Synthesis, Physico-Chemical Characterization and Interaction with DNA of Long Alkyl Chain Gemini Pyridinium Surfactants

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Nadia Barbero[a], Claudio Magistris[a], Pierluigi Quagliotto*[a], Luca Bonandini[a], Claudia Barolo[a], Roberto Buscaino[a], Carlotta Compari[b], Laura Contardi[b], Emilia Fisicaro[b], Guido Viscardi[a].

Dedicated to Prof. Ermanno Barni on the occasion of his 80th birthday

Abstract: Pyridinium *gemini* surfactants showing hexadecyl chains linked to nitrogen atoms and tuned aliphatic spacer bridging the two pyridinium polar heads in 2,2' positions have been synthesized and characterized. A multitechnique approach allowed to study the aggregation behaviour: conductivity, surface tension and fluorescence. Specific conductivity (κ) vs surfactant molar concentration (C) and molar conductivity (Λ) vs $C^{0.5}$ graphs suggest preaggregation phenomena of these amphiphiles at very low concentration. The trends of A_{min} as function of the spacer length confirm the hypothesis of a conformational change of the molecule with four methylenes as spacer, due to stacking interactions between the two pyridinium rings, mediated by the counterion. Moreover, the trends of A_{min} and β suggest that the spacer must be longer than eight carbon atoms to fold efficiently toward the micellar core. The opportunity to tune the surfactant structure and aggregation properties make those surfactants, in particular the long chain ones for which the DNA complexing ability was shown by AFM imaging, desirable candidates for gene delivery experiments.

Introduction

Gemini surfactants differ from the more conventional monomeric ones since they consist of two hydrophilic headgroups, each of them bearing one hydrophobic chain, connected by a spacer. Their properties are peculiar in comparison to monomeric surfactants owing to their increased surface activity, useful viscoelastic properties, lower C_{20} (molar surfactant concentration required to reduce the surface tension of the solvent by 20 mN/m)[1] and lower critical micelle concentration (CMC) of aggregates of different shapes.[2-10] Due to these properties, the interest in *gemini* surfactants, in particular cationic ones, increased recently in pharmaceutical field, both as non-covalent components in carbon nanotubes-based formulations[11] and as nonviral vectors in gene therapy.[12-20]

Gene therapy (GT) is finding more and more clinical applications for monogenic disorders, cancer, and infectious diseases.[21-23] The first step of GT is to compact and encapsulate the therapeutic desoxyribonucleic acid (DNA) into soft nanoparticles and to deliver it into the nucleus by means of a specific vector.[19] Retroviral vectors (RVs) have been widely used to deliver therapeutic genes with clear therapeutic benefit[24-31] but severe adverse events linked to insertional mutagenesis due to aberrant vector-on-host interactions occurred, e.g. in clinical trials for primary immunodeficiencies.[27, 32-35]

Non-viral gene vectors appear as a promising alternative. Cationic amphiphiles, also known as cationic lipids, showed DNA complexation properties useful for cell transfection.[13, 36-43] A recent review evidenced the great potential of these non-viral vectors, in particular the gemini surfactants, due to tunability of their molecular moieties: hydrophobic chains and hydrophilic headgroup, spacer, counterion.[9] However, efficient non-viral delivery systems must still be realized.

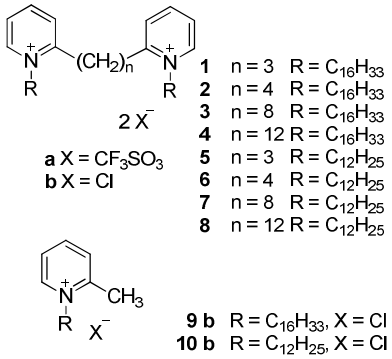
For some time, we have been collecting physicochemical and biological data concerning *gemini* surfactants with the idea of correlating in a quantitative way their structure with their biological activity, particularly transfection ability, and so to drive the synthesis of more and more efficient vectors.[17, 44-47] Encouraging results in gene delivery have been obtained by some of us by using ammonium *gemini* surfactants, as derivatives of N,N-bisdimethyl-1,2-ethanediamine with general formula $C_nH_{2n+1}OOCCH_2(CH_3)_2N^+CH_2CH_2N^+(CH_2)_2CH_2COOC_nH_{2n+1}/2Cl^-$ (bis-C_nBEC) and where the subscript *n* stands for the number of carbon atoms of the alkyl chain bound to the carboxyl group, when formulated with DOPE [L-α-phosphatidylethanolamine dioleoyl (C18:1,[cis]-9)].[17] Then, we synthesized cationic *gemini* surfactants containing two pyridinium groups showing dodecyl[10] and fluoroalkyl chains.[48] The pyridinium group should account for better interaction with DNA phosphate group due to higher charge dispersion with respect to ammonium headgroups; chloride counterion is normally used since it is non toxic.[9]

In this paper we describe the synthesis of pyridinium counterparts bearing hexadecyl chains and the study of their aggregative properties by conductivity, surface tension and fluorescence measurements. In general, the use of long alkyl chains improves the DNA complexation and transfection ability. Purpose of this paper is also to define and optimize structure/biological activity relationships (i.e. the determination of the chain length to be used).

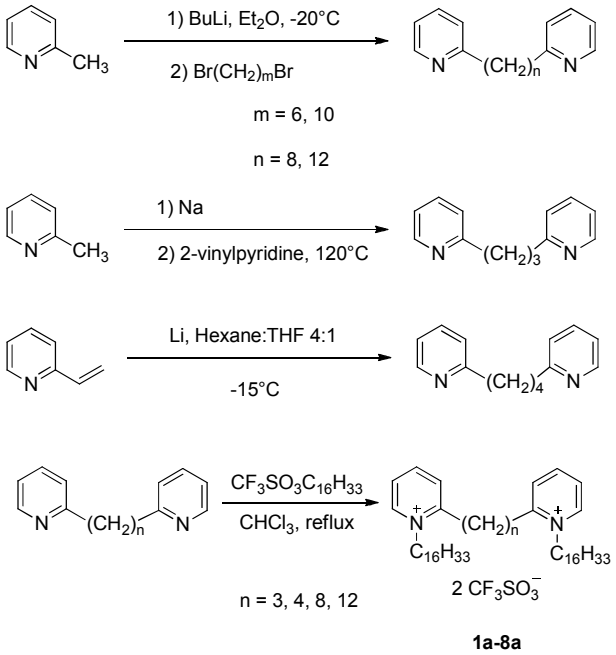
Pyridinium *gemini* surfactants, structurally different from the compounds here presented (showing the spacer bridging the heteroaromatic nitrogens), have been recently proposed in literature for transfection purposes.[20]

Results and Discussion

The structure of novel **1**-**4** surfactants (named 16Py-n-Py16-X, where X=CF_3SO_3, Cl) is described in Scheme 1, along with the previously reported surfactants **5**-**8** (named 12Py-n-Py12-X where X=CF_3SO_3, Cl)[10] and the structurally related monomers **9b**-**10b**.[49-50] Their synthesis (Scheme 2) was performed by the use of hexadecyltrifluoromethanesulfonate as quaternizing agents of suitable α,ω-bis(2-pyridyl)alkane, prepared as previously described.[10] Alkylhalides resulted not enough reactive, as already noted previously.[10, 48] Alkylmethanesulfonates resulted reactive in order to prepare **5**-**6** surfactants[10] while they were completely unsuccessful to obtain **1**-**4** and **7**,**8** ones.



Scheme 1. Surfactants prepared in the present work.



Scheme 2. Synthesis and quaternization reactions of α,ω-bis(2-pyridyl)alkanes.

The quaternization was performed in chloroform at 70°C by slow dropping of base into the hexadecyltrifluoromethanesulfonate solution. The product was obtained as a solid by simple cooling of the reaction mixture. After filtration, the solid was crystallized from acetonitrile/ethyl acetate mixture. The yields were generally very

high, sometimes nearly quantitative. This kind of alkylating agents allowed to quaternarize, for the first time, similarly crowded structures with long alkyl chains. The ionic exchange technique gave the desired chlorides (**1b**-**10b**) (See Supporting Information).[10]

Amphiphilic characterization.

A surfactant, in order to act as efficient non-viral vector of DNA, in primis should compact and encapsulate the DNA into soft nanoparticles, large and stable enough for preventing rapid leakage into blood capillary but small enough for escaping macrophages of the reticuloendothelial system.
Since the properties of DNA nanoparticles are heavily affected by the structure of the cationic surfactant and by its aggregation ability, it is fundamental to characterize the potential non-viral vectors from the amphiphilic point of view in order to find structure–activity relationships.[9] First of all, the critical micelle concentration (CMC), defined as the concentration at which a surfactant starts to form aggregates as micelles, has to be determined.
The CMCs of present gemini surfactants were determined by using specific conductivity (κ, measuring bulk properties of the system), surface tension (γ, related with surface properties of the system) and fluorescence measurements (determined by the probe-micelle interaction). In fact, the evaluation of the CMC in the case of Gemini surfactants is not so straightforward as for monomeric surfactants and discussion about this point is still open in literature.[9-10, 44-45, 47-48, 51-65]

Trifluoromethanesulfonate surfactants were not studied from the amphiphilic point of view owing to their high Krafft point: 80-85°C for the dodecyl gemini surfactants **5b-8b**, while for the hexadecyl ones the Krafft point resulted impossible to be measured because higher than 100°C.

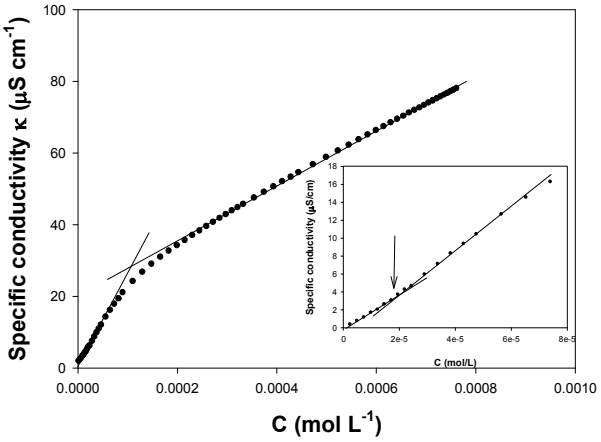


Figure 1. Specific conductivity (κ) vs C plot of surfactant 16Py-3-Py16 2 Cl (**1b**).

Table 1. Table 1. Amphiphilic properties of surfactants **1b**-**10b** at 25°C.

Surfactant	Chain	n[a]	CMCc [b]	β (%)[c]	CMCs [d]	CMCc/CMCs	CMC [e]	CMC [f]	I_1/I_3 [g]
1b	$C_{16}H_{33}$	3	8.51	61	1.2	7.1	8.87	195	1.11
2b	$C_{16}H_{33}$	4	9.42	53	2.1	4.5	10.2	143	1.08
3b	$C_{16}H_{33}$	8	8.38	49	0.15	55.9	8.34	40.4	1.05
4b	$C_{16}H_{33}$	12	5.95	56	0.078	76.3	5.91	54.6	1.07
5b	$C_{12}H_{25}$	3	145	68	111	1.3	132	166	1.19
6b	$C_{12}H_{25}$	4	107	69	28	3.8	124	137	1.11
7b	$C_{12}H_{25}$	8	106	44	12	8.8	103	97.2	0.96

8b	$C_{12}H_{25}$	12	21	70	2.3	9.1	26.2	35.7	1.44
9b	$C_{16}H_{33}$	-	102	55	----	----	101	139	1.34
10b	$C_{12}H_{25}$	-	1270	61	----	----	1570	991	0.94

[a] number of methylene groups in the spacer. [b] CMC x10^5 (molL^{-1}), obtained by specific conductivity (κ) vs C data following the non linear fit method. [c] Degree of counterion binding. [d] CMC x10^5 (molL^{-1}), obtained by surface tension measurements. [e] CMC x10^5 (molL^{-1}), obtained by Coumarin 6. [f] CMC x10^5 (molL^{-1}), obtained by Pyrene. [g] I_1/I_3 ratio of the intensity of the first (373 nm) and third (384 nm) vibronic bands of pyrene fluorescence at CMC.

Figure 1 (see also Fig. SI1 - SI11 of Supporting Information) shows for surfactant **1b**, as example, the dependence of the specific conductivity (κ) on surfactant molar concentration (C).[66] An evident inflection of plot allows to extrapolate the CMC value.

Two different methods were applied to extract the CMC values working on specific conductivity (κ) vs C data. The first more classical approach allows the CMC to be determined by the intersection of the two linear regimes at low and high concentrations.[66] A few years ago, a nonlinear fit method was proposed by Ruiz et al.,[51] that became a standard method to deal with smooth specific conductivity vs C data plots. Recently, we also applied it successfully to some monomeric and gemini surfactants.[10, 52-53] Both methods gave consistent results, but the nonlinear fit method was straightforward in obtaining reliable CMC values, since sometimes the transition between the two linear regimes was gradual and the two lines were difficult to be extrapolated. For details about Ruiz et al.[51] extrapolation method see Supporting Information.

The CMC values, determined by both methods for the gemini surfactants and their related monomers, are reported in Table 1. The values of CMC obtained by surface tension measurements, shown also in Figure 2, are also reported in Table 1. They are taken as the concentrations at the point of intersection of the two linear portions of the γ vs logC plots (dotted straight lines in Figure 2).

Since the CMC values extrapolated from specific conductivity (κ) and surface tension appear significantly different we evaluated this aggregative parameter also by the fluorescent probes coumarin 6 and pyrene.[52-53, 67-70] Extrapolated CMC values are reported in Table 1.

Coumarin 6 is a highly hydrophobic and insoluble in water fluorescence probe, normally allocated in the interior part of the micelle palisade, relatively away from the polar heads of the pyridinium cation.

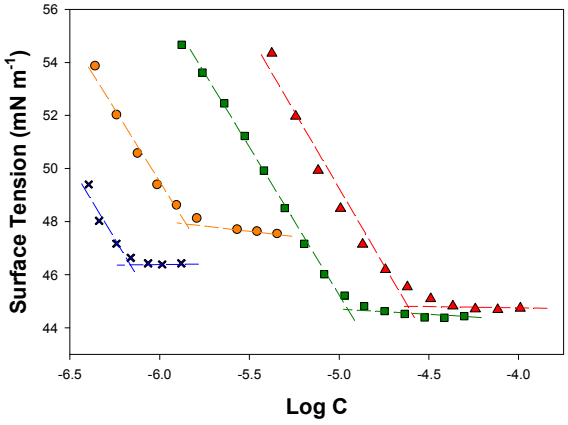


Figure 2. Surface tension as function of the logarithm of surfactant concentration for the gemini surfactants 1b (■), 2b (▲), 3b (●) and 4b (×) at 25°C.

Sigmoidal plots are obtained by plotting the Emission Intensity (I_{em}) vs C and the CMC values, extrapolated by using Boltzmann sigmoidal fitting developed by Ruiz et al.[70] are reported in Table 1. The I_{em} vs C plot for the surfactant **1b** (16Py-3-Py16-Cl), taken as an example, is reported in Figure 3a. CMC values obtained by using coumarin 6 as probe are in agreement with those obtained by conductivity supporting the use of conductivity as technique to study micelle formation phenomena.

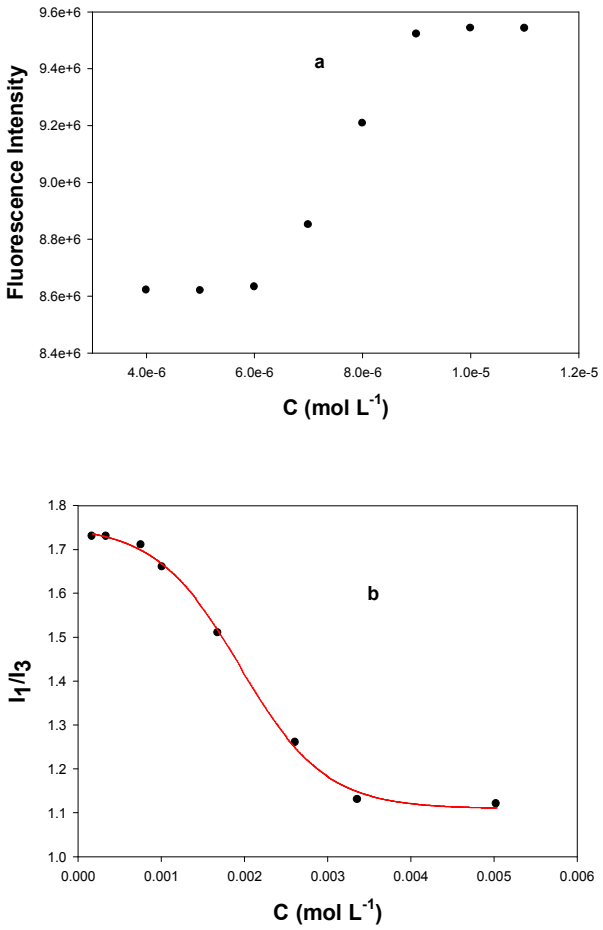


Figure 3. Surfactant **1b** (16Py-3-Py16 Cl): a) I_{em} vs C plot by using coumarin 6 as probe; b) I_1/I_3 vs C plot by using pyrene as probe.

The use of pyrene needs to consider fluorescence quenching properties of pyridinium cations owing to pyrene localization in the micelle near the cationic headgroups, following the interaction of the pyrene aromatic electrons with the positive charge of pyridinium.[55, 71-72] Kalyansundaram et al.[73] demonstrated that, while being a diffusional quenching mechanism operative among pyridinium cation and pyrene, the quenching does not influence the I_1/I_3 vibronic band ratio. By applying Kalyansundaram et al. conditions, here described in Experimental Section, the measurements were performed without particular problems.

The pyrene intensities of emissions at 373 nm (I_1) and 384 nm (I_3) (first and third vibronic peaks respectively) are influenced by the polarity of its solubilization environment. Below the CMC, the pyrene I_1/I_3 ratio value corresponds to the water polar environment; as the surfactant concentration increases, the pyrene I_1/I_3 ratio decreases rapidly, indicating that the pyrene is sensing a more hydrophobic environment, reaching a roughly constant value due to probe incorporation into the micelles. A typical sigmoidal curve is obtained by plotting the I_1/I_3 ratio as a function of the total surfactant concentration; the CMC was extrapolated by the Ruiz et al. method reported in reference 70. In Figure 3b the curve obtained for surfactant **1b** (16Py-3-Py16-Cl) is reported as an example.

The exam of CMC values obtained by specific conductivity (κ), surface tension and Coumarin 6 fluorescence, and reported in Table 1, confirms the higher efficiency of pyridinium gemini surfactants to form micelles in comparison to corresponding monomeric ones and suggests that, as usually found in the literature,[5, 56] longer chains (16Py-s-Py16 series) stabilize the micellar aggregates and reduce CMC. From the literature it seems that this stabilizing effect would be obtained for chain having length between 16 and 18. A similar effect was observed for ammonium gemini surfactants,[56] but the effect of hexadecyl versus dodecyl chain is about halved for pyridinium gemini surfactants series than ammonium gemini ones,[56] probably because the pyridinium ring reduces the conformational freedom. Its planar shape and large dimension can hinder the micellar surface, increasing the CMC.[10] This lower efficiency of pyridinium gemini surfactants than ammonium ones to form micelles is supported also by the $CMCs/C_{20}$ parameter reported in

Supporting Information, whose values appear higher for pyridinium gemini surfactants in comparison to values of ammonium gemini ones.[56, 59]

Noteworthy is the effect of spacer length on CMC: the spacer seems to act as a third hydrophobic chain affecting the aggregation and the packing parameter.[45-46] The lowering of the CMC in presence of hexadecyl chains seems less evident rather than in presence of dodecyl chains. This hydrophobic effect of spacer has been already observed for ammonium gemini surfactants and is confirmed by the pC_{20} values (see Supporting Information) that in general becomes higher when in a series both the alkyl chains linked to nitrogen atoms and the spacer length increase.[10] This suggests that the spacer elongation can heavily affect also the properties of surfactants-DNA lipoplexes.

The values of the CMC extrapolated from surface tension are in general lower with respect to those extrapolated from specific conductivity (κ), in particular for surfactants with hexadecyl chains and long spacers (8-12 methylenes); i.e. for more hydrophobic surfactants. This phenomenon has been already reported in literature.[48, 57-58, 74-75] Rosen et al. tentatively introduced the CMCc/CMCs ratio to describe the extent of the difference of the CMC determined by different methods.[74] Analogously, we reported this parameter in Table 1.

In agreement with literature,[48, 54, 57-61, 74-75] we ascribed the difference between CMC data obtained by conductivity and surface tension to formation of premicellar aggregates, not surface active, such as dimers, trimers an so on. When the surfactant concentration is increased, the premicellar aggregates turn progressively into conventional micelles. To confirm our hypothesis, the exam of conductivity measurements in diluted solutions was meaningful. For a monomeric surfactant in dilute solution, an increasing and a decreasing linear correlation is obtained in specific conductivity (κ) vs C and molar conductivity (Λ) vs $C^{0.5}$ plots in very diluted solutions (Figure 4). Otherwise, anomalous higher values of specific conductivity (κ) than that expected, (inset of Figure 1 and Figures SI1-SI11 of Supporting Information) or maxima in molar conductivity (Λ) (Figure 5 and Figures SI12-SI22 of Supporting Information)) plots are due to preaggregation phenomena.[57, 62]

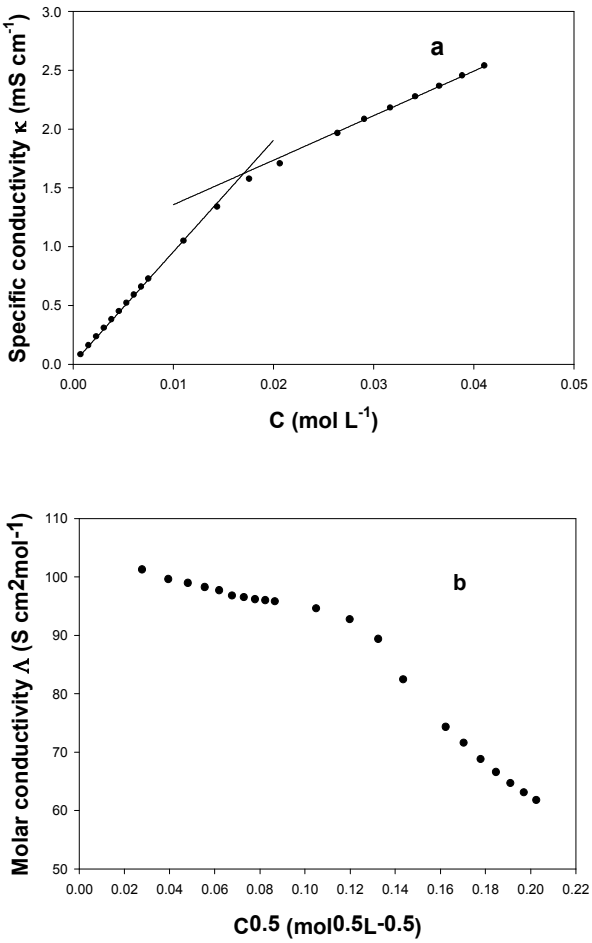


Figure 4. Specific conductivity (κ) vs C (a) and molar conductivity vs $C^{0.5}$ (b) plots for the N-dodecylpyridinium chloride.

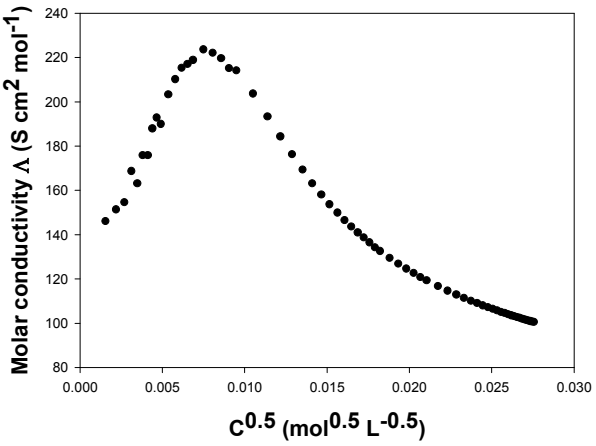


Figure 5. Molar conductivity (Λ) vs $C^{0.5}$ plot for surfactant **1b** (16Py-3-Py16 2 Cl).

Counterion Binding and Surface Minimum Area.

The DNA complexation and DNA release in the cell is mediated also by optimal interaction of cationic vector with negative charges of DNA phosphate group.[12] The study of the degree of counterion binding (β), i.e how many counterions are tightly bound to the micelle in the Stern layer, is meaningful to evaluate the potentiality of a surfactant to form lipoplexes. A high β value implies the presence of a high charge density on the micellar surface, that requires counterions to be neutralized. This parameter can also allow to evaluate how much the polar headgroups are close to each other, i.e. the micelle surface compactness.[10, 48, 52-54]
Besides, the presence of a delocalized charge, in pyridinium surfactants, influences positively the aggregation properties and the DNA transfection activity. The DNA complexation and its release in the cell is mediated also by optimal interaction of cationic vector with negative charges of DNA phosphate group.[12] A headgroup having delocalized positive charge is a softer ion than the ammonium one and thus should better interact with a reasonably soft anion, like the phosphate that we can find into the DNA.[9]
The degree of counterion binding (β) is calculated by the expression $\beta=1-\alpha$ where α is the degree of counterion dissociation obtained by the ratio of the two slopes directly determined from the definition of the two linear regimes after and before cmc of specific conductivity (κ) vs C plot.

$$\alpha = (dK/dC)_{C>CMC}/(dK/dC)_{C<CMC} \qquad (1)$$

In Figure 6, β values, obtained by the slopes evaluated following the nonlinear fit, are reported; β values obtained by the slopes evaluated following also the classical method are reported in Supporting Information.[51]
The β values vary along with spacer length showing a minimum for n=4, 8 in the case of hexadecyl surfactants and n=8 for dodecyl ones. For short spacers, i.e. three methylenes long, the two charged headgroups are quite close and thus the surface charge density in the micelles is high, and counterions are recruited from the solution to neutralize the positive charges and stabilize the aggregate showing a relatively high β value. When the spacer has four methylenes, we already suggested[44-45] that the molecule can double up surrounding the counterion and taking it firmly in between the pyridinium rings. The difference in β values between dodecyl and hexadecyl surfactants could be due to the larger hydrophobic interaction of hexadecyl chains.
The surfactants having a spacer made of eight methylenes has sufficient conformational freedom and they occupy a larger space to the micellar surface, and the charge density is decreased and β is lower.
When the spacer is long enough to fold towards the micellar core, and thus the two pyridinium rings can stay a bit closer and the surface charge density increases along with the β value (surfactants **4b**, **8b**).

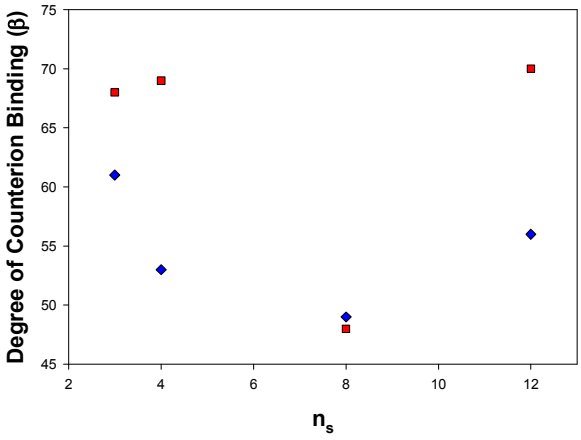


Figure 6. Degree of counterion binding (β) as a function of the spacer length (n) for C12 (■) and C16 (◆) pyridinium gemini surfactants.

The degree of counterion binding is related to compactness of the micellar surface.[1, 52-53] The values of β suggests that the micellar surface of 16Py-s-Py16 series has a lower compactness than 12Py-s-Py12 one. The use of pyrene is informative in this sense. The CMC determined with this fluorescent probe for hexadecyl surfactants (**1b**-**4b**) was not in agreement with the values determined by conductivity (from 5 to 22 times higher). In fact, their less compact micellar surface allows pyrene to interact with cationc headgroups remaining still hydrated. As a consequence the I_1/I_3 ratio does not change significatively until the micelles show an adequately high compactness and this occurs at higher surfactant concentration. Noteworthy is to compare I_1/I_3 ratio of pyridinium and ammonium gemini surfactants. Ammonium gemini surfactants show higher values ranging between 1.21-1.35.[53-54, 67] This means that the presence of a flat pyridinium ring as the headgroup instead of trimethylammonium can better help to accommodate and dehydrate the pyrene probe.
Literature suggests that the counterion can be bound to a single surfactant molecule before micelles formation; i.e ion pair formation occurs.[62] The analysis of conductivity data is informative also to evidence the ion pair formation that determines a departure of experimental points from linearity of specific conductivity (κ) vs C plot in diluted solutions before CMC; for example compare Figure 1 (surfactant **1b**) with Figure 4a (N-dodecylpyridinium chloride). Zana defined this behaviour as a curvature towards the C axis and assigned it to ion pair association, easily achieved for gemini surfactants since the two cationic headgroups can keep a counterion tightly between them.[62, 76] Similar behaviour for all the gemini surfactants **1b**-**8b** was observed (see Supporting Information). The A_{min} (surface minimum area, i.e. area occupied by a single molecule at the air-solution interface) was extrapolated from surface tension measurements and the maximum surface excess concentration Γ_{max} through the equation 2 and 3 (Gibbs model).[1, 59]

$$A_{min} = 10^{16}/(N \cdot \Gamma_{max}) \qquad (2)$$

$$\Gamma_{max} = -(1/2.303nRT)\,(\partial\gamma/\partial \log C)T \qquad (3)$$

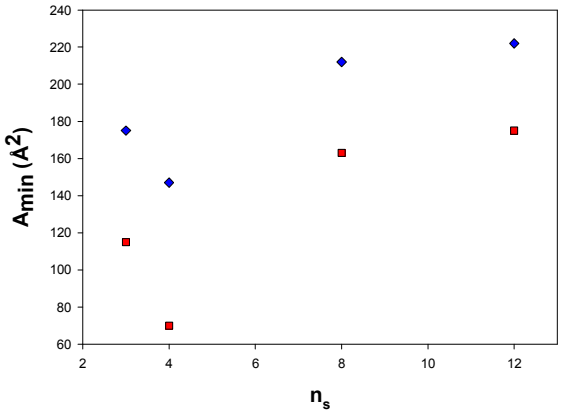


Figure 7. A_{min} as a function of the spacer length (n) for C12 (■) and C16 (◆) pyridinium gemini surfactants.

Before discussing the relationships about minimum area and behaviour of surfactants, it is worth mentioning that the uncertainty in the surface purity can provoke great errors in the evaluation of the physico-chemical parameters such as the A_{min} that can be derived from the surface tension.[77-78] Absolute methods to determine the concentration of surfactant molecules at the surface, the so-called "surface excess" (Γ) such as neutron reflectivity (NR) were already assessed at the end of nineties[76]. Their use allows to compare their results with those obtained by surface tension. In very recent years the reliability of the Gibbs model (eq. 1) was questioned on the basis of some apparent discrepancies and the question was debated. [79-83] Careful use of NR technique gave sufficient evidences that, at least in case of non ionic surfactants the Gibbs model was found valid, while in the case of ionic surfactants the ionic nature of the molecules made the results more complicated to be interpreted.[83] Notwithstanding, the debate on always better techniques and careful revision on physico-chemical models is useful for research and knowledge progress. Since our more hydrophobic surfactants **3b** and **4b** show cmc ranging at a concentration of about 10^{-5}-10^{-6} M, they could also be affected by phenomena such as depletion of the adsorbed water-air surface layer due to adsorption of surfactants to the solid-liquid surface, changing considerably the bulk surfactant concentration and also kinetic effects could be possible to take part to the process of surface tension determination.[83] However, the resort to an absolute method such as NR to make research about adsorption phenomena is difficult since it requires not easily available instrumentations and the urge for more simple and cost-effective benchtop instrumentation that could permit to correctly study and describe adsorption even at low concentrations is evident. The results we presented, however show a trend in reasonably agreement with literature, apart from peculiar behaviour for compound **2b**.

In Figure 7, the A_{min} values as function of the spacer length (n) for both series are reported. 16Py-s-Py16 surfactants show greater values of A_{min} than 12Py-s-Py12 series in agreement to literature.[65] The A_{min} values increase with increasing of spacer length. This increase seems to reach a plateau going from eight to twelve methylenes of spacer length, suggesting that, similarly to gemini ammonium surfactants, the spacer tends to fold toward the micellar core.[59] This seems to be slightly anticipated, around spacer length of eight methylenes with respect to ammonium gemini surfactants, for which the same behaviour seems to occur around a spacer length of 10-12. Noteworthy is the minimum value of A_{min} for surfactants whose spacer shows four methylenes, in agreement with analogous effect observed for dodecyl pyridinium gemini surfactants having methanesulfonates as counterions.[44-45] The conformational freedom of four methylenes spacer allows the molecule to doubles like a book, giving rise to stacking interactions between the two pyridinium rings, mediated by the counterion whose charge screens the positive rings from each other.

Surfactant-DNA interaction.

In order to check the ability of the new gemini surfactants to induce structural changes in the DNA, we have used the atomic force microscopy (AFM) technique. The experiments were done using plasmid DNA imaged in air in the tapping mode before and after incubation with surfactants **1b**-**8b**. In Figure 8, as an example, some images of the circular DNA and of the structures obtained after addition of the surfactants are shown. They suggest a very nice structure-activity relationship, being the compacting ability strictly related to the length of the spacer. The AFM images (Figure 8) show that only the **2b** is able to condense the DNA in nearly spherical particles. **3b** could succeed in a minor extent, giving rise also to some particles not homogeneous in size nor in shapes. The **1b** and **4b** hexadecyl surfactants along with the **5**-**8** dodecyl surfactants gave no DNA collapse or only partial collapse without achieving full DNA condensation. For a more detailed discussion about the gene delivery ability of the compounds here presented, see ref. 64. [64]

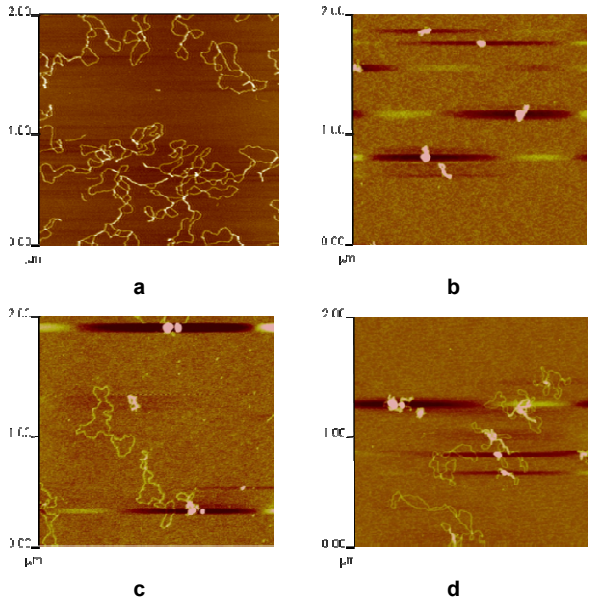


Figure 8. AFM images showing the effect of the interaction of different hexacecyl surfactants on the DNA compaction: a) native plasmid DNA; b) plasmid DNA with compound **2b**; b) plasmid DNA with compound **3b**; b) plasmid DNA with compound **4b**. The behaviour of **1b** and dodecyl surfactants **5b**-**8b** (not shown) is similar to **4b**.

Transfection experiments where pyridinium gemini surfactants have been formulated with DOPE ([L-α-phosphatidylethanolamine dioleoyl (C18:1,[cis]-9)]), seem to indicate better performance for hexadecyl surfactants series with a particular dependence from the spacer length.[64] Higher activity has been observed when the transfecting agent has four or eight methylene groups suggesting a possible correlation of biological activity with counterion binding (β) and A_{min} parameter and also with the visual inspection of AFM images for which only those surfactants showed ability to compact DNA.[64]

The variation of the spacer length is known to modify the packing parameter[46, 59] and to tune the aggregation properties[84-85] (CMC, micellar aggregation number, micellar shape, etc.) and these preliminar results indicate that it affects the properties of the surfactants-DNA lipoplexes. This structural feature, that determines the peculiar behaviour of surfactants **2b** and **3b** for A_{min} and the degree of counterion binding β, seems to be also highly relevant for their activity in transfection experiments. In our recent work also the DNA shift, the interaction with membranes were studied and always the uncommon peculiarities, shown by the surface tension, conductivity and fluorescence techniques confirmed that the behaviour of those surfactant could be related to particular activity.[64]

In view of these results it appears interesting to explore similar structures bearing some hydrolysable bond such as amide or ester,[88] to improve the biodegradability and possibly the viability to the cells, since normally quaternary compounds show bactericidal activity and limited biodegradability which however do not hamper their use as gene carrier.

Conclusions.

A series of hexadecyl pyridinium gemini surfactants was prepared by extending the generality of a quaternization method based on alkyl trifluoromethanesulfonates. Peculiar aggregation behaviours have been studied by a multitechnique approach: conductivity, surface tension and fluorescence. The CMC values, in the order of 10^{-5} M, suggest the higher efficiency of pyridinum gemini surfactants in comparison to corresponding monomeric ones. Long alkyl chains (16Py-s-Py16 series) stabilize the micellar aggregates.

The comparison of CMC values extrapolated by coumarin 6 and pyrene as fluorescent probe and specific conductivity (κ) vs C and molar conductivity (Λ) vs $C^{0.5}$ plots suggest premicellar aggregate occurrence for pyridinium gemini surfactants with hexadecyl chains (16Py-s-Py16 series). The data suggest also that pyrene is a less useful fluorescent probe to determine CMC for the present series of surfactants but it helps to evidence the lower micellar compactness of hexadecyl series with respect to dodecyl ones. In general, the application of fluorescent probes can give very useful information also for surfactant systems showing non conventional behaviour, improving the knowledge of complex aggregation of surfactants.

For some terms of surfactants series, a more complex behaviour was found. Formation of an ion pair and of pre-aggregates has been supposed.

The study of the degree of counterion binding (β) could be an interesting parameter for the structure-activity relationships study, also for activity as gene vector. In this context, the pyridinium ion is a softer cation than ammonium one and its delocalized charge can better interact with a reasonably soft anion such as the phosphate, influencing the aggregation properties and the DNA transfection activity. For DNA complexation and its release in the cell, an optimal interaction of cationic vector with negative phosphate group of DNA is needed.

The degree of counterion binding decreased with the lengthening of the alkyl chains and was strongly affected by the spacer length. A minimum for the degree of counterion binding was found for molecules having an eight methylenes long spacer, irrespective of the differences in the alkyl chains. This peculiarity was related to the compromise of the conformation that these gemini surfactants can adopt at the micellar surface and the electrostatic interactions between the pyridinium rings, giving a poorly packed micellar structure as a result.

The trends of A_{min} as function of the spacer length for the didodecyl and dihexadecyl gemini surfactants are in perfect agreement with what we reported for the same didodecyl gemini surfactants having methanesulfonates as counterions, confirming the hypothesis of a conformational change of the molecule when the spacer is four methylene long, due to stacking interactions between the two pyridinium rings, mediated by the counterion. Moreover, the trends of both A_{min} and β suggest that the spacer must be longer than eight carbon atoms to fold towards the micellar core.

The opportunity to tune the surfactants aggregation properties, such as the CMC towards lower values, the whole molecular hydrophobic character, the spacer length and the degree of counterion binding make those surfactants, in particular the long chain ones, desirable candidates for gene delivery experiments. Preliminary study of transfection activity shows that some terms of the present series of surfactants, formulated with DOPE, have high DNA transfection activity. The chain length dependence study towards the interaction with DNA was important, since dodecyl analogs did not show DNA complexing ability and only two hexadecyl analogs formed lipoplexes in an efficient way. The effect of the spacer length, for compounds having four and eight methylenes, on both A_{min} and β is peculiar not only for surfactant properties but also on the transfection activity.

While several structural features contributes to the surfactant behaviour, this relationship cannot be disregarded and should enforce research to establish structure-activity relationships.

Experimental Section.

General Procedures and Materials. See the Supporting Information too.

The 2,2'-alkybipyridines were prepared as described in the literature[10] and the products **5**-**10** were prepared according previously established procedures.[10, 48]

Hexadecyl triflate.[10, 86] The product was obtained following previously stated procedures.[10] Chloroform was found to perform better than dichloromethane in the solubilization of the hexadecyl alcohol, thus improving the procedure. After flash chromatography on silica with petroleum ether, the product was obtained by solvent evaporation and used immediately for quaternizations.

Yield 6.27 g. (85 %). ^{1}H NMR ($CDCl_3$): δ 0.88 (t, 3H, CH_3), 1.26 (bs, 26H, $13CH_2$), 1.83 (t, 2H, $CF_3SO_3CH_2\mathit{CH_2}$), 4.53 (t, 2H, $CF_3SO_3\mathit{CH_2}CH_2$).

General procedure for the quaternization of α,ω-bis(2-pyridyl)alkanes. A solution of hexadecyl triflate in anhydrous chloroform was introduced in a three-necked flask under argon, and warmed at 70°C under magnetic stirring. A solution of the proper α,ω-bis(2-pyridyl)alkane in anhydrous chloroform was slowly dropped into the warm solution. The reaction was stopped after 0.5-1.5 hours. The resulting solution was treated with ethyl acetate, giving a white precipitate, that was filtered giving the pure product. The chlorides **1b**-**4b** were obtained from products **1a**-**4a** by ion exchange on an anionic exchange resin as previously described.[10]

1,1'-dihexadecyl-2,2'-trimethylenebispyridinium ditrifluoromethanesulfonate (1a). Yield 87%; m.p. 206-209°C; R_f 0.31 on silica (eluent BAW: Butanol/Acetic Acid/water 4:1:5, organic phase); ^{1}H NMR (DMSO-d6): δ 0.85 (t, 6H, $2CH_3$), 1.23 (m, 52H, $26CH_2$), 1.86 (m, 4H, $N^+CH_2\mathit{CH_2}$), 2.19 (m, 2H, $PyCH_2\mathit{CH_2}$), 3.29 (t, 4H, $PyCH_2$), 4.58 (t, 4H, N^+CH_2), 8.02 (t, 2H, H_{meta}), 8.11 (d, 2H, H'_{meta}), 8.54 (t, 2H, H_{para}), 9.03 (d, 2H, H_{orto}); ^{13}C NMR (DMSO-d6): δ 14.20 (CH_3), 22.35, 26.01, 28.83, 28.97, 29.19 (several C), 29.33 (several C), 30.65, 30.98, 31.55, 57.34, 126.09 (Ar), 128.83 (Ar), 145.52 (Ar), 146.03(Ar), 157.26 (Ar); FT-IR (KBr): 3086, 2918, 2850, 1630, 1512, 1468, 1226, 1166, 786, cm-1. UV/Vis (ethanol) λ_{max} 269 nm, logε 3.99; MS (ESI+): m/z 797.66 (M^+ - CF_3SO_3, calcd. 797.58); Anal. Calcd. for $C_{47}H_{80}F_6N_2O_6S_2$: C, 59.59; H, 8.51; N, 2.96. Found: C, 59.62; H, 8.53; N, 2.90.

1,1'-dihexadecyl-2,2'-trimethylenebispyridinium dichloride (1b). Yield 99%; m.p. 200-201°C; R_f 0.35 on silica (eluent BAW); ^{1}H NMR (DMSO-d6): δ 0.84 (t, 6H, $2CH_3$), 1.23 (m, 52H, $26CH_2$), 1.86 (m, 4H, $N^+CH_2\mathit{CH_2}$), 2.19 (m, 2H, $PyCH_2\mathit{CH_2}$), 3.33 (t, 4H, $PyCH_2$), 4.67 (t, 4H, N^+CH_2), 8.02 (t, 2H, H_{meta}), 8.22 (d, 2H, H'_{meta}), 8.55 (t, 2H, H_{para}), 9.16 (d, 2H, H_{orto}); ^{13}C NMR (DMSO-d6): δ 13.96 (CH_3), 22.12, 25.75, 28.65, 28.74, 28.98, 29.05 (several C), 29.11 (several C), 30.59, 30.77, 31.32, 57.09, 125.77 (Ar), 128.78 (Ar), 145.19 (Ar), 145.84 (Ar), 157.20 (Ar); FT-IR (KBr): 2918, 2851, 1630, 1514, 1466, 1173, 789 cm-1. UV/Vis (ethanol) λ_{max} 269 nm, logε 4.18; MS (ESI+): m/z 683.75 (M^+ - Cl, calcd. 683.60); Anal. Calcd. for $C_{45}H_{80}Cl_2N_2$: C, 75.06; H, 11.20; N, 3.89. Found: C, 75.06; H, 11.16; N, 3.87.

Conductivity measurements. Conductivity measurements were performed on a conductivity meter equipped with a conductivity cell having cell constant of 0.943 cm^{-1}, except for surfactants **1b**-**4b** for which a conductivity cell with a cell constant of 9.895 cm^{-1} was used, as already reported.[10, 60] The addition of concentrated surfactant solutions by a titrator to pure water and the collection of the conductivity data were performed by using a computer controlled automated system, working with a homemade program, written in Quick Basic, available from the author.

Fluorescence measurements. The steady-state intensity fluorescence measurements were performed on a Perkin Elmer LS55 spectrofluorimeter. Sample solutions were prepared by dilution of a surfactant stock solution, to which 10 μl of the probe stock solution was added. Pyrene and Coumarin 6 were dissolved in ethanol. Pyrene spectra were collected in the 360-600 nm range, checking carefully that no excimer was formed: final concentration $5 \cdot 10^{-7}$ M, excitation wavelength 320 nm; excitation slit 5 nm and emission slit 2.5 nm. Coumarin 6 spectra were collected in the 475-650 nm range, final concentration $1.5 \cdot 10^{-7}$ M, excitation wavelength 465 nm; excitation slit 2.5 nm and emission slit 5.0 nm. In order to fulfill the conditions for a correct measurement of the CMC of pyridinium surfactants with pyrene, it is better to try to adjust the pyrene concentration in order to have enough fluorescence signal after the CMC, since pyridinium surfactants are known to quench pyrene fluorescence. Besides too concentrated pyrene could give excimers when it transfer into the micelles.

Surface tension measurements. The surface tension was measured by using a Lauda (TE1C/3) digital tensiometer. Measurements were made at 25±0.1°C using the Du Noüy ring (Pt/Ir alloy (80/20); circumference: 60 ± 0.2 mm, wire diameter 0.4 mm, weight: 1.6 g). Sample temperature was controlled to 0.1°C by using a circulating water thermostatic bath (ISCO GTR 2000 IIx). The data were corrected according with Zuidema and Waters method.[87] The instrument was calibrated against double-distilled, previously deionized water, equilibrated against atmospheric CO_2, each time measurements were done.

Because the dicationic surfactants adsorb onto negatively charged glass surfaces, all glassware was thoroughly soaked with the solution to be measured; soaking solutions were discarded. The fresh solution was aged for several hours before surface tension measurement. Sets of measurements were taken at 15 min intervals until no significant change occurred. Standard deviation of the surface tension measurements is less than 0.15 mN/m. The absence of a minimum in the surface tension vs log concentration plot in the post-cmc region (see Figure 2) showed that there was very little or no surface active impurity present in the final products.

Sample preparation and AFM imaging

DNA samples were prepared by diluting the plasmid DNA to a final concentration of 0.5 nM in deposition buffer (4 mM Hepes, 10 mM NaCl, 2 mM $MgCl_2$, pH = 7.4) either in the presence or in the absence of surfactants **1b**-**8b**. The mixture was incubated for 5 minutes at room temperature, then a 20 μl droplet was deposited onto freshly-cleaved ruby mica (Ted Pella, Redding, CA) for one minute. The mica disk was rinsed with milliQ water and dried with a weak stream of nitrogen.

AFM imaging was performed on the dried sample with a Nanoscope IIIA Microscope (Digital Instruments Inc. Santa Barbara, CA) operating in tapping mode. Commercial diving board silicon cantilevers (NSC-15 Micromash Corp., Estonia) were used. Images of 512×512 pixels were collected with a scan size of 2 μm at a scan rate of 3-4 lines per second and were flattened after recording using Nanoscope software.

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[a] Dr. N. Barbero, C. Magistris, Dr. P. Quagliotto*, L. Bonandini, Dr. C. Barolo, Dr. R. Buscaino, Prof. G. Viscardi
Dipartimento di Chimica, Interdepartmental "Nanostructured Surfaces and Interfaces" NIS Centre - Università di Torino.
Via Pietro Giuria 7 - 10125 Torino – Italy
E-mail: pierluigi.quagliotto@unito.it
[b] Dr. C. Compari, Dr. L. Contardi, Prof. E. Fisicaro
Department of Pharmacy - Università di Parma
Parco Area delle Scienze 27/A – 43124 – Italy

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