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Nonviral Gene-Delivery: Gemini Bispyridinium Surfactant-Based DNA Nanoparticles

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ABSTRACT

The interaction with model membrane, the formation of DNA nanoparticles and the transfection ability of a homologous series of bispyridinium diexadecyl cationic gemini surfactants, differing for the length of the alkyl spacer bridging the two pyridinium polar heads in 1,1’ position (P16-n with n =3, 4, 8, 12), has been studied by means of differential scanning calorimetry (DSC), atomic force microscopy (AFM), electrophoresis mobility shift assay (EMSA) and transient transfection assays measurements. The results here presented show that their performance in gene delivery is strictly related to their structure in solution. For the first time the different transfection activity of the compounds can be explained by referring to their thermodynamic properties in solution, previously studied. The compound with spacer formed by four carbon atoms, showing unexpected enthalpic properties vs. concentration in solution, is the only one giving rise to a transfection activity comparable to that of the commercial reagent, when formulated with L-α-dioleylphosphatidylethanolamine (DOPE). We suggest that P16-4 behaves like molecular tongs able to grip basic group near each other, so allowing the formation of compact and near spherical DNA particles. The compound with the longest spacer gives rise to loosely condensed structures by forming a sort of bows, not able to give rise to transfection notwithstanding the double positive charge of the molecule. On the other hand, DSC measurements on synthetic membranes show that the compounds with the shortest spacer (3 and 4 methylene groups) practically do not interact with the 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) membrane, while the compounds P16-8 and, particularly, P16-12 induce the formation of surfactant-rich and surfactant-poor domains in the membrane, without showing any peculiarity for the compound P16-4. This could suggest that the mechanisms involved in the interaction with model membrane and in gene delivery are substantially different and could strike a blow for an endocytosis mechanism for the internalization in the cell of the DNA nanoparticles.
**Keywords:** synthetic vectors for gene delivery, gemini surfactant-membrane interaction, atomic force microscopy, DPPC model membranes, transient trasfection assay
INTRODUCTION

By the name of “gemini” surfactants are indicated those surfactants in which at least two identical moieties are bound together by a spacer at the polar head level. They show peculiar properties in comparison with the monomeric ones owing to their increased surface activity, lower critical micelle concentration (CMC), and useful viscoelastic properties.\(^1\)\(^-\)\(^8\) Recently a comprehensive review has been published, focused on positively charged heterocyclic gemini surfactants: their major synthetic access routes are presented and the impact of structural elements on their physicochemical and aggregation properties is examined.\(^8\) In recent years, the scientific and applicative interest in cationic gemini surfactants has increased, also because of their potentialities in pharmaceutical field, both as non-covalent functionalization agents for carbon nanotubes-based formulations for drug delivery\(^9\) and as non viral vectors in gene therapy\(^10\)\(^-\)\(^18\). Gene therapy is used to treat diseases caused by a missing, defective or overexpressing gene. It is based on the delivery of therapeutic by means of a specially designed vector. To reach this goal, the DNA must be packaged in a viral or nonviral delivery system.\(^17\) Since viral vectors, currently the most efficient, are not without the risk of adverse or immunogenic reaction, or replication, depending on the virus being used, cationic lipids have in many cases become the preferred means of gene delivery into eukaryotic cells. Cationic lipids constitutes the building blocks to compact and encapsulate the DNA into soft nanoparticles, delivered to specific sites by size-dependent passive targeting or by active targeting, this is to say by means of ligands attached onto their structure to achieve cell surface specificity. Moreover nanoparticles are able to protect the DNA from enzymatic degradation and their tunable size allows for building up nanoparticles large enough for preventing rapid leakage into blood capillary but small enough for escaping macrophages of the reticuloendothelial system. However, nonviral delivery systems with high transfection efficiency must still be realized. We have obtained encouraging results in gene delivery by using very simple bisquaternary ammonium gemini surfactants, derivatives of N,N-bisdimethyl-1,2-ethanediamine of general formula

\[
/C_nH_{2n+1}OOCCH_2(CH_3)_2N^+CH_2CH_2N^+(CH_3)_2CH_2COOC_nH_{2n+1}/2Cl^- \text{ (bis-C}_n\text{BEC)}, \text{ where the}
\]

\(n\) is an integer between 0 and 8.
subscript $n$ stands for the number of carbon atoms of the alkyl chain bound to the carboxyl group, when formulated with DOPE [L-$\alpha$-phosphatidylethanolamine dioleoyl (C18:1,[cis]-9)]. These results urged us to design and characterize new gemini compounds starting from those having, as polar head, two pyridinium groups, bridged together by aliphatic chains of different length. In this paper we report for the first time physicochemical and biological data concerning this new homologous series of gemini surfactants, with the aim to achieve a better insight about the interaction of the cationic surfactants with membranes and DNA. In fact, we are for some time collecting these data with the idea of correlating in a quantitative way the structure of the surfactants with their biological activity, particularly transfection ability. In this perspective, synthetic vectors present the advantage that their constituent parts can be quite easily modified, thereby facilitating the elucidation of structure-activity relationships. A kind of gemini pyridinium surfactants, structurally different from the compounds here presented with the spacer bridging the heteroaromatic nitrogens, has been recently proposed in literature for transfection purposes.

EXPERIMENTAL METHODS

Compounds

The series of bispyridinium cationic gemini surfactants under study was prepared by us, as described in ref. 19 for the dodecyl compounds (see also SI). In this paper the compounds having chloride as counterion, are named $P m-n$, were $m$ indicate the number of carbon atoms of the alkyl chain and $n$ the spacer length and $P$ stands for bispyridinium. The compounds studied are the following: 1,1′-diexadecyl-2,2′-trimethylenebispyridinium dichloride (P16-3); 1,1′-diexadecyl-2,2′-tetramethylenebispyridinium dichloride (P16-4); 1,1′-diexadecyl-2,2′-octamethylenebispyridinium dichloride (P16-8); 1,1′-diexadecyl-2,2′-dodecamethylenebispyridinium dichloride (P16-12). The structure of the compound P16-3 is shown in Figure 1.
Moreover, P12-8 (1,1′-didodecyl-2,2′-octamethylenebispyridinium dichloride) and P12-8MS (1,1′-didodecyl-2,2′-octamethylenebispyridinium dimethanesulfonate) were also synthesized by us, as described in ref. 19.

Purity was checked by NMR, elemental analysis and TLC: eluent BAW (butanol:acetic acid:water = 4:1:5-organic phase) on silica gel plate (Merck). The solutions were prepared by weight using freshly boiled bi-distilled water, stored under nitrogen. Solution concentrations are expressed as molality, \( m \) (mol kg\(^{-1}\)).

1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) was purchased from Sigma Aldrich, Steinheim, Germany. Lipid purity was greater than 99%.

**DNA Preparation and Storage**

Plasmid DNA was purified through caesium chloride gradient centrifugation. A stock solution of the plasmid 0.7 \( \mu \)M in milliQ water (Millipore Corp., Burlington, MA) was stored at -20 °C. Linearized plasmid DNA (pEGFP-C1) was obtained by cutting with EcoRI restriction enzyme (Roche), column
purified (Genomed) and alcohol precipitated. Linearized plasmid DNA pellet was washed with 70% of ethanol, air dried and dissolved in distilled H₂O at a final concentration of 1 μg/μl.

**Cell Culture**

The human rhabdomyosarcoma cell line RD-4, obtained from David Derse, National Cancer Institute, Frederick, Maryland, were maintained as a monolayer using growth medium containing 90% DMEM, 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 10 μg/ml streptomycin. Cells were subcultured to a fresh culture vessel when growth reached 70-90% confluence (i.e. every 3-5 days) and incubated at 37 °C in a humidified atmosphere of 95% air/5% CO₂.

**Electrophoresis Mobility Shift Assay (EMSA)**

Binding reactions were performed in a final volume of 14 μl with 10 μl of 20 mM Tris/HCl pH 8, 1 μl of plasmid (1 μg of pEGFP-C1) and 3 μl of P16- with \( n =3, 4, 8, 12 \) at different final concentrations, ranging from 25 to 200 μM. Binding reaction was left to take place at room temperature for 1 hour, 5 μl of 1g/ml in H₂O of glycerol was added to each reaction mixture and loaded on a TA (40 mM Tris-Acetate) 1% agarose gel. The gel was run for 2.5 hours in TA buffer at 10 V/cm, EDTA was omitted from the buffers because it competes with DNA in the reaction.

**Transient Trasfection Assay**

Transfections were performed in 6 well plates, when cells were 80% confluent (approximately \( 3 \times 10^5 \) cells) on the day of transfection. 3 μg of plasmid, P16- with \( n =3, 4, 8, 12 \) were added to 1 ml of serum-free medium at final concentration of 15 μM, mixed rapidly and incubated at room temperature for 20 minutes. Each mixture was carefully added to the cells following the aspiration of
the culture medium from the cells. Lipoplex formulations were performed adding DOPE to plasmid-surfactant mixture at different surfactant:DOPE molar ratios (1:1, 1:2 and 2:1); where the surfactant concentration was kept to 15 µM. GenePORTER transfection reagent, a neutral lipid transfection reagent was used as positive transfection control.

Mixture and cells were incubated at 37 °C in a humidified atmosphere of 95% air/5% CO₂ for 5 hours. Finally, 1 ml of medium containing 20% of FBS was added to each transfected well and left to incubate for 72 hours.15,22

Transfected cells were observed under fluorescence microscope for EGFP expression. Five random fields were examined from each well and each experiment was repeated three times. Statistical differences among treatments were calculated with Student’s test and multi factorial ANOVA.

**Differential Scanning Calorimetry (DSC) Measurements**

DSC studies were performed according to the protocol described earlier using the Mettler Toledo Thermal Analysis System D.S.C. 821e.15 The cycles were performed three times. The experimental uncertainty of temperature was ± 0.2 °C.

**Sample Preparation and Atomic Force Microscopy (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 MgCl₂, pH = 7.4) either in the absence or in the presence of P16-n with n =3, 4, 8, 12 in the same DNA/surfactant ratio used in transient transfection assay experiments. 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.

**Modeling**

The geometry optimization for dications of the compounds P16-\(n\) with \(n = 3, 4, 8, 12\) and P12-8 was performed using semiempirical quantum computations with the MOPAC2012 ver. 12.309W\(^{23}\) program via the VEGA ZZ package\(^{24}\). The computations were done for the dications in vacuum.

**RESULTS**

**Interactions with Model Lipid Membrane and Modeling**

The influence of *gemini* surfactants on the thermotropic phase behavior of dipalmitoylphosphatidylcholine bilayer is shown in Figure 2. In the absence of a surfactant, DPPC exhibited two endothermal transitions upon heating, the pretransition at 35 °C and the main gel-to-liquid-crystalline phase transition at 41 °C. The compounds P16-3 and P16-4 have virtually no effect on the transitions. For the compound P16-8 the main phase transition temperature \((T_m)\) remains practically unchanged, but the values of transition enthalpy \((\Delta H_m)\) and the half-maximum width \((T_{1/2})\) increase (Table 1). The main transition is slightly broadened and asymmetrical which suggests the lateral phase separation. Probably surfactant-rich and surfactant-poor domains are formed. The compound P16-8, like P16-3 and P16-4, does not suppress the pretransition. The compound P16-12 affects both phase transitions much more than the previous compounds. At molar ratio 0.01 on the left side of the main phase transition (I) appears a shoulder. This suggests that also compound P16-12 causes phase separation and surfactant-rich and surfactant-poor domains are formed. At molar ratio 0.03 the shoulder transforms into a new peak (II) and the pretransition disappears. At higher
concentrations both peaks are shifted to lower temperatures, their half-maximum widths increase and enthalpy of peak I decreases. At molar ratio 0.1 the peaks overlap.

Figure 2. DSC heating curves of MLVs with increasing molar ratios of the compounds under study to DPPC. The curves are normalized for the amount of DPPC; scan rate 2 °C/min.
Table 1. Temperature $T_{m}$, half-width of the peaks $T_{\frac{1}{2}}$ and enthalpy change $\Delta H$ of the main phase transition of the lipid bilayer vs. surfactant-to-lipid molar ratio ($n_{X}/n_{DPPC}$). The values marked I and II are related to the peaks marked I and II in DSC thermograms.

<table>
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<th>compound</th>
<th>$n_{X}/n_{DPPC}$</th>
<th>$T_{m}$/°C</th>
<th>$T_{\frac{1}{2}}$/°C</th>
<th>$\Delta H$/kJ/mol</th>
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The interaction of compound P16-12 is stronger and phase separation is much more enhanced in comparison to the effects of compound P16-8. The compounds with shorter alkyl chains P12-8 and P12-8-MS (having methanesulfonate as counterion) affect the phase transitions in a similar way. They both abolish pretransition at the lowest concentration and decrease $T_m$ and $\Delta H_m$ much more than former ones showing strong fluidization effect.

The dications of the compounds P16-$n$ with $n=3, 4, 8, 12$ and P12-8 (the same as P12-8-MS) were prepared in different initial conformations for the quantum calculations. Then, they were optimized using the two parametrizations PM7 and RM1. An example of the optimized geometry is shown in Fig. 1 for P16-3 (Other examples can be found in Supporting Information). The main effect observed in the calculations is that in vacuum the pyridinium rings tend to position away from each other, which is understandable given that they bear the positive charge in the vicinity of the nitrogens. In some cases for the compounds with long spacer (8 and 12 carbon atoms) the hydrocarbon tails tend to more or less align with the spacer chain. This can take place especially in the water solution, considering the hydrophobic interactions between the alkyl chains. However, one can expect that the dications entering the lipid bilayer will take conformations with the alkyl tails immersed in the hydrophobic layer of the membrane, and the pyridinium rings placed at the level of the negatively charged phosphate groups. The calculated distance between the pyridinium rings of the optimized dications, measured as the average distance between nitrogens, is about 0.69 nm, 0.81 nm, 1.27 nm, 1.5–1.7 nm and 1.25 nm for the compounds P16-$n$ with $n=3, 4, 8, 12$ and P12-8, respectively.

**Biological Assays**

The interaction of P16-$n$ with $n=3, 4, 8, 12$ with plasmid DNA pEGFP-C1 (Clontech) was monitored by agarose gel electrophoresis mobility shift assay (EMSA, Figure 3). The shift activity was observed for all the compounds investigated, able to modify the mobility of DNA at the lowest
concentration tested (25 µM). Finally, to test the capability of the same compounds to deliver DNA inside the cells, a transient transfection assay was performed with a plasmid carrying an EGFP expression cassette under the control of the CMV immediate early promoter (pEGFP-C1, Clontech) to monitor EGFP expression under a fluorescence microscope.

RD-4 cells were chosen among a large panel of several cell lines because they are a good compromise between very difficult to transfect cells and very easy to transfect cells with traditional methods (electroporation, lipofection and calcium phosphate precipitation). Moreover they are easy to handle, fast growing and derived from a human nasty cancer. This later reason makes the data achieved from the molecules employed in the present paper relevant for oncology gene transfer studies. P16-4 was also the only one able to deliver DNA inside the cells, as shown by EGFP expression, albeit at an efficiency much lower than that obtained with a standard commercial transfection reagent (GenePORTER, Gene Therapy System), which we used as a positive transfection control.

**Figure 3.** EMSA experiments showing complexation of P16-\(n\) \((n = 3, 4, 8, 12)\) with circular plasmid pEGFP-C1. Shifting is observable as a function of concentration (µM). As a negative control, only the plasmid was used, which is completely unshifted.
It appears that DOPE enhances the transfection activity of cationic formulations through the stabilisation of DNA/lipid complex\textsuperscript{26-27} and facilitates the transfer of DNA in the context of endosomal escape, owing to its fusogenic property. Previous studies have demonstrated that DOPE significantly affects the polymorphic features of lipoplexes promoting transition from a lamellar to a hexagonal phase, thus facilitating endosomal escape.\textsuperscript{28} We therefore tested \textit{in-vitro} the transfection efficiency of P16-\textit{n} with \textit{n} = 3, 4, 8, 12 formulated with DOPE at different P16-\textit{n}: DOPE molar ratios (1:1, 1:2 and 2:1). P16-\textit{n} concentrations were fixed at 15 µM. The addition of DOPE increases a little the transfection efficiency of P16-4 and P16-8, but it is ineffective with P16-3 and P16-12 (Figure 4). In particular P16-4 shows, when formulated with DOPE, a transfection ability comparable with that of the standard commercial transfection reagent (Figure 4). In contrast, absence of transfection is observed only for DOPE transfection control.

\textbf{AFM Experiments}

To verify the ability of the compounds under investigation to induce structural changes in the DNA, we employed AFM.\textsuperscript{29-30} This technique has been successfully used to study the interaction of both synthetic ligands\textsuperscript{31-32} and proteins\textsuperscript{33} with DNA. AFM experiments were carried out using circular DNA imaged in air with the tapping mode. Figure 5a shows a typical image of the plasmid DNA.
alone deposited onto freshly cleaved mica. Single plasmids and concatamers are seen in their plectonemic form with several supercoils which cause the double helix to cross itself a number of times. Besides the topological constraint, plectonemes appear well extended all over the mica surface. Figures 5b-d show the plasmid DNA after incubation with P16-n with n = 4, 8, 12, respectively. According to the EMSA results, only P16-4 (Figure 5b) is able to condense all DNA in near spherical nanoparticles less than 0.1 µm in diameter. After addition of P16-8 (Figure 5c) only part of the DNA condense in nanoparticles homogeneous neither in size nor in shape. In the case of P16-12, AFM images show that partially condensed structures, if any, are formed, looking like bows (Figure 5d).

P16-3 (not shown), interacting with DNA as well, is unable to condense the DNA in nanoparticles, but only reduces the extension of the loops formed by plasmid DNA, by overlapping of the opposite sides.
**Figure 5.** AFM images showing the effect induced on DNA plasmid by incubation with P16-\(n\) (\(n = 4, 8, 12\)). Each image represents a 2 x 2 \(\mu\)m scan (scale bar = 0.2 \(\mu\)m). All images were obtained with supercoiled 0.5 nM pEGFP-C1 plasmid deposited onto mica and with the microscope operating in tapping mode in air. (a) DNA plasmid alone. Plasmid incubated with (b) 2 nM P16-4; (c) 2 nM P16-8; (d) 2 nM P16-12. For P16-3 see SI. The chromatic bar inside the figure is referred to the thickness of the objects.

**DISCUSSION**

The use of *gemini* surfactants as non viral vectors in gene therapy has been proposed, on account of the possibility of taking advantage of their multiple cationic charge, necessary for binding and compacting DNA, and of their superior surface activity.\(^8\)\(^{-15}\) DNA nanoparticles, in fact, are generally composed by specific cationic lipids to ensure efficient DNA condensation and cellular uptake of the complexes and by helper lipids such as dioleoylphosphatidylethanolamine (DOPE).\(^17\) As said before, we have obtained encouraging results in gene delivery by using very simple bisquaternary ammonium *gemini* surfactants, bis-C\(_n\)BEC, when formulated with DOPE.\(^15\) Keeping on like this, we designed and characterized new *gemini* compounds having, as polar head, two pyridinium groups, bridged together by an aliphatic chain, with the idea that the presence of two aromatic pyridinium rings could enhance the interaction with DNA and their ability of compacting and encapsulating DNA into nanoparticles readily internalized by cells.\(^19\) The cmc of the hexadecyl derivatives having chloride counterion was found to be lower than 1 \(\cdot\) 10\(^{-4}\) M by means of conductometric techniques. As an example, the cmc of P16-3 resulted 8.51 \(\cdot\) 10\(^{-5}\) M, which is about 17 fold lower than the corresponding dodecyl derivative P12-3 (cmc: 1.45 \(\cdot\) 10\(^{-3}\) M). The cmc is little affected by the addition of a methylene group in the spacer. It is worth noting that the compound with 4 carbon in the spacer (P16-4) has a cmc value of 9.4 \(\cdot\) 10\(^{-5}\) M, a little higher than P16-3 (see SI). With the aim of enriching the fundamental understanding on self-aggregation thermodynamics of this class of surfactants, we
have measured the apparent and partial molar enthalpies at 298 K of the aqueous solutions of the homologous series of cationic *gemini* surfactants 1,1’-didodecyl-2,2’-alkylenebispyridinium chlorides and methanesulfonates, differing for the spacer length.\textsuperscript{20-21} We measured the dilution enthalpies of the didodecyl compounds because the diexadecyl compounds have a too low cmc not allowing for an accurate determination both in the premicellar and postmicellar concentration regions.\textsuperscript{20-21} We were amazed by their very peculiar behaviour as a function of the spacer length, never found before in literature, not allowing for the determination of a $-\text{CH}_2-$ group contribution when this group is added to the spacer. In fact, as a rule, the inclusion of a methylene group in the structure of the surfactant give rise to a monotonic change in the trends of the apparent and partial molar thermodynamic quantities as a function of the concentration of the solute, so that it is possible to extract a $-\text{CH}_2-$ group contribution of the solute, useful to foresee the properties of new compounds. This is not the case for the homologous series of *gemini* compounds under study in which the methylene groups are added to the spacer. In fact, the curve of the compound with spacer formed by four carbon atoms lies between those of the compound with spacer of three carbon atoms, not below the latter, as expected, and this behaviour, not affected by the counterion, suggests that something happens in the structure of the molecule in solution when the spacer is four carbon atoms long.\textsuperscript{21} We interpreted this surprising behaviour as evidence of a conformation change of the molecule caused by stacking interactions between the two pyridinium rings, mediated by the counterion and appearing at an optimum length of the spacer. The hypothesis was also supported by the data obtained from the surface tension vs. log $c$ curves, showing that $A_{\text{min}}$, the minimum area taken on the surface by the molecule, is significantly lower for the compound with spacer 4 carbon atoms long than that of the other compounds of the same homologous series, and that the same compound has a greater tendency to form micelles instead of adsorbing at the air/water interface.\textsuperscript{20-21} It is interesting to understand if this different behaviour outlined by the trend of apparent and partial molar enthalpies vs. concentration, affects also the biological properties of the surfactants. This has
been done by studying the interaction of the gemini surfactants with model membranes and their gene delivery ability.

**Interactions with Model Membranes**

Measurements show that the compounds P16-3 and P16-4 with the shortest spacer (3 and 4 methylene groups, respectively) practically do not alter the ordering of the DPPC membrane. The other compounds interact with the DPPC membrane differently, depending on the length of the spacer. The compounds P16-8 and P16-12 induce the formation of surfactant-rich and surfactant-poor domains in the membrane but the process is much more pronounced for P16-12.

We have evaluated also the effect on the membrane ordering of the alkyl chain length of the surfactants by comparing the compounds P16-8 and P12-8 and of the counterion by comparing P12-8 and P12-8MS, having methanesulfonate (MS) instead of chloride as counterion. P12-8, with dodecyl chains, shows much more destructive effect on the membrane than the compound with hexadecyl tails. The compounds P12-8MS and P12-8 show very similar effects on membranes and hence the kind of counterions has almost no effect on the interaction with model membranes, although this is not a general case.\textsuperscript{5-7, 34-36}

The effect of the compounds under study on the phase transitions of the DPPC bilayer can be elucidated in part in view of commensurability of the dications with the structure of the bilayer. The calculated distance between the pyridinium rings of the compounds is given in the Results section above. For P16-12, the spacer is most flexible, thus the N–N distance in the optimized geometries varies over a fairly wide range. In the crystalline state, the average distance between the DPPC molecules is about 0.8 nm, which is close to the distance between the pyridinium rings of P16-4, and about a half of that of compound P16-12. The calculated N–N distance for the compounds P16-8 and P12-8 is incommensurate with the bilayer lattice constant, thus it can effect in the stronger
disturbance of the bilayer structure. Moreover, the alkyl tails of the compound P12-8-MS and P12-8 are distinctly incommensurate with the palmitoyl chains of DPPC molecules, in contrast to the tails of compound P16-\(n\) with \(n=3, 4, 8, 12\). This is another factor which may explain the stronger impact of compounds P12-8MS and P12-8 than compound P16-8 on the bilayer ordering.

**Gene Delivery Ability**

To obtain DNA nanoparticles, cationic lipids (cytofectins)\(^{15, 27, 37}\) or cationic polymers\(^{38}\) are needed to neutralise the anionic charges of the DNA phosphate groups, thus compacting the DNA and obtaining complexes of approximately spherical shape having a small dimension when compared to the naked DNA. The amphiphilic nature of such molecules allows to interact strongly with membrane phospholipids and to release the DNA in due time. DNA nanoparticles are therefore composed of specific cationic lipids to ensure efficient DNA condensation and cellular uptake and helper lipids such as dioleoylphosphatidylethanolamine (DOPE).\(^{39}\) We expect that the compounds under investigations have to interact with the DNA, owing their double positive charges carried by their heterocyclic polar heads. Moreover, the aromatic nature of the polar heads could give rise to stacking interaction with DNA bases by intercalation.

We have chosen to use for gene delivery studies the compounds with alkyl chain of 16 carbon atoms, according with our previous experience on this subject.\(^{15, 22}\) It is reported that the biological activity of cationic surfactants increases with the chain length up to a critical point.\(^{39}\) In the case of the homologous series of alkanediyl-\(\alpha,\omega\)-bis(dimethylalkylammonium bromide), the term with two alkyl chains of 16 carbon atoms is generally the most biologically active.\(^{40}\) The same seems to hold also for single-chained sugar based cationic surfactants for example with glucopyranosyl\(^{32}\) and gluconamide\(^{41, 42}\) moieties in the head group.

The results of biological assays show that the compounds here studied are all able to interact specifically with the DNA, completely shifted at concentration above 50 \(\mu\)M. This is particularly true
for P16-4 and P16-8, doing so at halved concentration. It is very surprising to compare the DNA condensation ability of the compounds under study. In fact, as shown in figure 4 and 5, it is enough the addition of one methylene group in the spacer to go from a compound unable to efficiently compact the DNA to a compound able to transform plasmid DNA in nanoparticles and give rise to transfection. Without thermodynamic data, it would be very difficult to understand this sudden change in behaviour. As outlined before, P16-4 shows an anomalous behaviour as far, for instance, enthalpic properties are concerned. In order to explain it, we have assumed a conformation change of the molecule: when the spacer reaches the right size, the molecule in solution doubles up, like a book, due to stacking interactions between the two pyridinium rings and to the hydrophobic interactions of the alkyl chains, independently on the counterion.\textsuperscript{20-21} This arrangement, not envisaged by quantum mechanical calculation in vacuum in absence of counterions and hydrophobic interactions, because the electrostatic repulsion prevails, is possible neither when the spacer is too short (2 or 3 carbon atoms), because of the lack of enough conformational freedom, nor when the spacer is too long, because the pyridinium rings are too far apart. In this way, P16-4 behaves like molecular tongs, able to grip the aromatic bases of the DNA or phosphate groups. The distance between two residues of DNA is 0.34 nm: if the phosphate groups substitute the counterions between the two aromatic rings, reducing the electrostatic repulsion, the molecule could assume the stacked conformation suggested by thermodynamic data, with a medium distance between pyridinium rings much lower than that calculated in vacuum (0.81nm) for the dications. Then, the hydrophobic interactions between the chains of the surfactants cause the efficient formation of nanoparticles. When the length of the spacer increases, this arrangement becomes more difficult and the pyridinium rings are prone to interact with DNA sites far from each other. This explains why P16-8 is still able to form some less compact nanoparticles and to give rise to a little transfection, while the compound with spacer 12 carbon atoms long is not. In fact, the latter probably assumes an extended conformation in solution with the pyridinium ring far apart, also because the hydrophobic interactions in solution could involve the spacer, and the positive polar heads interact with DNA bridging remote bases and so giving rise to
bows, as shown in Figure 5d. In this way the structures formed are not enough compact to be able to penetrate in the cellular membrane, the first step of transfection. Similarly, we suggested the mechanism of cytotoxicity of gluconamide-based single-chained cationic surfactants involving an early effect on the metabolic activity of the cells.\textsuperscript{42}

The coformulation with DOPE does not change considerably the gene delivery ability of the compounds.

In short, it results that P16-4, showing the highest gene delivery ability, is unable to affect the ordering of DPPC membranes. The above results could suggest that the mechanisms involved in the interaction with model membrane and in gene delivery are substantially different and could strike a blow for an endocytosis mechanism for the internalization in the cell of the DNA nanoparticles. This is to say that, to enhance transfection ability, not necessarily we have to look for carriers giving rise to strong interactions with cell membrane, but we have to optimize the electrostatic interactions between cationic lipid and DNA.

CONCLUSIONS
The results here presented show that pyridinium \textit{gemini} surfactant could be a valuable tool for gene delivery purposes, but also that their performance is strictly related to their structure in solution. For the first time the transfection activity of the compounds results strictly related to their thermodynamic properties in solution. The compound with spacer formed by four carbon atoms, showing unexpected enthalpic properties \textit{vs.} concentration in solution is the only one giving rise to a transfection activity comparable to that of the commercial reagent, when formulated with DOPE. We suggest that P16-4 behaves like molecular tongs able to grip basic group near each other. The compound with the longest spacer gives rise to loosely condensed structure by forming a sort of bows, not able to give rise to transfection notwithstanding the double positive charge of the molecule. On the other hand, DSC measurements on synthetic membranes show that the compounds
with the shortest spacer (3 and 4 methylene groups) practically do not alter the ordering of the DPPC membrane, while the compounds P16-8 and, particularly, P16-12 induce the formation of surfactant-rich and surfactant-poor domains in the membrane, without showing any peculiarity for the compound P16-4. This could suggest that the mechanisms involved in the interaction with model membrane and in gene delivery are substantially different and could strike a blow for an endocytosis mechanism for the internalization in the cell of the DNA nanoparticles.

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Supporting Information Available: The complete list of authors of ref. 11 and 22. Schematic description of the synthesis of the compounds under study together with details of the paper under consideration, reporting synthesis and tensidic characterization of P16-n Gemini surfactants. Examples of optimized conformations of the compounds under study. This material is available free of charge via the Internet at http://pubs.acs.org.
REFERENCES


