Chiral pH-Sensitive Cyclobutane β-Amino Acid-Based Cationic Amphiphiles: Possible Candidates for Use in Gene Therapy

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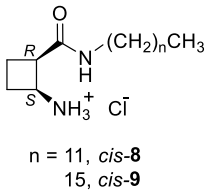
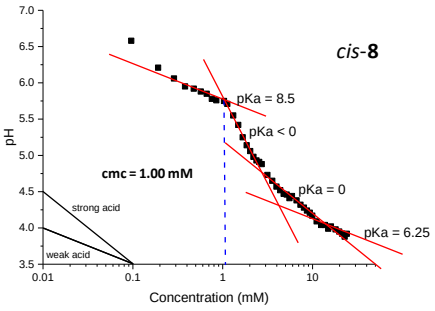
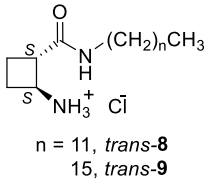
ABSTRACT.

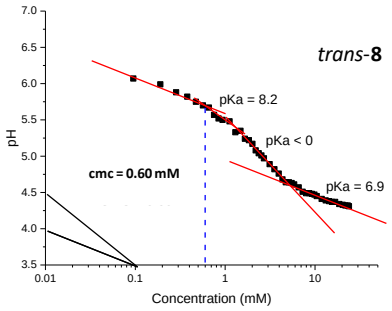


n = 11, *cis*-**8**
15, *cis*-**9**





n = 11, *trans*-**8**
15, *trans*-**9**



Chiral *cis/trans* diastereomeric cationic amphiphiles have been synthesized and studied. They are based on β-amino acids and contain C_{12}- and C_{16}-alkyl chains, respectively, as hydrophobic tails while the polar head consists of an ammonium cation linked to a cyclobutane ring. Their physicochemical properties, such as the cmc (critical micellar concentration), the pK_a, the ratio of cationic versus non-ionic species, and the surface tension are strongly dependent on the pH of the medium. At the same time the aggregation state influences on the apparent pK_a values of the aggregates, with release of protons at the cmc whose values, as well as those of the adsorption effectiveness, account for their efficient surfactant behavior. A tail-length effect is manifest because surfactant cmc of compounds with a C_{16}-alkyl chain are smaller than the C_{12}-ones, although not as small as expected. On the other hand, while for C_{12}-surfactants the role of the stereochemistry on the physicochemical parameters is patent, it is not very clear for C_{16}-surfactants. The stereochemistry also determines the predominant mode of self-

assembly since the trend for *cis*-isomers is to form micelles or vesicles while *trans*-isomers preferably form fibers. CD spectroscopy confirmed the aggregation through the formation of intermolecular hydrogen bonds between the amide groups of the monomers. An alternative method to determine the cmc of these surfactants is provided by considering the relationship of the CD λ_{max} with concentration, although it is restricted to those surfactants in which the chromophore is located in a chiral environment. Furthermore, the non- toxicity of the surfactants has been verified by the MTT assay. This characteristic, jointly with the efficiency and the good properties shown, the fact that the cationic species are present in a high concentration at physiological pH, as well as the weak acid behavior of the aggregates, confirm these amphiphiles as promising candidates to be used as non-viral vectors for gene therapy applications.

1. INTRODUCTION

Gene therapy has gained significant attention over the past two decades as a potential method for treating genetic disorders [1-5] as well as an alternative way to traditional chemotherapy used in treating cancer [6]. It mostly involves the use of DNA fragments that encodes a therapeutic protein or gene in order to replace a mutated gene. In these cases, DNA is packed within a vector forming DNA-vector complexes in order to protect the information of the chain and to get inside specific cells within the body [7]. Depending on the temperature, the pH, or the ionic strength, a single DNA chain in solution could adopt different structures.

The role of chemists in the field of gene therapy is to design and prepare new non-viral vectors thus avoiding the risks derived of the work with virus materials. Special attention has been paid to cationic surfactants due to their properties [8]. Cationic polar heads can interact with negatively charged phosphate groups of the DNA while hydrophobic chains can stabilize the aggregates. In order to transfect into cells it is important to control DNA compaction and the neutralization of negative charges to avoid repulsive interactions with phospholipids in the cell membrane. One of the most studied surfactants is commercial CTAB (hexadecyl trimethylammonium bromide) [9-10]. It shows high efficiency compacting DNA compared with other shorter surfactants, but most trimethylammonium derivatives show cytotoxicity [11].

Amino acid-based surfactants are getting importance due to their good levels of biodegradability and biocompatibility [12-16]. The combination of amino acids or peptides with hydrocarbon chains of variable length has given rise to a variety of compounds with an amphiphilic structure and with good surfactant properties. Although most examples use natural α-amino acids, the design of pH-sensitive amphiphiles can be achieved from synthetic amino acids. The possibility to tune the properties of the

surfactants depending on the pH of the medium confers on these compounds a great potential for biomedical applications [16-17]. Otherwise, the presence of amide groups can help the self-assembly of surfactants through intermolecular hydrogen bonding [18].

In our laboratories [19], different cyclobutane-containing anionic, **1** [20], or non-ionic, **2** [21], β-amino acid-based amphiphiles were synthesized and studied. Results pointed out the relevance of the relative stereochemistry and of the stereochemical constraints imposed by the cyclobutane ring on the aggregation properties of these compounds (Figure 1).

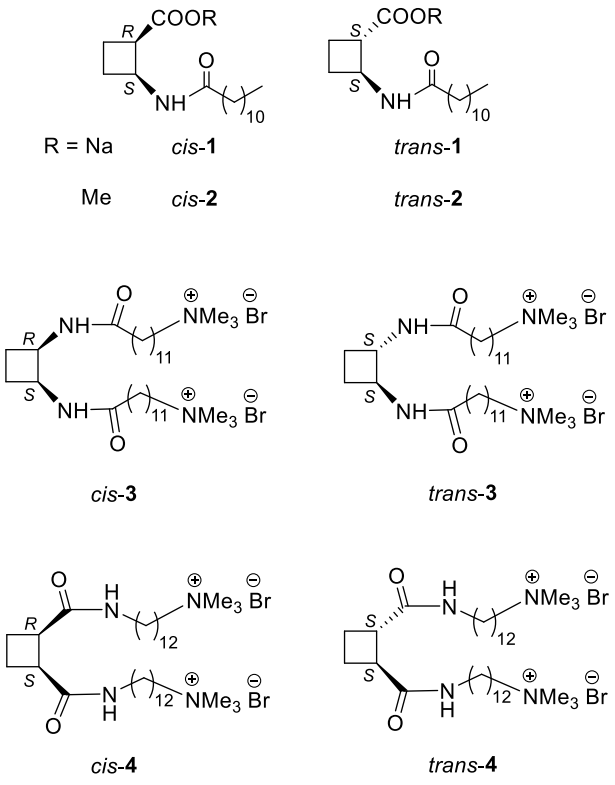
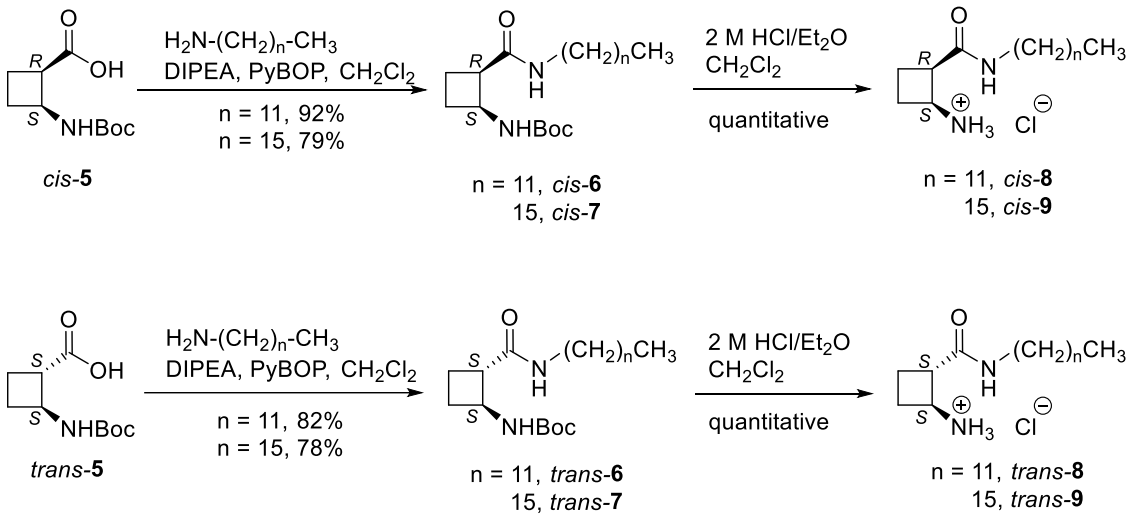


Figure 1. Anionic (**1**), non-ionic (**2**), and cationic (**3** and **4**) amphiphiles previously investigated in our laboratories.

Cationic bolaamphiphiles based on chiral cyclobutane scaffolds, **3** and **4**, were also prepared and investigated concluding that regiochemistry (*N*- versus *C*-centered

derivatives) plays a minor role on the cmc value but determines the morphology of the supramolecular aggregates formed [22] (Figure 1).

In this paper, we describe the synthesis of four new cationic β-amino acid-based surfactants *cis*- and *trans*-**8**, and *cis*- and *trans*-**9** (Scheme 1). All these compounds are chiral and include two pairs of *cis/trans* diastereomers; in turn, **8** and **9** differ in the alkyl-chain length (C_{12} and C_{16}, respectively). Both the influence of the relative stereochemistry and of the chain length have been considered in their physicochemical characterization and in the analysis of the chiral supramolecular systems formed as surfactants. Special attention has been paid to the influence of the pH on these properties. Furthermore, bearing in mind the possibility of the future use of these surfactants as non viral vectors for gene therapy, preliminary studies on their cytotoxicity have been carried out. We present and discuss herein all the results obtained.



Scheme 1. Synthesis of the cationic amphiphiles *cis*- and *trans*-**8**, and *cis*- and *trans*-**9.**

2. EXPERIMENTAL SECTION

2.1 Synthesis of the amphiphiles.

The detailed synthesis and characterization of surfactants *cis*- and *trans*-**8**, and *cis*- and *trans*-**9** and their precursors (Scheme 1) are reported in the Supporting Information.

2.2 pK_a Measurements.

The pK_a values were determined from the potentiometric titration of 1 mL of 5 mM aqueous surfactant solutions by using 5 mM NaOH aqueous solutions. The pH electrode was an ORION 8103SC semimicro and the potentiometer was a Thermo Orion model 720A+. All titrations were conducted at 25 ± 0.1 °C and under nitrogen atmosphere. pK_a was determined as the pH of the corresponding semi equivalence point. Once the titration is finished, a new titration with 5 mM HCl is done to determine the reversibility. De-ionized water (milli-Q) was used to prepare the solutions.

The pH values of different concentrations of surfactant water solutions under nitrogen were measured using a pH electrode (model ORION 8103SC Ross Semimicro). Measurements were made at increasing concentrations of surfactant to minimize errors from possible contamination from the electrode. The acid−base equilibrium was modelled by Equation 1 for a weak base, assuming dilute ideal behavior and complete salt dissociation. This equation was obtained taking into account the equilibrium constant definition, mass and charge balances, and the ionic product of water.

$$C_a = \left(1 + \frac{[H^+]}{K_a}\right)\left([H^+] - \frac{K_w}{[H^+]}\right) \quad (1)$$

The pK_a values both below the cmc (monomeric surfactant) and above the cmc (apparent pK_a) were evaluated by using this equation.

2.3 Surface Tension Measurements.

The surface tension was measured using a home-made pendant drop tensiometer as in reference 22. Surface tension in acidic (0.01 M HCl) and basic conditions (0.01 M NaOH) have been compared with unbuffered aqueous solutions. When using Gibbs isotherm the values of n to estimating the area per molecule at cmc are 1 for both buffered conditions and undefined for the unbuffered conditions. We are aware of the problems with estimating area per molecule from

surface tension, however, we think that these values have a strong comparative meaning when compared to other in the literature, please refer to reference 22 and literature cited herein.

2.4 Cryogenic Transmission Electron Microscopy (cryo-TEM).

A drop of the surfactant solution with 2.5 mM concentration was placed on a carbon-coated copper grid. Then, the sample-grid assembly was rapidly frozen using liquid ethane and kept at -180 °C during the imaging using liquid nitrogen. The images were acquired with Hitachi H-7000 microscope operating at 200 kV.

2.5 Computational Calculations.

The structure of the surfactants in solution was optimized with DFT in water solution using M06-2X density functional [23] and 6-31G(d) level of theory. These calculations have been carried out using the Gaussian-09 program [24].Circular dichroism spectra were calculated by taking the optimized structure of the monomer in water solution and calculating 30 excited states with Gaussian-09 using the TDDFT method [25-27] at the M06-2X/6-311++G(2df,2pd) level of theory.

2.6 Circular Dichroism (CD) Spectroscopy.

0.5-5.0 mM samples of surfactants were prepared in water and measured in a 1 cm width quartz cuvette. CD spectra were recorded with JASCO-715 spectropolarimeter and were processed using Spectra Manager Software.

2.7 Ultraviolet – visible (UV - vis) Spectroscopy.

The absorption was measured in a Hewlett Packard 8453 spectrophotometer in aqueous solutions in a range between 180 and 500 nm.

2.8 Biological Experiments.

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [28], was used to analyze the cell viability and, proportionally, the surfactant toxicity. MTT (yellow color) is reduced by the mitochondrial enzymes to formazan, an insoluble purple crystal which absorbance can be read with a spectrophotometer after its solubilization by an organic solvent. The higher the absorbance value, the higher the enzymatic activity, and therefore the more number of living cells are present. In separate experiments, *HeLa* cells [29] were incubated in the presence of the different surfactants at about 50 μM concentration in water and MTT (Sigma-Aldrich) in a 0.1 mg/mL concentration was added. After 3 h of incubation at 37 ºC in darkness, the MTT was removed and formazan crystals were dissolved using DMSO. The absorbance was recorded at 540 nm in an X3 Multilabel Plate Reader coupled to a PerkinElmer

2030 Manager control software. The experiments were replicated 4 times for each surfactant. The control experiment was performed under the same conditions but in the absence of a surfactant.

The normalization of the results was achieved considering the average of the absorbance value of the control population as 100% of cellular proliferation (Equation 2).

$$\%\ Cell\ viability = \frac{Average\ Absorbance\ of\ treated\ cells}{Average\ Absorbance\ of\ non-treated\ cells} x\ 100 \quad (2)$$

3. Results and Discussion

3.1 Synthesis of the amphiphiles.

cis- and *trans*-Diastereoisomers of compounds **8** and **9** were synthesized from the appropriate and partially protected amino acids *cis*- and *trans*-**5**, respectively, by means of analogous synthetic routes (Scheme 1). These amino acids were prepared, in turn, in an enantioselective manner as previously described [30-31].

Condensation of acid *cis*-**5** **[**30] with dodecylamine, in the presence of diisopropylamine (DIPEA) and (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) as a coupling agent, afforded *cis*-**6** in 92% yield for the two steps. Similarly, condensation with hexadecylamine led to *cis*-**7** in 79% yield. Subsequently, quantitative deprotection of the respective amines by acidolysis of the Boc carbamate with 2 M HCl in Et_2O provided cationic amphiphiles *cis*-**8** and *cis*-**9**, respectively. Following similar synthetic routes starting from *trans*-**5** **[**31], the diastereomers *trans*-**8** and *trans*-**9** were prepared in 82 and 78% yield, respectively.

3.2 Determination of the pK_a of the surfactants and study of the pH dependence.

The pK_a of the surfactants investigated was determined by using two different and complementary techniques: (a) Measurement of the pH at various surfactant concentrations; (b) acid-base titration with sodium hydroxide and retro-titration with hydrochloric acid. Surfactants **8** and **9** present an acid-base equilibrium in aqueous medium that is the average of several equilibria involving ionic or non-ionic monomers and aggregates, which depend on the pH of the medium and the concentration of the surfactant.

3.2.1 Measurement of the pH at diverse surfactant concentrations.

First, the dependence of the pH on the concentration of surfactant was investigated at 25 ºC. The obtained plots are shown in Figure 2 and from data therein, the relation of pK_a with respect to the concentration of surfactant was determined according to Equation 1 in the Experimental Section.

For pH-sensitive amphiphiles, at the extremes of concentration, the pH values follow the slope of 0.5 expected for weak acids, with pK_a corresponding to the monomers at low concentrations and to the average of monomers and micelles above the cmc (Figure 2). The onset of micellization is marked by the first change of slope, which corresponds to the critical micellar concentration (cmc). Indeed, when the formation of micelles occurs, a release of protons is produced due to the apparent pK_a change of the micelle with respect to the monomer [17] The dependence of the pK_a values on the concentration, for each surfactant, is detailed in the Supporting Information (figure S1) .

Comparing diastereomeric surfactants *cis*- and *trans*-**8**, both with a C_{12}-alkyl chain, the pK_a values before the cmc are very similar: 8.5 for *cis*-**8** and 8.2 for *trans*-**8** (Figure 2). Therefore, it seems that *cis/trans* stereochemistry does not exert a significant

influence on the behavior of the monomeric surfactants. Nevertheless, the slightly weaker acidity of the *cis* isomer could be understood by the possibility of hydrogen bonding between a proton of the ammonium cation and the oxygen of the carbonyl group in the amide (see Figure 6 below). This possibility does not exist in the *trans* stereoisomer. These values show that both surfactants are weak acids and their structure, cationic or non-ionic, will depend on the pH of the solution. However, these surfactants are more acid than alkylammonium chlorides (DAC), of use in gene therapy, which pK_a has been determined at several concentrations dodecylamine pK_a=8.8 at 5 mM, or pK_a=9.16 at 2 mM, while tetradecylamine has a pK_a=7.56 at 2 mM [32]. Taking into account that the pH of the human blood is around 7.4, we can expect that, under physiological conditions, *cis*- and *trans*-**8** will be mostly in the protonated form.

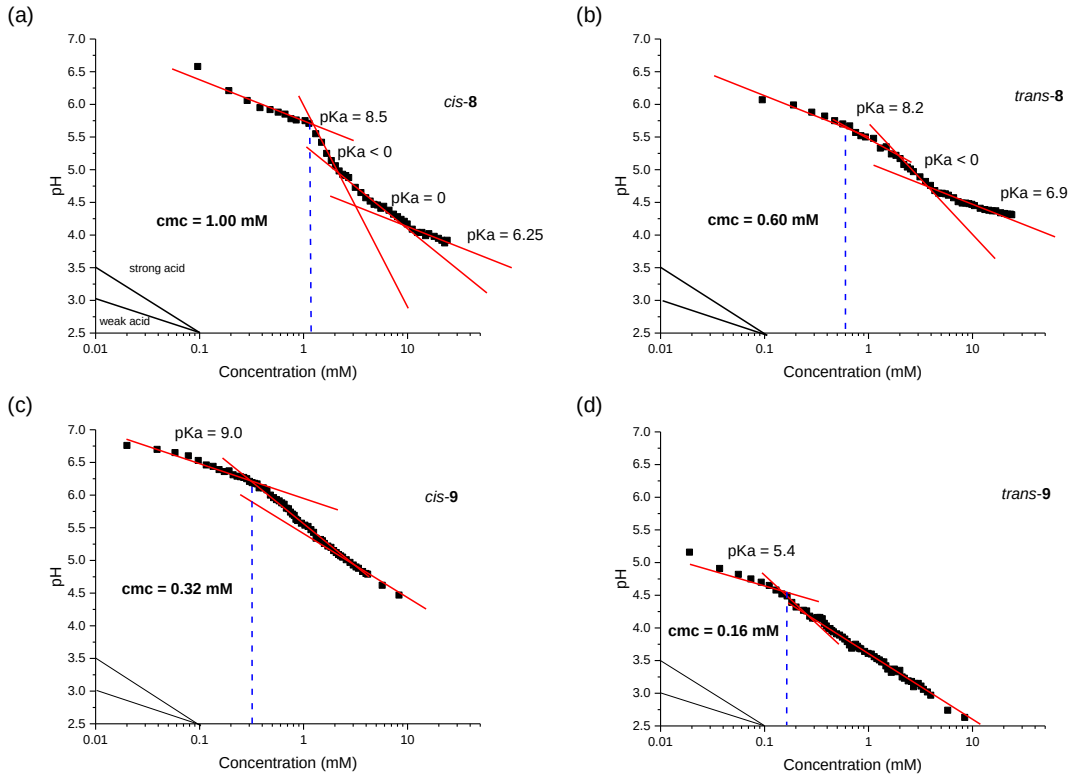


Figure 2. Plots of pH versus surfactant concentration for: (a) *cis*-**8**, (b) *trans*-**8**, (c) *cis*-**9**, (d) *trans*-**9**. Measurement at 25 ºC.

The situation is different for *cis*- and *trans*-**9** bearing a C_{16}-alkyl chain. Indeed, *cis*-**9** presents a pK_a 9.0 before the cmc while *trans*-**9** shows a very low pK_a, around 5.4. This unexpected behavior could suggest that, in this case, stereochemistry plays a very important role in the acidity of the proton, in contrast with surfactants *cis*-and *trans*-**8** with a shorter carbon chain. Surfactant *trans*-**9** was the less soluble and, indeed, some turbidity was observed at high concentration (5 – 10 mM) while making the measurements. This suggests the possible formation of oligomers involving both cationic and non-ionic species.

Otherwise, a pK_a shift depending on the surfactant concentration was observed in all cases (Figure 2 and Figure S1 in the Supporting Information). After the cmc, the pK_a value decreases, *cis*-**8** presenting a higher pK_a shift than that shown for *trans*-**8**. Thus, these compounds can behave as two different weak acids, as monomeric molecules before the cmc and as micelles after the cmc. A similar behavior was observed for diastereomers *cis*- and *trans*-**9**, although their lower solubility made difficult the study at high concentration.

The pK_a shift of molecules undergoing aggregation has been extensively studied [33-34], and two main contributions to this fact were identified. One contribution comes from the virtual charge effect due to a discontinuity in dielectric constant at the micelle surface [35] and a second contribution comes from the polar heads interaction where some protons are released to the solution in order to stabilize the aggregates.

Once molecules begin to self-assemble, the pH of the solution starts to decrease with a higher slope (Figure 2). From the intersection of the two lines, the cmc value is obtained: 1.0 mM and 0.60 mM for *cis*-**8** and *trans*-**8**, respectively. Smaller values were obtained for longer-chain surfactants **9** with cmc 0.32 mM and 0.16 mM for *cis*-**9** and *trans*-**9**, respectively. DAC, a cationic surfactant with similar chain length, presents a

cmc of 9 mM (pH 7) and 12 mM (pH 5) at 25 °C [36], which are higher values than those for **8** and **9**. Therefore, the presence of the cyclobutane unit and the amide bond seems to have a significant role on those values. This finding seems to contradict what was found in the case of anionic surfactants with polar heads also containing cyclobutane and amide bond but which cmc value did not change appreciably with respect to the a carboxylate of the same chain length (i.e. 12 mM for sodium laurate).

Moreover, the charge distribution in function of the concentration was determined for each surfactant by plotting $[NH_3^+]/[R\text{-}NH_2]=[H^+]/K_a$ versus concentration (see Figure S2 in the Supporting Information). These graphics provide information about the behavior of these surfactants as acids. All of them have a maximum of charge at the cmc point. Before the cmc, each surfactant behaves as a normal weak acid but, once the cmc is reached, the amount of cationic species divided by the amount of non-ionic species ($R\text{-}NH_3^+/R\text{-}NH_2$) starts to decrease in order to stabilize the aggregates and to reduce electrostatic repulsion between the molecules. It is interesting to note that, for *trans*-**9**, the charge ratio presents values between 3 and 5 showing that the concentration of the cationic surfactant is only 3-5 times higher than the concentration of the non-ionic surfactant. This ratio is much higher for the other surfactants studied.

The behavior of these amphiphiles could be interesting in medicinal chemistry, because changes in the protonation state would lead to changes the in headgroup area and, as a result, in their aggregation state. Surfactants with this type of properties were shown to be efficient vectors for gene therapy because the release of DNA into the cells is improved and, consequently, the level of gene transfection may be increased [37].

3.2.2. Acid-base titrations.

As a second methodology to determine the pK_a, acid-base titration of the surfactant aggregates with aqueous NaOH and subsequent retro-titration with HCl were carried out. The resultant curves for each surfactant are shown in Figure 3.

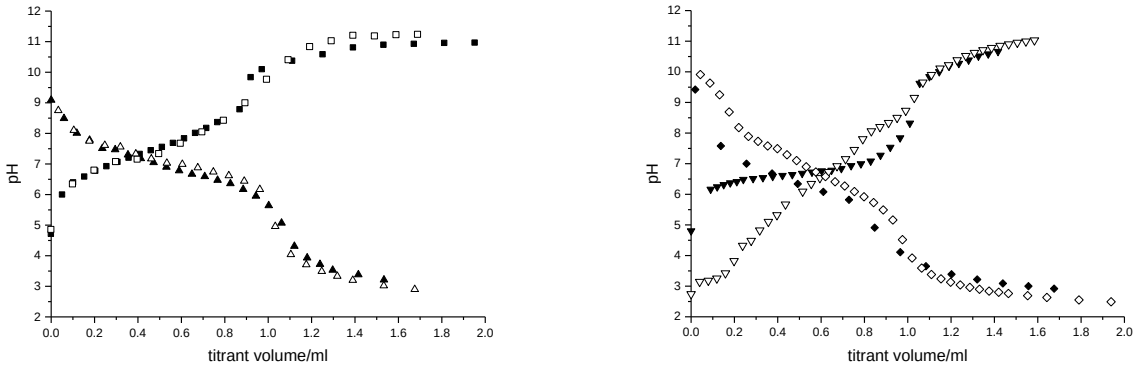


Figure 3. Titration curves of 5 mM aqueous solutions of *cis*-**8** (full squares), *trans*-**8** (open squares) and the corresponding back-titrations (full and open triangles respectively), *cis*-**9** (full down triangles) and *trans*-**9** (open down triangles) and the corresponding back-titrations (full and open diamonds respectively), at 25 °C.

The pK_a values can be obtained from the pH at the half-equivalence point, and refer to the aggregate, which contains a 1:1 ratio of cationic and non-ionic species. At the end of the first titration with NaOH, the obtained aggregate consists mostly of non-ionic surfactants, while at the end of the retro-titration with HCl, the aggregate will be mainly made by cationic surfactants.

We can observe that the pK_a of these aggregates depends mainly on the length of the chain. The pK_a of the half-charged aggregates for the C_{12}-alkyl chain surfactants **8** is 7.2±0.2 without significant influence of the stereochemistry while the pK_a for the C_{16} -surfactants is 6.6±0.1 for the *cis* stereoisomer and smaller (5.9 in the direct titration and 7.1 for the back-titration) for the *trans*- stereoisomer.

Looking at the profiles of the titration curves, we can conclude that for *cis*-**8,** *trans*-**8** and *cis*-**9** there is a clear equivalence point while for *trans*-**9** the equivalence point is less defined. Moreover, there is not a buffering effect with this molecule and the pH changes at almost constant slope (with some unexpected buffering effect at pH around 3-3.5). This different behavior could be due to the presence of strongly different aggregates than in the other cases. That means that the starting aggregate is different from the others, as was observed in the previous pK_a measurements. Furthermore, once the non-ionic aggregate is obtained and the retro-titration is carried out, the profile of all the surfactants is quite similar, showing perfectly the two equivalence points. As observed in Figure 3, once the non-ionic aggregate has been formed, surfactant *trans*-**9** behaves as the others remarking the strong influence of the starting aggregate on the pH measurements. This difference seems a bit contradictory in the present case, since the passage through an insoluble phase would allow to reaching a thermodynamic state.

3.3 Surface tension measurements.

As stated before, these pH-sensitive surfactants are not purely cationic, but they are a mixture of cationic and non-ionic species depending on the pH. Therefore, in order to study their surfactant behavior, different measurements of the surface tension (γ) were carried out at fixed pH values under buffered conditions or unbuffered (pH changing with surfactant concentration) conditions (see the Experimental Section). Plots of the surface tension versus surfactant concentration at three different pH values are shown in Figure 4. From these graphics, the parameters characterizing the surface properties of these surfactants, at 25 ºC and different pH values, could be determined (Table 1).

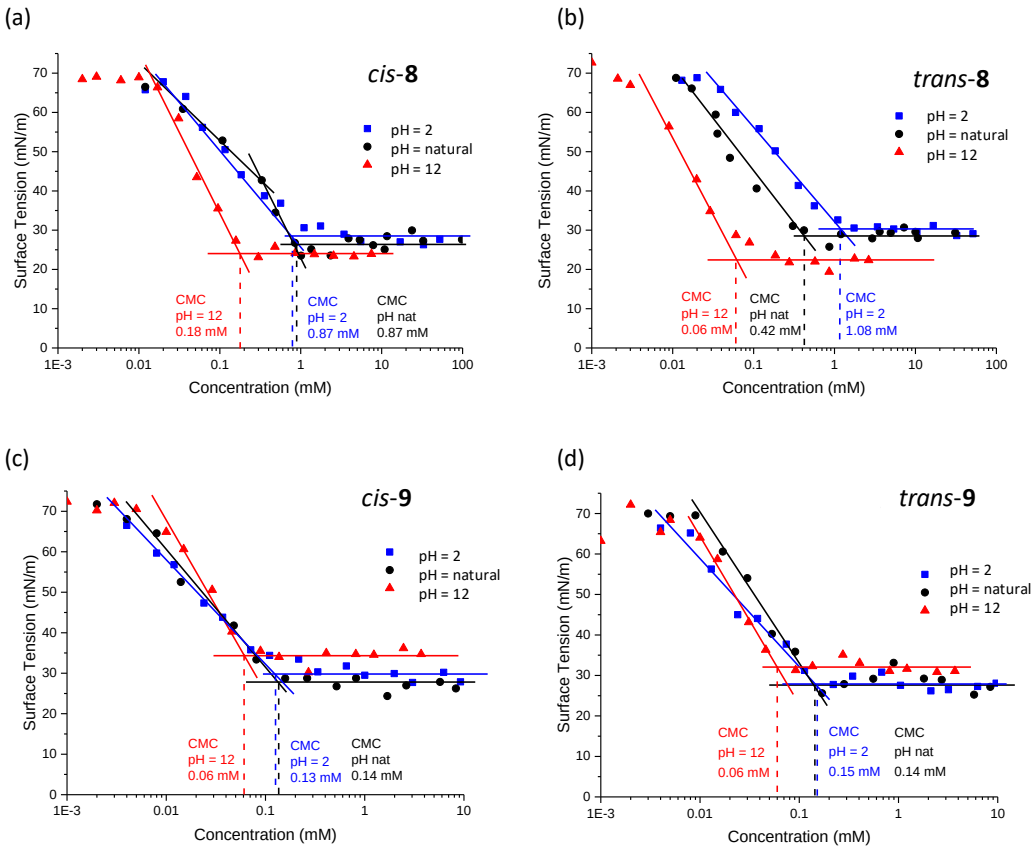


Figure 4. Plot of surface tension versus surface concentration for *cis*-**8** (a), *trans*-**8** (b), *cis*-**9** (c), and *trans*-**9** (d) at pH 2 (blue), natural (black) and 12 (red).

The four compounds behave as good surfactants because they reduce the surface tension from 70 to less than 40 mN/m, which has been taken as an indication of saturation of the water-air interface with hydrocarbon tails [38]. In Figure 4, the cmc values at each pH were determined, for each surfactant, as the intersection of the two fitting straight lines in the plots of γ versus concentration. From the slope of the curve just before the cmc, the Gibbs surface excess (Γ_{max}) at the air-surface corresponding to the cmc was obtained. From this value, the area per molecule (A_m) at the surface could be calculated at pH=2 and pH=12. (See the Experimental Section).

Table 1. **Surface Properties of Surfactants *cis*- and *trans*-8, and *cis*- and *trans*-9 in Water at 25 ºC.**

surfactant	pH	surface excess Γ_{max} (μmol/m^2)	area A_m (Å^2)	Effectiveness Π_{cmc} (mN/m)	cmc (mM)
	2	4.1±0.2	41±4	41.5±1	0.87±0.15
cis-**8**	natural	6.7-3.4	---	46.8±2	**0.87**±0.15
	12	7.3±0.3	23±2	48.0±1	0.18±0.03
	2	4.2±0.2	40±4	40.8±1	1.08±0.10
trans-**8**	natural	4.6-2.3	---	44.3±2	**0.42**±0.05
	12	7.6±0.3	22±2	49.9±1.5	0.08±0.04
	2	4.3±0.2	39±4	44.6±1	0.13±0.02
cis-**9**	natural	5.0-2.5	---	44.6±2	**0.14**±0.02
	12	7.5±0.3	22±2	37.4±1	0.06±0.01
	2	4.2±0.2	39±4	43.6±1	0.14±0.02
trans-**9**	natural	6.2-3.1	---	44.4±2	**0.15**±0.02
	12	7.8±0.3	21±2	40.2±1	0.06±0.01

At natural pH, for each surfactant, a mixture of species adsorbed at the surface is expected. Then, at this pH, A_m has not been determined, because of the lack of physical meaning. Looking at the obtained values at pH 2 and 12, we can observe that all the surfactants in the cationic state occupy around 40 Å^2 while, when non-ionic, the area is around 22 Å^2, which corresponds to the minimum area that a tail alkyl chain occupies at the surface [39]. This result shows that in all cases they are able to saturate the surface of the solution with the corresponding carbon chains. This implies a high density of methyl terminal groups, which agree with the very low surface tension. In the case of C_{16}-alkyl chain surfactant, the area per molecule of the neutral compounds is also low. However, it does not correspond to a so low surface tension and the reasons for this behavior are probably related to the previously observed anomaly for these C_{16}-compounds, that is, premicellar aggregation. The area per molecule of the cationic

surfactants is bigger than that of the non-ionic surfactants due to the repulsion between the charges. We can compare the values obtained here with the values obtained for trimethyl ammonium bromides and chlorides. For instance, the area per molecule determined for tetradecyl trimethyl ammonium bromide is 61 with a small reduction to 59 in the presence of NaBr at 0.1 M. Chloride salts produce smaller area per molecule as deduced for dodecyl trimethyl ammonium chloride in 0.1M NaCl (38 $Å^2$) or the longer chain hexadecyl trimethyl ammonium chloride in 0.1 M NaCl (46 $Å^2$) [38]. Surprisingly, the present surfactants show an area per molecule at full protonation comparable to the dodecyl trimethyl ammonium chloride with small or nil increment due to the bulky cyclobutane scaffold.

Special attention has to be paid to the obtained cmc values because they will mark the working concentrations for a possible further study of the interaction with DNA. At pH 2, both *cis* and *trans* diastereomers have a very similar cmc value: around 1 mM for *cis*- and *trans*-**8** and around 0.15 mM for *cis*- and *trans*-**9**. The behavior of these cationic surfactants can be compared with commercial dodecyltrimethylammonium bromide (DTAB), with cmc 16 mM at 25 °C [40], and CTAB, with cmc 0.98 mM, at 25 °C [41]. For surfactants **8** and **9**, the difference in the cmc value while increasing the alkyl chain length from 12 two 16 carbon atoms is smaller than in the case of DTAB and CTAB. This is probably due to the presence of the cyclobutane moiety and points out that surfactants **8** and **9** are more efficient than the commercial ones. Therefore, at pH 2, having only cationic surfactants, stereochemistry seems not to play any significant role. Nevertheless, at pH 12, the cmc values of the compounds give the impression of being more dependent on the stereochemistry and on the chain length. For the non-ionic surfactants, the cmc is 0.18 mM for *cis*-**8**, while cmc is 0.06 mM for *trans*-**8**. Then, the *trans* isomer seems to be more efficient than the *cis* one. However,

for both *cis*- and *trans*-**9** the cmc is around 0.06 mM. So, in this case, the length of the chain has more influence on the solubility and, consequently, on the cmc value than the stereochemistry. Therefore, both diastereomers behave similarly as non-ionic surfactants at this pH. Finally, at natural pH, where there is a mixture of cationic and non-ionic surfactant, the cmc values appear to have the same behavior as at pH 2, as observed in Table 1, and can be compared with the obtained ones when measured in the bulk of the solution. It is noteworthy the different behavior of *trans*-**9** with respect to pH and surface tension. While in surfactants **8** former there are strong differences between both stereoisomers, for surfactants **9** there are not very significant differences. Anomalous trends in the dependence of cmc with chain length have been previously observed, particularly in the case of gemini surfactants [42]. In those cases, the decrease of cmc with chain length is smaller than for classical surfactants and, even increases of cmc have been observed for C_{16}-alkyl chain surfactants compared with C_{12}-ones. This anomalous behavior has been attributed to the formation of premicellar aggregates. Although the present structures are by no means gemini, it is not unconceivable that a dimer could behave in similar way as to a gemini surfactants.

3.4 Cryo-TEM Studies.

The morphology of the aggregates formed by these four surfactants above the cmc, at three different pH values (2, natural and 12), was studied by using cryo-TEM at –180 °C. Selected cryo-TEM images are shown in Figure 5. (For a more complete set of cryo-TEM micrographs, see the Supporting Information, figures S3-S6). The images obtained showed irregular aggregates along with some vesicles and/or fibers with different size. Nevertheless, some trends were perceived allowing us to conclude that, independently of the pH, stereochemistry has some influence on the morphology of the aggregates and that *cis* surfactants seem to self-assemble preferably forming vesicles,

sometimes as multilayer aggregates, while *trans* surfactants, in addition to vesicles, form fibers at basic pH.

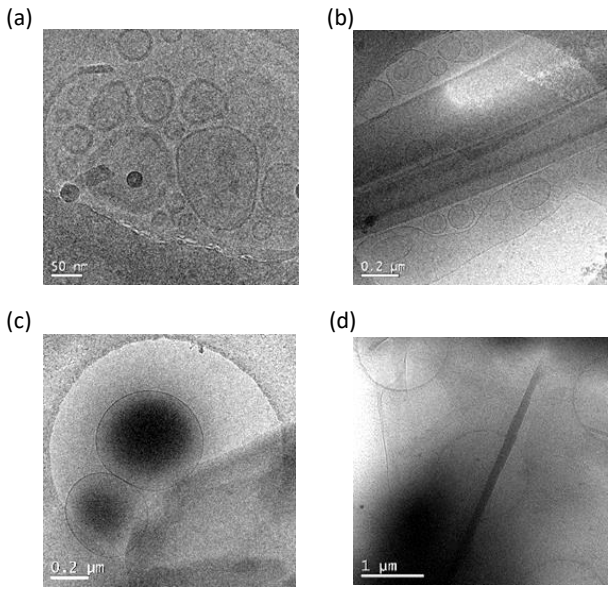


Figure 5. Selected cryo-TEM micrographs of a) *cis*-**8** at natural pH, b) *trans*-**8** at pH 12, c) *cis*-**9** at pH 2, d) *trans*-**9** at pH 12.

3.5 Computational calculations.

In order to gain insight in the structural features of monomeric surfactants and their ability to form intramolecular hydrogen bonds, computational calculations were carried out. The predicted structures are shown for *cis*- and *trans*-**8** in Figure 6. The same geometry of the polar head was found for *cis*- and *trans*-**9**, respectively. Distances of hydrogen bonds between the carbonyl oxygen of the amide and hydrogen from the free amine or the ammonium cation are listed in Table 3. Cationic *cis*-isomers form stronger intramolecular hydrogen bonds than non-ionic surfactants *cis*-**8** and *cis*-**9**. As expected, the *trans* diastereomers present very weak intramolecular hydrogen-bonding both in the cationic and non-ionic species The length of the alkyl chain also plays a role in agreement with the lower solubility in water of surfactants **9** with respect to that of **8**.

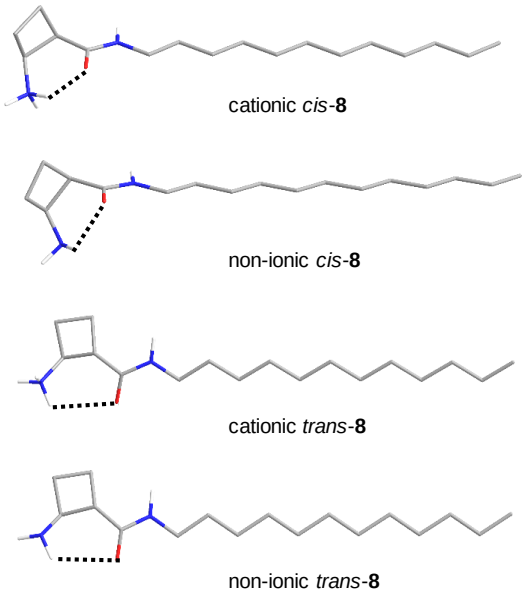


Figure 6. Calculated structures of cationic and non-ionic species for surfactants *cis*- and *trans*-**8**. Non-polar hydrogen atoms are omitted for clarity.

Table 3. **Hydrogen-bond Distances in Monomeric Surfactants *cis*- and *trans*-8, and *cis*-and *trans*-9**.

Surfactant	C=O···H-N (cationic) (Å)	C=O···H-N (non-ionic) (Å)
cis-**8**	1.979	2.469
trans-**8**	2.853	2.863
cis-**9**	2.042	2.639
trans-**9**	2.858	2.869

3.6. Circular Dichroism (CD) studies.

Aggregation of chiral surfactants into chiral aggregates can be followed using CD spectroscopy [20,43-44]. Therefore, the analysis of the corresponding spectra between 200 and 250 nm would afford information about the chirality of the system [45] and one

could expect different CD spectra before and after the cmc. This assumption lies on the fact that Cotton effects are extremely sensitive to changes in the chiral environment close to the chromophore and to the interchromophoric interactions in supramolecular aggregates [46]. The resultant CD spectra are shown in Figure 7. The shape and sign of the CD signal was the same than that predicted by the theoretical CD spectrum of each monomer of *cis*- and *trans*-**8** in solution (See the figure S7 in Supporting Information).

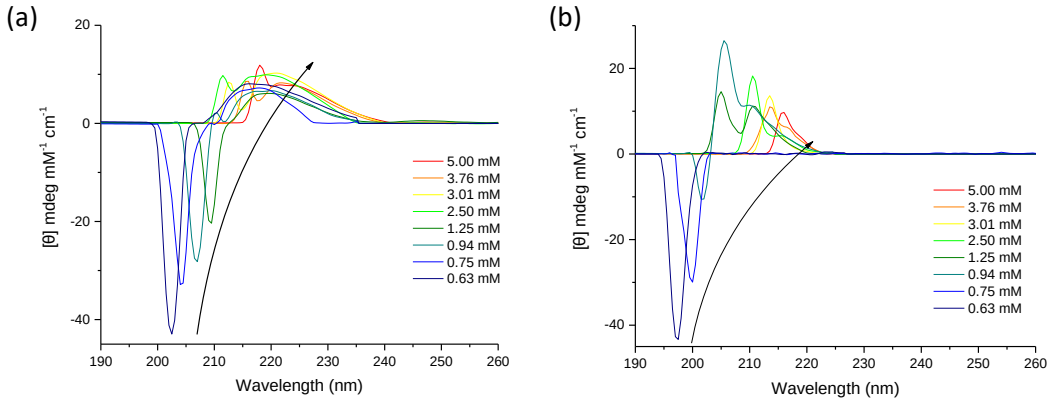


Figure 7. Experimental CD spectra at different concentrations of surfactants *cis*-**8** (a) and *trans*-**8** (b).

When increasing the concentration of surfactant, the signal of the monomer is shifted to longer wavelengths. The red shift could be attributed to the formation of aggregates through the creation of hydrogen bonds involving the chromophore group, i.e. the amide, that provokes a change in the transition energy [18,46].

Measurements of the UV-vis absorption of each surfactant in aqueous solution were carried out (see figure S8 in the Supporting Information) and, in fact, a slight red shift can be observed from low to higher concentrations, in agreement with CD results. However, λ_{max} in UV-vis is not the same as λ_{max} in CD because there is a change from single molecules to aggregates at the cmc, as can been appreciated in the representation of CD λ_{max} values in function of the surfactant concentration (Figure 8).

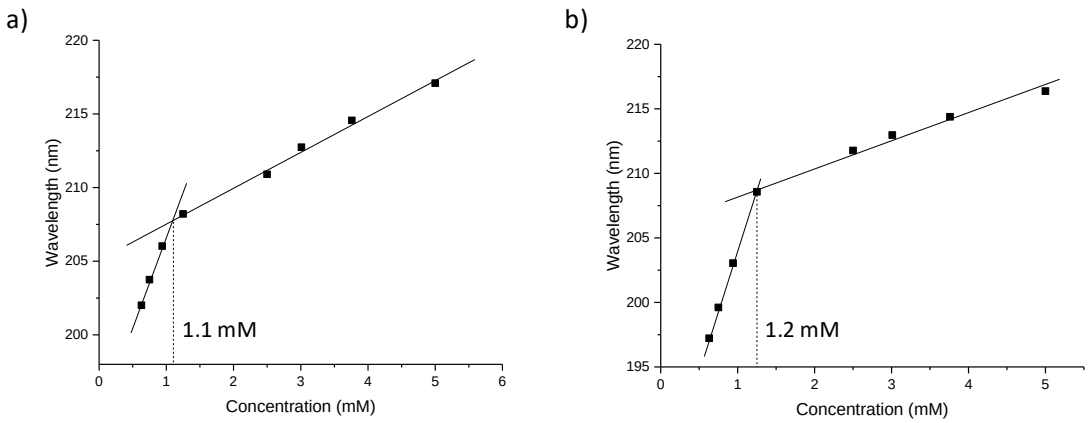


Figure 8. Plots of CD λ_{max} versus concentration of surfactant *cis*-**8** (a) and *trans*-**8** (b).

Both graphics show a sharp change in the slope at values closely related to those determined for the cmc in solution (see previous sections in this manuscript). Therefore, in these cases, CD spectroscopy can be used for the determination of the cmc because it is known that this parameter contains a range of concentrations and its true value can vary up to one order of magnitude depending on the method used and on the measured physical property [17,20]. The determination of the cmc of a surfactant by the change in UV-vis absorption related to the formation of aggregates had been already reported [47-49]. However, these techniques are based only on the intensity of the absorption and not on the λ_{max} value and a clear peak in the spectra should be observed in order to determine the maximum. Even so, this technique is restricted to surfactants that self-assemble through a chromophore located in a chiral environment.

Surfactants *cis*- and *trans*-**9** were also investigated (see figure S9 in the Supporting Information). Nevertheless, due to their low solubility and, consequently, their low cmc value (around 0.1-0.2 mM) the method became limited to the study of a narrow range of concentrations and reliable conclusions could not be obtained.

3.7. Cytotoxicity of the surfactants.

Encouraged by the interesting properties found for surfactants **8** and **9** and focusing on the possibility to use them as non viral vectors for gene therapy, a preliminary investigation of their cytotoxicity was carried out by means of the MTT assay. Standard HeLa cells were used for this study. The 100% living cells was considered as the result of a control experiment performed in absence of the surfactants and under the same conditions as explained in the Experimental Section. The average toxicity results of four replicates for each surfactant are shown in Figure 9. We can observe that surfactants **8** and **9** are non-toxic in contrast to DTAB and CTAB, which are commonly used in biological experiments.

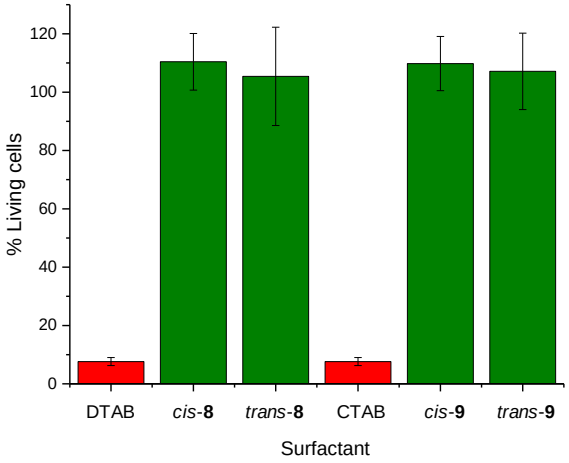


Figure 9. Toxicity results of the MTT assay with surfactants DTAB, *cis*- and *trans*-**8**, CTAB, *cis*- and *trans*-**9** using HeLa cells. Error bars represent standard deviations (SD) of four independent experiments.

4. Conclusions

Two pairs of *cis/trans* diastereomeric β-amino acid-based cationic amphiphiles have been synthesized and studied. They contain C_{12}- and C_{16}-alkyl chains, respectively, as hydrophobic tails and the polar head consists of an ammonium cation linked to a

cyclobutane ring. The pH of the medium modulates their physicochemical properties, such as the cmc, the ratio of cationic versus non-ionic species and the surface tension magnitude. At the same time the aggregation state influences on the apparent pK_a values of the aggregates, with release of protons at the cmc. The cmc for all of them is lower than that for commercial DTAB and CTAB, which is probably due to the steric restrictions imposed by the cyclobutane unit. Moreover, the adsorption effectiveness is around 40 mN/m in all cases, which is in accordance with an efficient surfactant behavior. The tail-length influences the surfactant properties and those with a C_{16}-alkyl chain are better than those with a shorter tail. In terms of surface tension there is some effect of stereochemistry regarding the position of cmc for the C_{12}- derivatives, while there is no effect for C_{16}- or on the area per molecule. Aggregation influences on the apparent pK_a of these molecules with *trans*-**9** behaving as a much more acidic molecule than expected, but this particular behavior does not translate to other properties. The stereochemistry also determines the predominant mode of self-assembling since the trend for *cis*-isomers is to form micelles or vesicles while *trans*-isomers preferably form fibers. CD spectroscopy supports the formation of aggregates through intermolecular hydrogen-bonding between the amide groups of the monomers. Moreover, by considering the relationship of the CD λ_{max} with the concentration, an alternative method to determine the cmc of these surfactants is provided. This methodology could be general but is restricted to those surfactants in which the chromophore is located in a chiral environment. Finally, the non- toxicity of the surfactants has been verified by the MTT assay. This characteristic, jointly with the efficiency and the good properties shown, the fact that the cationic species are present in a high concentration at physiological pH, as well as the weak acid behavior of the aggregates, confirm these

amphiphiles as promising candidates to be used as non-viral vectors for gene therapy applications. Further investigation in this field is being carried out in our laboratories.

ASSOCIATED CONTENT

Supporting Information

Synthetic procedures, characterization, and ^{1}H and ^{13}C NMR spectra of the new compounds; charge distribution at different concentration of the surfactants; cryo-TEM micrographs of the surfactants at different pH values; calculated CD spectra for *cis*- and *trans*-**8**; CD and UV-vis absorption spectra of *cis*- and *trans*-**9**.

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Notes

The authors declare no competing financial interest.

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