.71)	1.60 (4.82)	−23.4

Note: Values in brackets refer to critical concentrations in mmol L^{-1}.

The high values obtained for CAC_{app} may reflect complex coacervation due to self-assembly of a LAA-DNA complex formed at lower LAA concentrations. These LAA concentrations were not detected by conductivity measurements under the experimental conditions. The buffer used had a high ionic strength, which hides the conductivity of highly diluted ionic solutions, precluding further studies of more diluted LAA-surfactant solutions. Often, CAC values that occur at very low surfactant concentration cannot be detected by conductometry. This was the case for inulin-alkyltrimethylammonium surfactants (Takeuchi et al., 2002), gelatine-DTAB (Mitra et al., 2008), NaCMC-alkyltrimethylammonium surfactants (Takeuchi et al., 2002), and NaCMC-cationic gemini surfactant tetramethylene-1,4-bis(dimethyltetradecylammonium bromide) (14-4-14) systems (Das et al., 2016). For the octadecyltrimethylammonium (OTAB)-inulin system, CAC values, although determined by tensiometry, could not be detected by either conductivity, turbidity, or viscometry (Dan et al., 2009). Surface tension measurements are sensitive mainly to the concentration of the monomeric form of the surfactant while conductometric methods depend on the electrical conductivity of all the ionic species present in solution. Regarding polymer-surfactant systems, tensiometry can reveal the nature of the interaction of the surfactant with the polymer both at the interface and in the bulk, while conductivity is restricted to bulk phase properties, being less sensitive for detection of self-assembly processes at low surfactant concentrations. Surface tension measurements were performed for the studied LAA solutions; however, the long equilibration times did not allow for a constant surface tension value to be reached due to separation of a minor amount of coacervate from the solution upon standing for a long time. It is known that surfactants with high surface activity (i.e., with equilibrium surface tension ≤30 mN m^{-1}) and low CMC can lead to long equilibration times due to low adsorption dynamics and may require several hours of measurement for surface tension to reach a constant value (Bhadani and Singh, 2009; Branco et al., 2015; Faustino et al., 2010; Mitra et al., 2008; Zana, 2002).

Mixtures of polyelectrolytes and surfactants of opposite charges often show strong tendency to form insoluble complexes, leading to phase separation either by coacervation

or by precipitation due to reduced electrostatic repulsions between the polymeric chains upon charge neutralization (Das et al., 2016; Dias et al., 2008). Compensation of the electric charges of the polyelectrolyte by increasing the concentration of oppositely charged surfactant eventually leads to the formation of electrically neutral complexes of increased hydrophobicity that can separate out from the solution (Das et al., 2016; Dias et al., 2008). Further surfactant addition leading to charge overcompensation may result in redissolution of coacervates or formation of turbid colloidal dispersions (Dan et al., 2009; Das et al., 2016; Mitra et al., 2008). Free micelle formation in the bulk phase upon reaching CMC* can also result in coacervate (or precipitate) solubilization with depletion of visible turbidity (Mitra et al., 2008). Coacervation is mainly dependent on the balance between electrostatic, hydrophobic, and solvent interactions (Das et al., 2016). Several factors are known to influence complex coacervation, such as polyelectrolyte composition and charge density, chain length and flexibility, polymer molecular weight, micelle size, shape and surface charge density, temperature, pH, and ionic strength of the medium (Das et al., 2016).

The behavior of the studied LAA-DNA systems was similar to the one found for cationic Gemini surfactant 14-4-14 in the presence of anionic polymer NaCMC (Das et al., 2016). The tensiometric profile in the cited study system showed that 14-4-14 monomers interacted with the polymer at very low surfactant concentration with a CAC value in the micromolar range (Das et al., 2016). For surfactant concentrations above CAC, the surfactant-NaCMC complex self-assembled to form a turbid coacervate phase once polymer saturation concentration, C_{sat}, was reached. Above C_{sat}, the system became a turbid colloidal solution and free surfactant micelles were formed above CMC* that coexisted with the coacervates, even at higher surfactant concentrations (Das et al., 2016). On the other hand, the conductivity profile for this system showed two distinct transitions at surfactant concentrations corresponding to the C_{sat} and CMC* values. No breakpoint was observed in the concentration range near tensiometric CAC in the experiments performed by tensiometry (Das et al., 2016). Thus, the two break points in the conductivity plots against surfactant concentration for the studied LAA-DNA systems, CAC_{app} and CMC*, likely correspond to the onset of coacervation and to free micelle formation in the bulk phase, respectively.

Similar behavior has also been found for single-chain cationic surfactant OTAB in the presence of inulin, with formation of a surfactant-polysaccharide complex at low OTAB concentrations, as revealed by tensiometry, which self-aggregated above the CAC, leading to coacervate formation at the polymer saturation concentration C_{sat} (Dan et al., 2009). At concentrations above CMC*, free surfactant micelles were formed in solution (Dan et al., 2009). For the OTAB-inulin system, the conductometric profile showed no transitions close to the CAC, similar to the gelatine-cetyl trimethylammonium (CTAB) system, but the breakpoints corresponding to C_{sat} and CMC* were observed and agreed with the values obtained *via* tensiometry (Dan et al., 2009). An increase of turbidity for OTAB-inulin solutions appearing above C_{sat}, due to coacervation, was not significantly reduced as the surfactant concentration was further increased, even above CMC*, suggesting the coexistence of coacervates and free OTAB micelles. This was also the case for the Gemini 14-4-14-NaCMC system and for the studied LAA-DNA systems, where turbidity occurred after CAC_{app} and did not disappear in the post-CMC* region upon micelle formation in the bulk, being more pronounced at higher surfactant concentrations. Moreover, for the studied LAA-DNA systems, as well as for the 14-4-14-NaCMC (Das et al., 2016) and OTAB-inulin (Dan et al., 2009) systems, a minor amount of surfactant-polymer complex separated from the solution after standing for a long time. On the other hand, gelatin-CTAB complexes also formed turbid colloidal dispersions due to coacervation but solubilization of the coacervates by free surfactant micelles led to depletion of visible turbidity at surfactant concentrations above CMC* (Mitra et al., 2008).

The conductivity profiles for the studied cationic LAA-DNA systems show a linear increase of electrical conductivity with surfactant concentration in the low surfactant region up to the first breakpoint (CAC_{app}),attributable to the release of DNA counterions (Na^+) into the bulk phase upon surfactant binding, which also contributes to an increase in the entropy of the solution that drives cooperative binding (Matulis et al., 2002). After the first break point, which corresponds to the onset of coacervation, a sharp increase in electrical conductivity with surfactant concentration was observed. In addition, an increase in the slope between the first and the second break points occurred, reflecting the contribution of surface conduction (Stern layer conduction). This contribution is typical of colloidal dispersions to the overall conductance of the coacervate-containing colloidal solution (Dan et al., 2009; Das et al., 2016). Above the second break point (CMC*), the slope decreased due to condensation of counterions from the bulk phase to the interface of DNA-free micelles (Das et al., 2016). The same behavior was observed in the conductometric profiles for the cationic Gemini surfactant 14-4-14 in the presence of NaCMC (Das et al., 2016) and for the OTAB-inulin system (Dan et al., 2009). On the other hand, short-chain alkyltrimethylammonium surfactants, such as DTAB and tetradecyl trimethylammonium bromide (TTAB), showed a very weak interaction with inulin and only a single break point was detected in the

conductivity profile, which corresponded to CMC* (Dan et al., 2009). The strength of the interaction of the quaternary ammonium surfactants with inulin increased with alkyl chain length, supporting the significant role played by surfactant hydrophobicity in the complexation process (Dan et al., 2009).

The CMC* values for the studied LAA-DNA systems (Table 2) are all higher than CMC values obtained in the absence of the polyelectrolyte (Table 1), which was expected since formation of the surfactant-DNA complex hinders the micellization process. This behavior is an indication of effective interaction between surfactant and DNA and it is typical of other surfactant-polymer systems, including mixed systems formed by the same Gemini LAA studied (but prepared by chemical synthesis) and BSA (Branco et al., 2015; Faustino et al., 2009a, 2009b).

Both CMC* and CAC_{app} for the studied LAA-DNA systems decreased with increasing LAA hydrophobicity (Table 2). CMC* has a direct correlation with surfactant hydrophobicity, as discussed above, for the pure LAA systems. Thus, $(C_{12}Cys)_2$, a Gemini surfactant, i.e., more hydrophobic than the surfactants investigated herein, has a lower CMC* and CAC_{app}, supporting the role of hydrophobic interactions in complex coacervation (Ansari et al., 2013; Matulis et al., 2002). Observing that the values obtained for ΔG°_{agg} were all negative (Table 2), coacervation was a spontaneous process favored over free micelle formation in the bulk phase in the biopolymer environment since $|\Delta G^{\circ}_{agg}|$ is slightly higher than $|\Delta G^{\circ}_{mic}|$ for micellization in pure LAA solutions (Table 1).

Formation of the LAA-DNA Complex

The binding of the studied cationic LAA with dsDNA was confirmed from fluorescence measurements in the EB exclusion assay. EB is a cationic dye commonly used as a probe for native DNA that shows enhanced fluorescence emission upon intercalation between the DNA base pairs in comparison to residual fluorescence emission in water (Bhadani and Singh, 2009; Dasgupta et al., 2007).

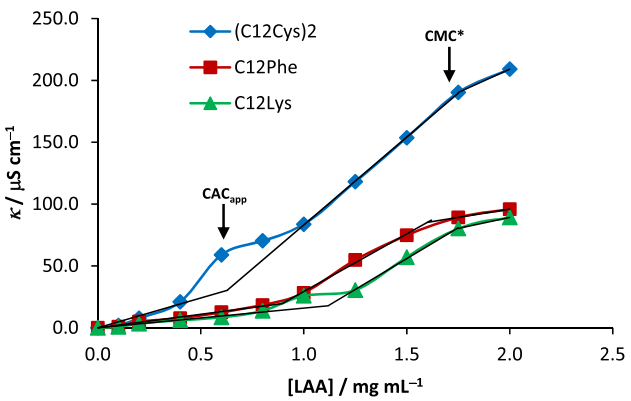

Fig. 4 Quencher efficiency (%) of the studied LAA at different concentrations (below CAC_{app}, above CAC_{app} and below CMC*, and above CMC*) in the ethidium bromide exclusion assay in 0.09 Mol L^{-1} Tris–HCl buffer solution (pH 8.0) and 0.01 g L^{-1} DNA

The extent of LAA binding to DNA can be determined by the ability of the surfactant to displace intercalated EB from the DNA-EB complex, which results in quenching of the fluorescence signal due to the formation of the surfactant-DNA complex (Dan et al., 2009; Zhao et al., 2008). The percentage of quenching (Q) observed from the displacement of EB from the intercalation complex with DNA upon addition of increasing amounts of cationic Gemini LAA was determined according to Equation (4):

$$Q\,(\%) = (1 - F/F_0) \times 100 \tag{4}$$

where F_0 and F are the fluorescence intensities at 600 nm of the DNA-EB complex in the absence and presence of LAA, respectively.

Although fluorescence quenching was observed at all surfactant concentrations tested, saturation of the fluorescence quenching was not achieved in the LAA concentration range studied. However, more concentrated solutions were not analyzed due to solubility problems. Thus, the accessibility of DNA to EB is preserved to a significant extent in the presence of the studied LAA, particularly for the more hydrophilic surfactant C_{12}Lys, as shown in Fig. 4.

The differences in the EB displacement shown by the different surfactants can be interpreted in terms of the strength of interaction between LAA and DNA. On the other hand, these differences can also be due to the type of self-assembly structure that the surfactant adopts in the vicinity of the DNA molecules. The fact that 50% fluorescence quenching was not achieved even at the highest LAA concentrations tested suggests a weak interaction of the LAA with DNA, which can be due to their relatively short dodecyl chain(s). Surfactant-DNA interaction is usually enhanced with increasing alkyl chain length, supporting the role of hydrophobic interactions in the process, in combination with the electrostatic interactions (Han and Wang, 2011; He et al., 2011; Husale et al., 2008). The CAC_{app} values decreased with increasing LAA hydrophobicity, reflecting the relevance of hydrophobic interactions in complex coacervation (Fig. 4). The same trend has been observed in other polyelectrolyte oppositely charged surfactant systems. Among the quaternary ammonium surfactants DTAB, TTAB, and CTAB, the one with the shorter (dodecyl) chain, DTAB, underwent very weak interaction with NaCMC (Ansari et al., 2013). Alkyltrimethylammonium surfactants with higher alkyl chain lengths were also more interactive with gelatin than their short-chain homologues (Mitra et al., 2008).

The high ionic strength of the buffer solution used can also weaken the LAA-DNA interaction due to the screening of electrostatic interactions between DNA and the oppositely charged LAA (Wang et al., 2013; Zhu and Evans, 2006). Addition of salt has been shown to markedly influence the formation of NaCMC/DTAB complexes, which is prevented at high ionic strength due to complete salt screening of the electrostatic attractions between DTAB and NaCMC (Wang et al., 2005). The $(C_{12}Cys)_2$-BSA system has been previously studied in a high ionic strength medium and a relatively weak interaction was observed (CAC/CMC ratio of 0.63 in phosphate buffer at pH 7.4 containing 0.15 mol L^{-1} NaCl; Branco et al., 2015).

The more hydrophobic LAA, $(C_{12}Cys)_2$ and $C_{12}Phe$, were able to quench 35.6% and 44.2% of the initial fluorescence intensity, respectively, while $C_{12}Lys$ only quenched 15.5% (Fig. 4). Thus, the efficiency of EB exclusion for the studied LAA decreased in the order $C_{12}Phe > (C_{12}Cys)_2 > C_{12}Lys$. Although enhanced EB exclusion from DNA has been associated with increased hydrophobic interactions due to increase in the alkyl chain length (Bhadani and Singh, 2009), the studied LAA all have dodecyl chains; therefore, the differences observed in the EB exclusion assay can be attributed to the polarity of the amino acid group and thus increased hydration resulting in larger effective size (Dasgupta et al., 2007). A similar trend has been observed for cationic surfactants with hexadecyl chains upon introduction of additional hydroxyl substituents in the head group (Dasgupta et al., 2007). Surfactants with larger head groups form smaller micellar aggregates and their higher curvature is associated with less efficient surface coverage of DNA, leaving parts of the polynucleotide structure accessible to EB binding (Singh et al., 2011). Among the studied LAA, $C_{12}Lys$ with a headgroup bearing two polar amine functions (Fig. 1) is the most hydrophilic, and it has been shown that surfactants with more hydrophilic and/or more hydrated headgroups are less efficient in expelling EB due to packing constraints (Dasgupta et al., 2007). In fact, Degusa et al. … showed that the accessibility of DNA to EB was preserved to a significantly larger extent for the more hydrophilic surfactants, in terms of surfactant packing, reflecting that the surfactants with more substituents have a larger headgroup and therefore form smaller micellar aggregates. Moreover, restrictive conformational freedom has also been related to better binding capability and enhanced quenching, e.g., cationic pyridinium surfactants have shown higher efficiency in EB displacement over quaternary ammonium surfactants of the same alkyl chain length (Bhadani and Singh, 2009). This fact can explain the higher quenching efficiency of $C_{12}Phe$ over Gemini LAA $(C_{12}Cys)_2$ due to the presence of the phenyl substituent in the former, which can provide additional stabilization of the $C_{12}Phe$-DNA complex by π-π interactions between the surfactant phenyl group and the nucleotide bases that impart conformational restrictions and block the accessibility to EB (Bhadani and Singh, 2009).

Conclusions

Cationic single-chain and dimeric surfactants derived from natural amino acids (LAA) were prepared by a green biotechnological approach using a lipase encapsulated in sol–gel and their supramolecular behavior in aqueous media (Tris buffer, pH 8.0) was characterized both in the absence and in the presence of dsDNA. Conductivity measurements showed that all LAA self-assembled to form micelles in solution which occurred at a higher LAA concentration in the presence of DNA, under conditions where coacervation occurs. The hydrophobicity of the LAA, which was mainly determined by the number of dodecyl chains and nature of the amino acid group, strongly influenced the self-assembly behavior and interaction with DNA. Formation of a surfactant-DNA complex was demonstrated from fluorescence measurements of the EB exclusion assay, although results suggest a weak interaction. The phenomenon can be attributed to the relatively short dodecyl chains, thereby supporting the major role played by hydrophobic interactions in the LAA-DNA association process in addition to electrostatic interactions between the negatively charged DNA phosphate groups and the positively charged amino groups of the LAA. Moreover, the high ionic strength of the buffer solution employed may have also contributed to screening of electrostatic interactions.

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

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