Nonviral gene-delivery by highly fluorinated gemini bispyridinium surfactant-based DNA nanoparticles

NONVIRAL GENE-DELIVERY BY HIGHLY FLUORINATED GEMINI BISPYRIDINIUM SURFACTANT-BASED DNA NANOPARTICLES

Emilia Fisicaro[*a], *Carlotta Compari*[a], *Franco Bacciottini*[a], *Laura Contardi*[a], *Erika Pongiluppi*[a], *Nadia Barbero*[b], *Guido Viscardi*[b], *Pierluigi Quagliotto*[b], *Gaetano Donofrio*[c], *Marie Pierre Krafft*[d]

[a] University of Parma, Department of Pharmacy, Parco Area delle Scienze, 27/A - 43124 Parma – Italy

[b] University of Torino, Department of Chemistry, Interdepartmental "Nanostructured Surfaces and Interfaces" NIS Centre, Via P. Giuria, 7 - 10125 Torino – Italy

[c] University of Parma, Department of Veterinary Sciences, Via del Taglio, 10 - 43126 Parma – Italy

[d] Institut Charles Sadron (CNRS), University of Strasbourg, 23 rue du Loess, 67034 Strasbourg Cedex, France

*Corresponding author e-mail: emilia.fisicaro@unipr.it

ABSTRACT

Fluorinated cationic lipids could be a sound option for efficient gene expression in biological fluids containing hydrogenated interfering surfactants, *e.g.* when genes have to be delivered to the respiratory or to the biliar epithelium. This is an essential requirement, for instance, in the treatment of cystic fibrosis and cystic fibrosis-associated diseases. Biological and thermodynamic properties of a new homologous series of highly fluorinated bispyridinium cationic *gemini* surfactants, differing for the length of the spacer bridging the pyridinium polar heads in 1,1' position, are here reported. Interestingly, it results that gene delivery ability is strictly connected with the spacer length, due to a structural change of the molecule in solution, allowed when the spacer is of the right size and suggested by the trends of apparent and partial molar enthalpies vs. molality. Results from agarose gel electrophoresis mobility shift assay (EMSA), MTT proliferation assay and Transient Transfection assays on RD-4 cells, derived from a human nasty cancer, for the biological assessment of the compounds, and from Atomic Force Microscopy for the morphological characterization of DNA nanoparticles, are discussed. Dilution enthalpies, measured at 298 K, allowed for the determination of apparent and partial molar enthalpies *vs.* molality. All the tested compounds (except FGP12), in different amount, can deliver the plasmid, when co-formulated with DOPE. The compound with spacer formed by eight carbon atoms gives rise to a gene delivery ability comparable to that of the commercial reagent. The compound with the longest spacer (FGP12) compacts DNA in loosely condensed structures by forming a sort of bows, not suitable for transfection. Therefore fluorination of gemini surfactants could produce new valuable compounds for biomedical applications. The trends of partial molar enthalpies *vs. m*. could provide the keystone for a deep understanding of their structure-activity relationships.

KEYWORDS: DNA nanoparticles; gene delivery; gemini surfactants; heterocyclic gemini cationic surfactants; synthetic vectors for gene delivery; atomic force microscopy; phase transition in solution; DNA-fluorinated surfactant interaction

INTRODUCTION

It is well known that perfluorinated compounds show peculiar properties, among which the most interesting for biomedical applications are the chemical and biological inertness, due to the strength of the C-F bond and their high hydrophobic and lipophobic characters.[1] These originate mainly from the structure of the fluorine atoms having a larger van der Waals radius and a lower polarizability than the hydrogen atoms.[1] As a consequence, the fluorocarbon chains show a rigid rod-like shape with a period of twist of 13 carbon atoms, while the hydrocarbon chains adopt the well known zig-zag structure. Fluorocarbon surfactants are much more surface active and stable against acidic, alkaline and oxidoreductive agents and elevated temperatures than their hydrogenated counterparts. These unique properties account for their practical relevance.[2-6] Several new fluorinated surfactants have been recently synthesized and studied in particular as drug carriers and as gene delivery systems with very promising results.[7-12] The introduction of highly fluorinated alkyl chains which are both hydrophobic and lipophobic, may also protect the lipoplexes from unwanted interactions with the biological medium. Fluorinated cationic lipids have been, in fact, proposed to obtain efficient gene expression in those biological fluids containing surfactants as pulmonary surfactants or bile salts, when genes have to be delivered to the respiratory or to the biliar epithelium.[7-10] This is an essential requirement, for instance, in the treatment of cystic fibrosis and cystic fibrosis-associated diseases.[8] At the same time also partially fluorinated surfactants (otherwise called "hybrid surfactants") appear to be interesting for biomedical applications, because in general, they show low to moderate acute toxicity and low haemolytic activity compared to their hydrogenated analogues.[12-13] Reverse water-in-fluorocarbon emulsions stabilized by a semifluorinated amphiphile have been proposed as drug delivery system for intrapulmonary administration of many drugs (insulin, pain killers, antibiotics, vaccines, etc.).[11]

In recent years, the interest in cationic *gemini* surfactants - i.e. surfactants in which at least two identical moieties are bound together by a spacer - has increased, especially in the pharmaceutical field.[14-15] It is well stated that *gemini* surfactants show very useful chemico-physical properties, in particular lower critical micelle concentration (CMC) and greater surface activity than the monomeric ones.[14-16] Owing to these properties, enhanced by fluorination[17], they have been proposed as components of new drug delivery systems and as non-viral vectors for gene delivery. They are in fact able to compact and encapsulate the DNA into soft nanoparticles of tunable size, able to protect the DNA from enzymatic degradation and to prevent rapid leakage into blood capillary but small enough for escaping macrophages of the reticuloendothelial system.[18-26]

In this field, we have obtained encouraging and at the same time surprising results from the study of a new class of hydrogenated *gemini* compounds having two pyridinium groups as polar heads. The two pyridinium moieties, each of which bears one hydrophobic chain, are bridged together by aliphatic chains differing for the number of carbon atoms.[27-31] We have shown that the measurement of their solution enthalpies constitutes the keystone for understanding their transfection activity. The compound with spacer formed by four carbon atoms shows unexpected enthalpic properties *vs*. concentration, which was explained by 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, mediated by the counterion. This compound, the only one giving rise to a transfection activity comparable to that of the commercial reagent, when formulated with 1,2-dioleyl-sn-glycero-3-phosphoethanolamine (DOPE), behaves like molecular tongs able to grip basic group near each other. The compound with spacer constituted by twelve methylene groups, 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.[30]

With the aim of bringing together the properties of gemini pyridinium surfactants with those of fluorinated ones, we have synthesized a class of highly fluorinated gemini pyridinium surfactants, whose synthesis and surface activity characterization are reported in ref. 32. In the present paper, the thermodynamic and biological properties of the aqueous solutions of this new homologous series of partially fluorinated *gemini* surfactants are reported for the first time, in comparison with those of the protiated counterparts.

EXPERIMENTALS

Compounds

The synthesis of the compounds under study (FGP*n*, where *n* = 3, 4, 8, 12 indicates the number of carbon atoms in the spacer), prepared by us, is reported in detail in ref. 32. In the following the IUPAC names of the compounds studied and in brackets the names in short by which they will be referred to in the paper are shown: 1,1'-bis(3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctyl)-2,2′-trimethylenebispyridinium dichloride (FGP3); 1,1'- bis(3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctyl)-2,2′-tetramethylenebispyridinium dichloride (FGP4); 1,1'- bis(3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctyl)-2,2′-octamethylenebispyridinium dichloride (FGP8); 1,1'-bis(3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctyl)-2,2′-dodecamethylenebispyridinium dichloride

(FGP12). The compounds with chlorides as counterions were obtained by ion exchange, after having synthesized the corresponding ditrifluoromethanesulfonate.[32] Purity was checked by NMR, elemental analysis and TLC: eluent BAW (only the organic phase separated from butanol:acetic acid:water = 4:1:5 biphasic system phase) on silica gel plate. Their general structure is shown in Figure 1.

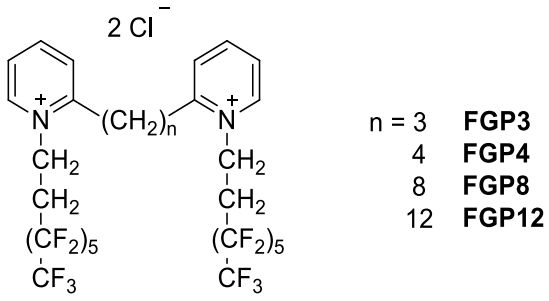

FIGURE 1. Compounds under study (n=3, 4, 8, 12), *n* is the number of carbon atoms in the spacer.

Dilution enthalpies measurements

The enthalpies of dilution were measured at 298 K by means of the Thermometric TAM (flow mixing cell) microcalorimeter, equipped with a 221 Nano Amplifier (Thermometric). The freshly prepared surfactant solutions, kept before injection at the experimental temperature by means of a Heto cryothermostatic bath, were diluted into the "mixing" measuring cell of the microcalorimeter in a ratio 1:1 by using CO_2-free water. A Gilson peristaltic pump, Minipuls 2 was used for injecting solutions and water. Their flows were determined by weight. For the dilution enthalpies measurements, the solutions were prepared by weight using freshly boiled bi-distilled water, stored under nitrogen. Solution concentrations were expressed as molality, *m* (mol kg^{-1}).

DNA preparation and storage

Plasmid DNA was purified through caesium chloride gradient centrifugation. A stock solution of the plasmid 0.7 μM 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 precipitated in alcohol. Linearized plasmid DNA pellet was washed with 70% of ethanol, air dried and dissolved in distilled water 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, is easy to handle and fast growing. The cells were maintained as a monolayer using growth medium containing 90% DMEM (Dulbecco's Modified Eagle Medium) , 10% FBS (Fetal Bovine Serum), 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% CO_2.

Electrophoresis Mobility Shift Assay (EMSA)

The binding reactions were performed in a final volume of 14 μl, obtained by adding to 10 μl of 20 mM Tris/HCl buffer at pH 8, 1 μl of plasmid (1 μg of pEGFP-C1) and 3 μl of FGP*n* with *n* = 3, 4, 8, 12 at different final concentrations, ranging from 6.5 to 200 μM. Binding reaction was allowed to take place at room temperature for 1 hour, 5 μl of 1g/ml in H_2O 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.

MTT proliferation assay

RD-4 cells were plated on 96-well plates (3000 cells per well) and different concentrations (2.5, 5, 10, 20, and 40 μM) of FGP*n* with *n* =3, 4, 8, 12 were added. The same experiments were carried out also by adding DOPE at a surfactant:DOPE ratio of 1:2. At 48 hours post treatment, the relative number of metabolically active cells was assessed by reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT)**.** Briefly, after 48 hours of treatments, 20 μl of MTT (5 mg/ml) were added to the culture for 4 h. Then, after addition of 100 μl of solubilization solution (10% SDS in HCl 0.01 M) cells were incubated at 37 °C overnight. Specific optical density was measured at 540 nm, using 690 nm as reference wavelength in microreader SLT-Lab (Salzburg, Austria). For the proliferation studies, each experiment was repeated three times and each treatment was performed with eight replicates. Statistical differences among treatments were calculated by Student's test and multi factorial ANOVA.

Transient transfection assay

Transfections were performed in 6 well plates, when cells were 80% confluent (approximately 3×10^5 cells) on the day of transfection. 3 μg of plasmid, FGP*n* 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 prepared by adding 1,2-dioleyl-sn-glycero-3-phosphoethanolamine (DOPE; SIGMA-Aldrich) to the plasmid-surfactant mixture at a surfactant:DOPE molar ratio of 1:2 where the surfactant concentration was kept to 15 μM. GenePORTER transfection reagent, a neutral lipid transfection reagent was used as positive transfection control.[22, 30]

Mixture and cells were incubated at 37 °C in a humidified atmosphere of 95% air/5% CO_2 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.

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.

Sample preparation and AFM imaging

DNA samples were prepared as described in ref. 30. The plasmid DNA was diluted 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 FGP*n* with *n* =3, 4, 8, 12 at the same N/P ratio as transfection. The N/P ratio is the ratio between the negative charges of the phosphate groups of DNA and the positive ones carried by the gemini pyridinium surfactants.

DOPE was dissolved in ethanol before addition. 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.

RESULTS

The thermodynamics of the solutions of highly fluorinated surfactants under investigation is given in terms of apparent and partial molar quantities of the solute, assuming the infinite dilution as reference state. These quantities are derived from the experimental data using methods stated in detail elsewhere. [28, 33-36]

For the sake of clarity, we recall that, with reference to the state of infinite dilution, the molar enthalpy of dilution, ΔH_d, is given by the difference between the apparent relative molar enthalpy in the final (after dilution) state, $L_{\Phi,f}$ and that in the initial (before dilution) state, $L_{\Phi,i}$:

$$\Delta H_d = L_{\Phi,f} - L_{\Phi,i} \qquad (1)$$

For ionic surfactants in the premicellar region, the apparent relative molar enthalpy can be expressed by means of a polynomial of $m^{1/2}$, in which the coefficients B_L and C_L are obtained interpolating by a least squares curve fitting the experimental points in the premicellar region. A_L is the limiting Debye-Hückel slope for relative enthalpies accounting for the long range electrostatic solute-solute interactions:

$$L_\Phi = A_L m^{1/2} + B_L m + C_L m^{3/2} \qquad (2)$$

In the micellar region, the apparent molar enthalpies are evaluated by means of eq. (1) and, when a value of L_Φ *vs*. m not experimentally measured is needed, by graphical interpolation. The trends of the apparent molar enthalpies for the compounds under investigation as a function of molality, m are shown in Figure 2.

The partial molar enthalpies L_2 are determined by drawing the best curve for the apparent molar enthalpies *vs.* m and then by calculating the partial molar quantities as $\Delta(mL_\Phi)/\Delta m$ from points interpolated at regular intervals. Results obtained for the fluorocarbon surfactants together with the same data for hydrocarbon analogs 12-Py(2)-*n*-(2)Py-12 Cl from ref. 29 are given in Fig. 3 for easy comparison

Dilution enthalpies, apparent and partial molar enthalpies *vs*. m for the compounds under

investigation, are available as supporting information.

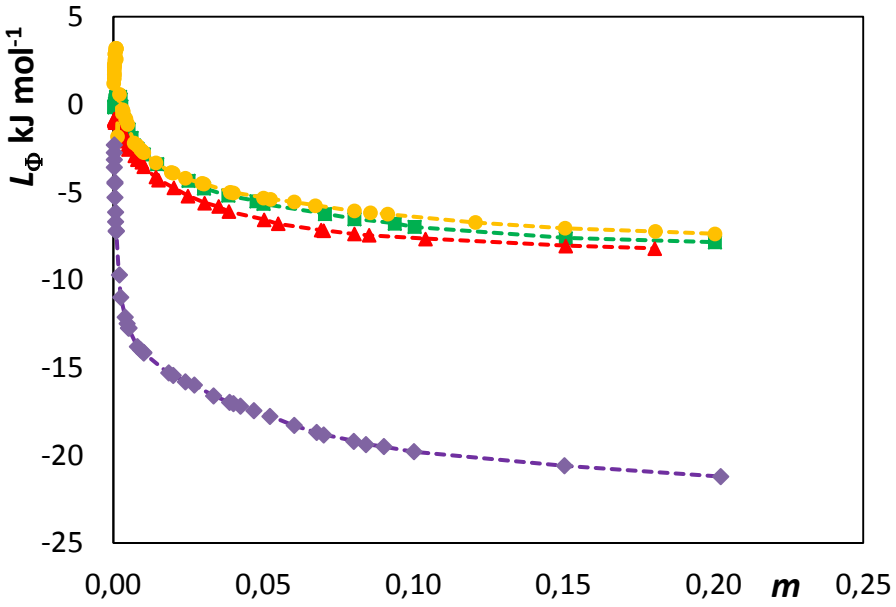

FIGURE 2. Apparent molar relative enthalpies of FGP3 (full squares); FGP4 (full triangles); FGP8 (full circles); FGP12 (full diamonds) as a function of surfactant molality, *m*.

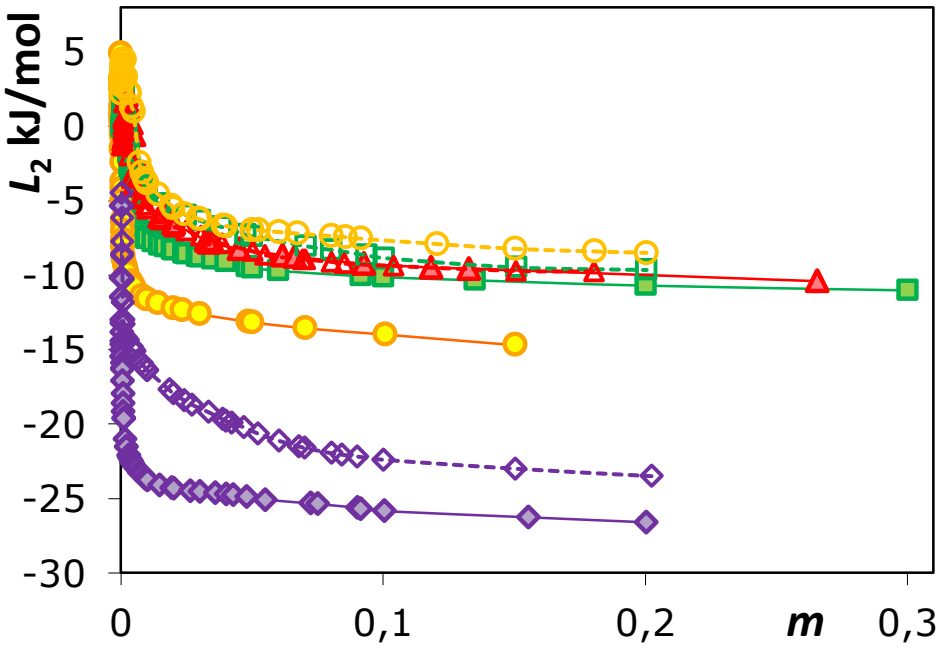

FIGURE 3. Comparison of partial molar relative enthalpies of hydrogenated gemini surfactants 12-Py(2)-*n*-(2)Py-12 Cl (full symbols, ref. 29) and fluorinated FGP*n* (empty symbols) with *n*=3 (squares), *n*=4 (triangles); *n*=8 (circles); *n*=12 (diamonds) as a function of surfactant molality, *m*.

Biological Assays

The interaction of FGP*n* with *n* =3, 4, 8, 12 with plasmid DNA pEGFP-C1 (Clontech) was monitored by agarose gel electrophoresis mobility shift assay (EMSA, Figure 4). All the compounds investigated are able to modify the mobility of DNA starting from a concentration of 100 μM, corresponding to a N/P ratio of 1/1.

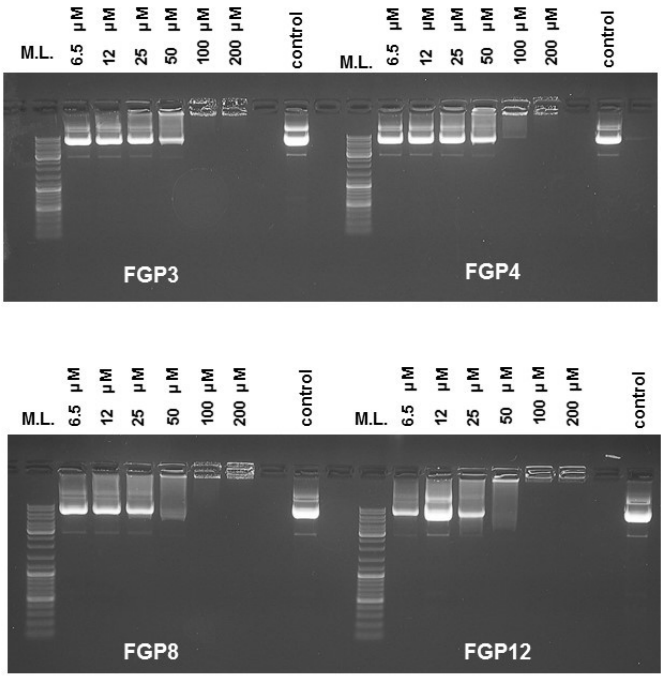

FIGURE 4. EMSA experiments showing complexation of FGP*n* (*n* = 3, 4, 8, 12) with circular plasmid pEGFP-C1. Shifting is observable as a function of concentration (μM), at N/P ratios 16/1 (6.5 μM); 8/1 (12 μM); 4/1 (25 μM); 2/1 (50 μM); 1/1 (100 μM); 1/2 (200 μM). As a negative control, only the plasmid was used.

The cytotoxicity of the molecules was also tested by an MTT proliferation assay to explore the possibility of exploiting their interaction with DNA to deliver DNA plasmid constructs inside the cells. Results are shown in Figure 5. In general, the cytotoxicity increases with the length of the spacer and, for spacers 3 and 4, also in presence of DOPE. Given that cytotoxicity has a strong impact in cell transfection, for each compound the concentration producing the best compromise between cell viability and transfection efficiency was used. At concentrations lower than those selected the cell viability was comparable or higher but the percentage of transfected cells was lower while at higher concentrations cytotoxicity was too high.

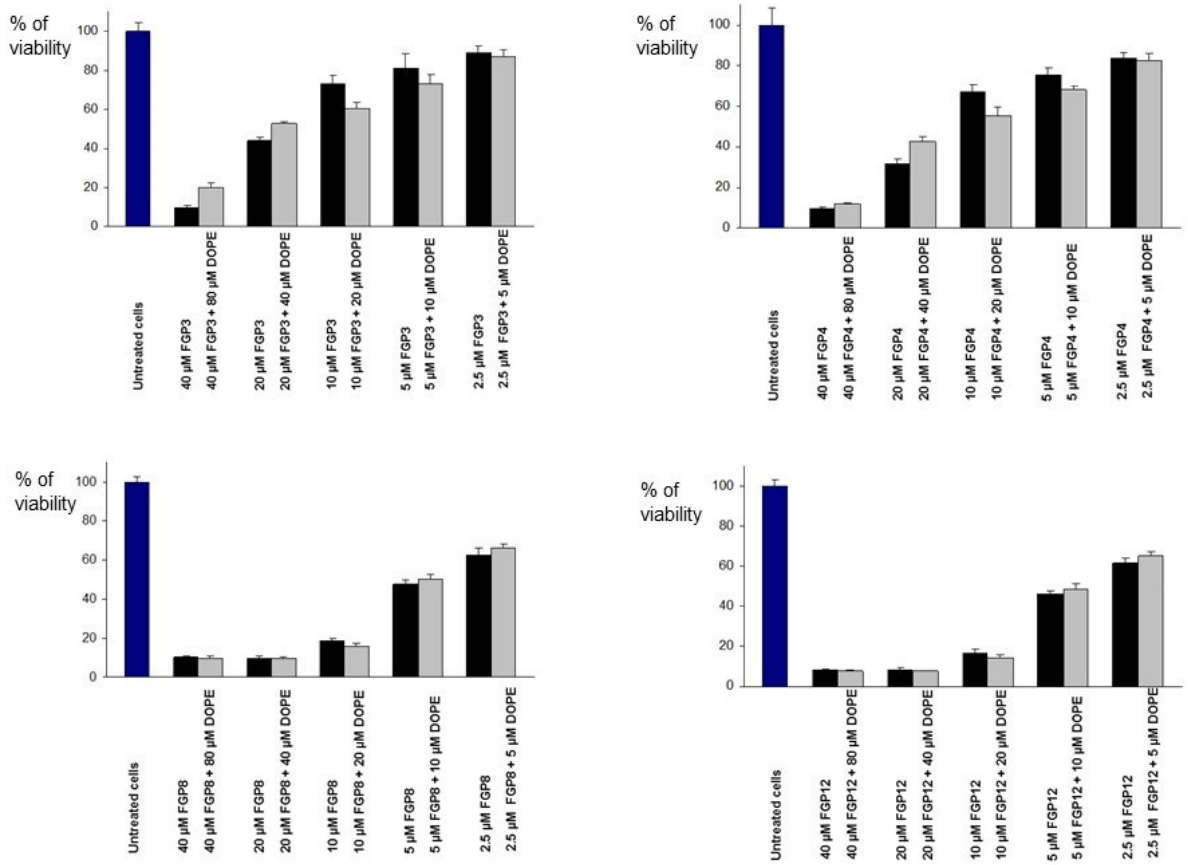

FIGURE 5. Effects of FGP*n* with *n* =3, 4, 8, and 12 on RD-4 cell line proliferation measured by MTT test after 48 h of incubation. Values are the mean ±S.D. of three independent experiments (n=8 per treatment, p<0.05).

The ability of the compounds under investigation to deliver DNA inside the cells was tested by a transient transfection assay. A plasmid carrying an EGFP expression cassette under the control of the CMV immediate early promoter (pEGFP-C1, Clontech) was used to monitor EGFP expression under a fluorescence microscope. When used without helper lipid none of the compounds tested was found able to deliver DNA inside RD-4 cells. We therefore tested *in-vitro* the transfection efficiency of FGPn with *n* = 3, 4, 8, 12 (15 μM), formulated with DOPE at FGPn:DOPE molar ratios of 1:2. The addition of DOPE increased the transfection efficiency of FGP3, FGP4 and FGP8, but it was ineffective with FGP12 (Figure 6).

In particular, FGP8 is the only one able to deliver DNA inside the cells, as shown by EGFP expression, at an efficiency comparable with that obtained with a standard commercial transfection reagent (GenePORTER, Gene Therapy System), used as a positive transfection control. FGP12 is unable to deliver DNA and, when formulated with DOPE, only 1% of the viable cells give rise to

EGFP expression. It has been shown that the absence of transfection is observed only for DOPE transfection control.[22]

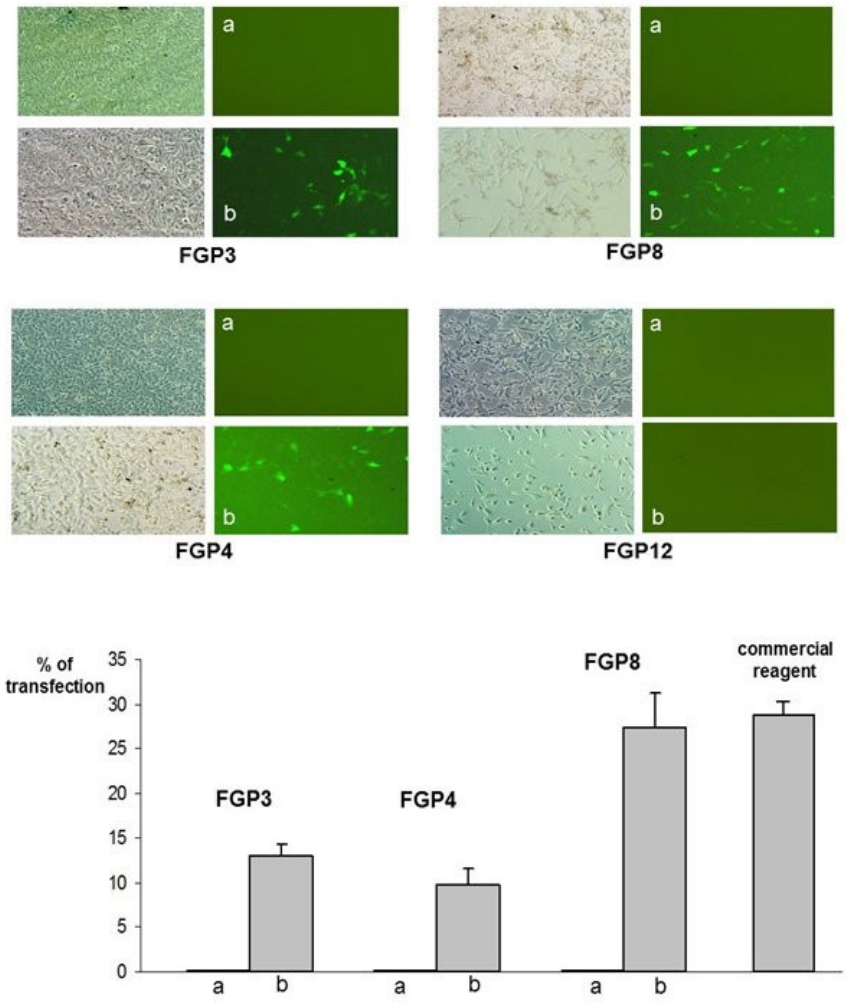

FIGURE 6. Transfection of RD-4 cells by FGP*n* (*n* = 3, 4, 8). For each surfactant, on the left, phase contrast and, on the right, fluorescence microscope observation of RD-4 transfected cells (as shown by green cells expressing EGFP) are shown. Pictures marked by a) refers to experiments done only with the surfactants and by b) with surfactant:DOPE ratio =1:2. Down below: efficiency of transfection obtained by FGP*n* (*n* = 3, 4, 8) and positive control by a commercial reagent. 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. FGP12 is not reported because it is unable to deliver DNA also when formulated with DOPE. Cells are not transfected with DOPE alone (see refs. 22 and 30).

AFM Experiments

The morphology of the structural changes induced in DNA by the compounds under investigation was studied by AFM,[37-40] a technique successfully used to study the interaction of synthetic

ligands[41], proteins[42] and cationic surfactants[30] with DNA. AFM experiments were carried out in air in the tapping mode using circular DNA.

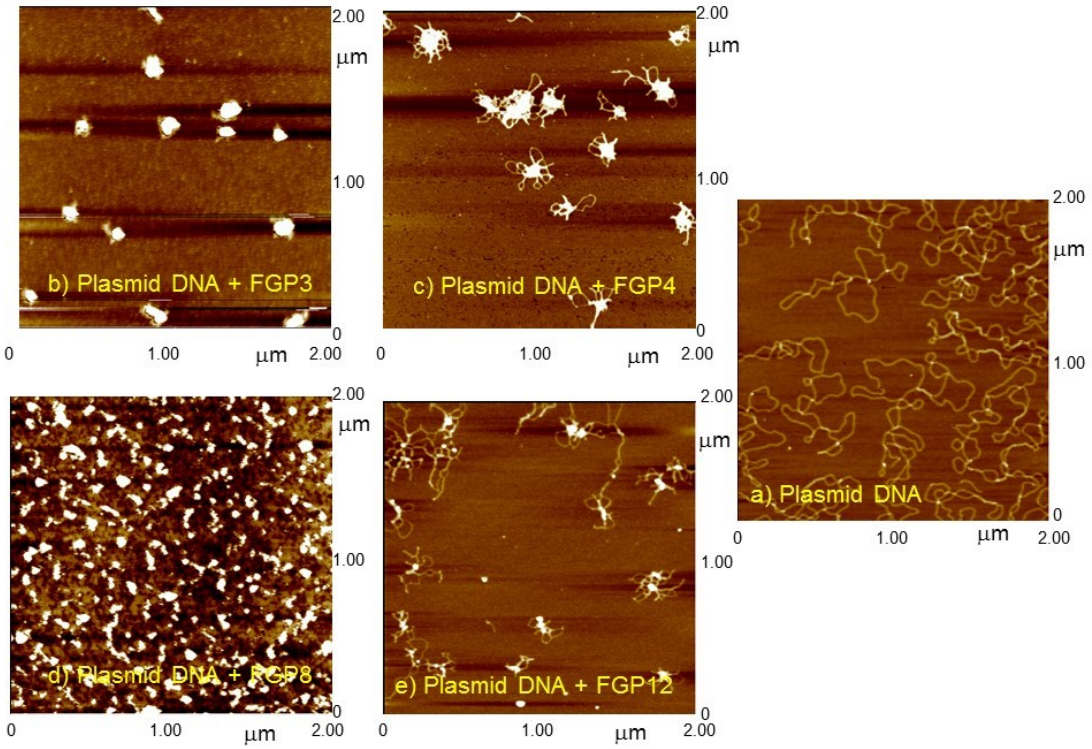

FIGURE 7. AFM images showing the effect induced on DNA plasmid by incubation with FGP*n* (*n* = 3, 4, 8, 12). Each image represents a 2 x 2 µm scan. All images were obtained with supercoiled 0.5 nM pEGFP-C1 plasmid deposited onto mica and with the microscope operating in tapping mode in air at the same N/P ratio as transfection.

First of all, the plasmid DNA alone deposited onto freshly cleaved mica was imaged (Figure 7a). Single plasmids and concatamers are seen in their plectonemic form with several supercoils which cause the double helix to cross itself a few times. In spite of the topological constraint, plectonemes appear well extended all over the mica surface. The plasmid DNA after incubation with 15 µM FGP*n* with *n* = 3, 4, 8, 12, is shown in Figures 7b-e, respectively. Only FGP8 seems to be able to condense all DNA in small near spherical nanoparticles (Figure 7d). FGP3 (Figure 7b) gives rise to larger and less homogeneous DNA nanoparticles. Incubation with FGP4 results in a not complete condensation of DNA in distinct structures (Figure 7c). AFM images of DNA incubated with FGP12 show that DNA is only partially condensed and the different structures, looking like bows, are interconnected (Figure 7e).

DISCUSSION

Apparent and partial molar enthalpies

We have reported before the apparent and partial molar enthalpies at 298 K of the aqueous solutions of the homologous series of protiated cationic gemini surfactants 1,1'-didodecyl-2,2'-alkylenebispyridinium dimethanesulfonates[28] and chlorides[29], differing for the spacer length. We were surprised by their very peculiar behaviour as a function of the spacer length, not allowing for the determination of a $-CH_2-$ group contribution when this group is added to the spacer.[28-30] The interest in finding out a group contribution is related to the possibility of predicting thermodynamic properties in solution of new designed surfactants. The deviation of the properties from those theoretically predicted could be an indication that something new happens in solutions, may be a phase transition or a change in conformation of the molecule. We ascribed the surprising behaviour of protiated gemini pyridinium surfactants, independent on the counterion, to a different arrangement of the molecule inside the micelle and on the interphase surface, when the spacer is four methylenes long.[28-30] On the contrary, the group contribution additivity for the counterion is respected, independently on the spacer length, analogously to what happens in the case of monomeric surfactants[34]. Because of the great scientific and applicative relevance in gemini surfactants, it would be very interesting to clarify the effect of the methylene group when added to the spacer, i.e. to the polar head of the surfactant, at least when the spacer is short. In the case of the monomeric surfactants, the addition of a $-CH_2-$ to the hydrophobic chain causes a lowering of the partial molar enthalpy curves of about –1.5 kJ mol^{-1},[34] but, if it is added to the polar head, the effect is exactly the opposite, close to that obtained by removing one methylene group from the hydrophobic chain[36]. This is true for very short alkyl chains bound to the polar head, but, if the chain length increases, the hydrophobicity effect prevails[29]. With the aim of obtaining new information about this topic, we decided to study the solution thermodynamics of analogous compounds in which the hydrophobic tail has been modified by partial fluorination. To make a meaningful comparison, we have taken into account that the hydrophobicity of a $-CF_2-$ group is about 1.5 times that of a $-CH_2-$ group, as shown by the values of the respective cmc[34]. This is why we have synthesized the corresponding octyl compounds having the last six carbon atoms fluorinated. As shown in Figure 2 and 3, the curves of the apparent and partial molar enthalpies *vs. m*, considered one by one, respect what is generally found for ionic surfactants: after increasing in the premicellar region, they tend to level off at concentrations above the critical micelle concentration (cmc), where they are almost parallel. The lowering of the curves in the micellar region is attributed to the electrostatic interactions in micellar solutions, and is proportional, for the same counterion, to the

number of carbon atoms in the alkyl chain or to the global hydrophobicity of the whole molecule. The trends for the fluorinated compounds with chlorides as counterions, having the same hydrophobic tail, but different alkyl spacer lengths, still show a very peculiar behavior, but different from that found in the case of the hydrogenated analogues.[28, 29] In fact, the effect of the addition of a $–CH_2–$ to the spacer still does not give rise to a monotonic change in the values of the enthalpies in the micellar region, not allowing for the evaluation of a group contribution for the $–CH_2–$ group for the shortest spacers. However, the trends are very close for the spacers with 3 and 4 carbon atoms, but the curve of FGP4 is still lower than that of FGP3: this means that the addition of a methylene group to the shorter spacer brings about a little increase in the hydrophobicity of the whole molecule. The inversion of the expected trend is obtained with the eight methylene long spacer: in fact, FGP8 shows the highest value of apparent and partial molar enthalpies in the plateau region. As figure 3 clearly shows, this behavior, indicative, in our opinion, of an important change in configuration of the molecule in solution, is found for the compounds with spacer four carbon atom long in the case of hydrogenated analogs and for FGP8 for the fluorinated ones. It is worth noting that the greatest gene delivery ability is just shown by the hydrogenated compound with n=4 and by the fluorinated one with n=8 (Figure 8), confirming the utmost importance of solution thermodynamic data for a deeper insight also of the biological data. In the case of the hydrogenated analogues, we supposed that, when the spacer reaches the right size, the molecule doubles up, like a book, due to stacking interactions between the two pyridinium rings, mediated by the counterion. This arrangement is possible neither when the spacer is too short, because of the lack of conformational freedom, nor when the spacer is too long, because the pyridinium rings are too far apart.[28-30]

The different behaviour of the fluorinated compounds is obviously ascribed to the presence of the fluorine atoms in the alkyl chains, due both to their greater volume (the volumes of CF_2 and CF_3 groups are ~38 $Å^3$ and ~ 92 $Å^3$; those of CH_2 and CH_3 groups are 27 $Å^3$ and 54 $Å^3$, respectively)[1] and stiffness and to the non-ideality of mixing between fluorocarbons and hydrocarbons. The FGP12 spacer is too long for bending towards the hydrophobic chain and, at the same time, avoiding the contact with the fluorinated moieties: the minimum of the configurational energy in solution is obtained when the pyridinium rings are kept away from each other. Biological data afterwards reported agree with this picture.

In order to obtain the enthalpy change upon micellization, ΔH_{mic}, we have applied a pseudo-phase transition model, in which the aggregation process is regarded as a phase transition, taking place at equilibrium. Therefore the micellization parameters are obtained by extrapolating at the cmc the

trends of the partial molar properties before and after cmc[28, 32-35]. In Table 1 a comparison of the thermodynamic parameters for micelle formation between fluorinated and hydrogenated gemini surfactants is reported.

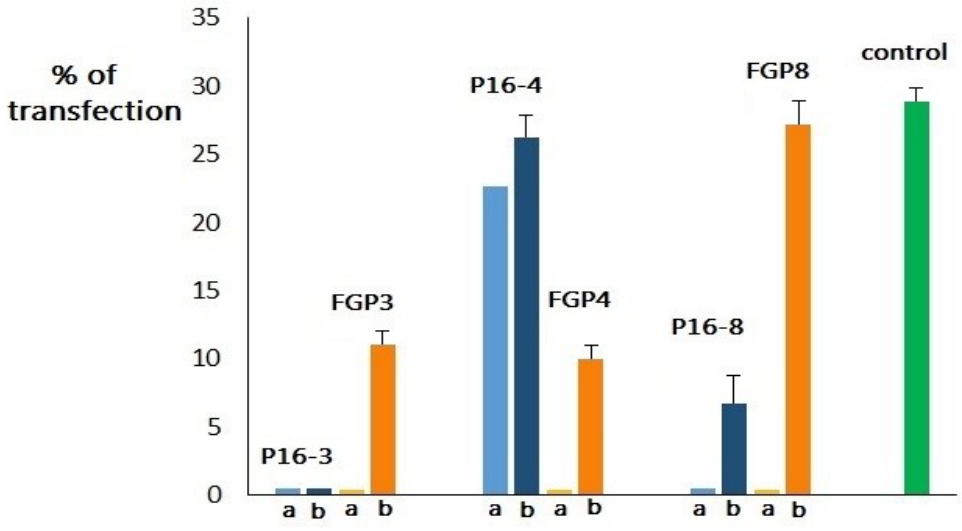

FIGURE 8. Comparison of the gene delivery ability between between partially fluorinated gemini surfactants (orange) here studied and P16-n hydrogenated gemini surfactants (blue) from ref. 30: a) experiments done only with the surfactants (light colours) and by b) with surfactant:DOPE ratio =1:2 (dark colours). In green, positive control by a commercial reagent.

TABLE 1[a]

n	Fluorinated Compound	cmc [b] [mmol/L]	β [b]	ΔH_{mic} [kJ/mol]	Hydrogenated Compound [c]	cmc [c] [mmol/L]	β [c]	ΔH_{mic} [kJ/mol] [c]
3	FGP3	0.28	63	-10.1	12-Py(2)-3-(2)Py-12 Cl	1.0	72	-11.2
4	FGP4	0.50	62	-10.5	12-Py(2)-4-(2)Py-12 Cl	1.15	40	-10.4
8	FGP8	0.25	52	-8.9	12-Py(2)-8-(2)Py-12 Cl	0.29	57	-16.8
12	FGP12	0.12	54	-23.0	12-Py(2)-12-(2)Py-12 Cl	0.11	57	-25.0

[a] *n*, number of carbon atoms in the spacer; cmc, from surface tension; *β*, degree of counterion association, determined from conductivity measurements; ΔH_{mic}, change in enthalpy upon micellization for the surfactants under investigation.

[b] Ref. 32

[c] Ref. 31

The micellization enthalpies, according to the data presented in figure 3, confirm the above considerations. They are all negative, as generally found for ionic gemini surfactants, and the lower absolute value belongs to FGP8. The values for FGP3, FGP4 are very near as for the corresponding hydrogenated compounds, without possibility to extract a group contribution for the $–CH_2–$ in the spacer. The absolute value increases sharply for the twelve carbon atoms spacer in both classes of compounds, suggesting that, when the spacer becomes long enough, it behaves as a further hydrophobic chain. Also the values of the cmc, obtained by surface tension measurements, show a non-monotonic decrease with the number of carbon atoms in the spacer (see Table 1) with a maximum for $n = 4$.

Biological properties

We have already studied the gene delivery ability of hydrogenated pyridinium *gemini* surfactants and we were able to show for the first time that their transfection activity is strictly related to their unexpected thermodynamic properties in solution.[30] These are explained by a conformation change dependent on the length of the spacer. In fact, the compound with a four carbon atoms spacer, having a surprising behaviour as far as the curves of apparent and partial molar enthalpies are concerned, is the only one giving rise to a transfection activity comparable to that of the commercial reagent, when formulated with DOPE. We suggest that this compound behaves like molecular tongs able to grip basic group near each other.[30] The compound with a 12 methylenes long spacer, on the contrary, 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. We have also shown that this behaviour is independent on the counterion.[29]

The interest for biological applications of fluorinated and partially fluorinated surfactants together with the above mentioned encouraging results urged us to study the gene delivery ability of the partially fluorinated analogues. Experimental results show that all the compounds under study are able to compact DNA starting from a N/P=1/1 ratio, as shown from EMSA results in Figure 4 and thus they could be employed to obtain DNA nanoparticles. Moreover, we expect that the interaction with DNA is made easier by the aromatic nature of the polar heads, able to give rise to stacking interaction with DNA bases by intercalation. In fact, cationic lipids[22, 30] or cationic polymers[43] are used to neutralise the anionic charges of the DNA phosphate groups. In this way complexes of approximately spherical shape, having a small dimension when compared to the naked DNA and therefore potentially able to penetrate inside the cells, are obtained. The interaction with DNA

seems not to be hindered by the presence of hydrophobic and lipophobic fluorinated moiety, because the positive charges are delocalized on the two pyridinium rings, bound together by an hydrogenated spacer. The fluorinated moieties are bound to the positive nitrogen through two -CH_2-CH_2- groups (see Figure 1), so that the positive charges interacting with the phosphate groups of DNA are in a hydrogenated environment. Results of the gene delivery experiments on RD-4 cells are reported in Figure 6 and in Figure 8 in comparison with the hydrogenated analogues. The RD-4 cells were chosen among a large panel of several cell lines because they constitute 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 the results obtained in the present study could be relevant for oncology gene transfer studies because they derive from a human nasty cancer. Figure 6 clearly shows that DNA nanoparticles obtained by using the compounds under study alone, whose structure is shown in figure 7, are unable to deliver gene inside the cells. The situation changes if transfection experiments are carried out in presence of the helper lipid dioleoylphosphatidylethanolamine (DOPE). DOPE enhances the transfection activity of cationic formulations through the stabilisation of DNA/lipid complex[44, 45] and facilitates the transfer of DNA in the context of endosomal escape, owing to its fusogenic property and significantly affecting the polymorphic features of lipoplexes[46]. In presence of DOPE, with the exception of FGP12, all the compounds are able to deliver genes inside the cells, with an efficiency increasing in the order FGP4< FGP3<< FGP8, the last one giving rise to a transfection efficiency comparable to that of the commercial reference agent. This behaviour could suggest that the fusogenic activity of DOPE helps to overcome the repulsion between the fluorinated DNA nanoparticles and the hydrogenated phospholipids constituting the cell membrane. The non-monotonic dependence on the spacer length is explained by two experimental facts: a) the trends of apparent and partial molar enthalpies vs. m; b) the AFM images. The AFM images, obtained in the same condition as transfection (Figure 7) suggest that FGP8 (Figure 7d) is able to give rise to small, compact, and quite uniform DNA nanoparticles. DNA condensation by FGP3 gives rise to near spherical particles, greater than those obtained by FGP8 but more compact than those made by FGP4. Nanoparticles obtained with FGP3 are able (Figure 8) to transfect about 12% of RD-4 cells when coformulated with the helper lipid. In the same figure, it is interesting to note that the hydrogenated analogue P16-3 do not give rise to transfection neither without nor with DOPE. This finding is explained by the AFM results compared in figure 9 a and b. The fluorinated surfactant results more efficient in compacting DNA with respect to the hydrogenated one. This observation is general: fluorinated gemini surfactants give rise to smaller and more compact DNA nanoparticles, notwithstanding the quite short length of the hydrophobic partially fluorinated chains.

In fact, it is reported that the biological activity of cationic surfactants increases with the chain length up to a critical point.[47] For instance, in the case of the homologous series of alkanediyl-α,ω-bis(dimethylalkylammonium bromide), the term with two alkyl chains of 16 carbon atoms is generally the most biologically active.[48] This is way we have chosen to carry out the gene delivery experiments with the hydrogenated analogues having the hydrophobic chains 16 carbon atoms long. On the contrary, we have chosen to start our studies on partially fluorinated compounds from compounds with shorter chains (corresponding approximately to the dodecyl hydrogenated compounds) showing a value of cmc not too much low to be able to measure thermodynamic properties also in premicellar region. The efficiency in gene delivery could be probably improved by increasing the hydrophobic tails length of the partially fluorinated compounds.

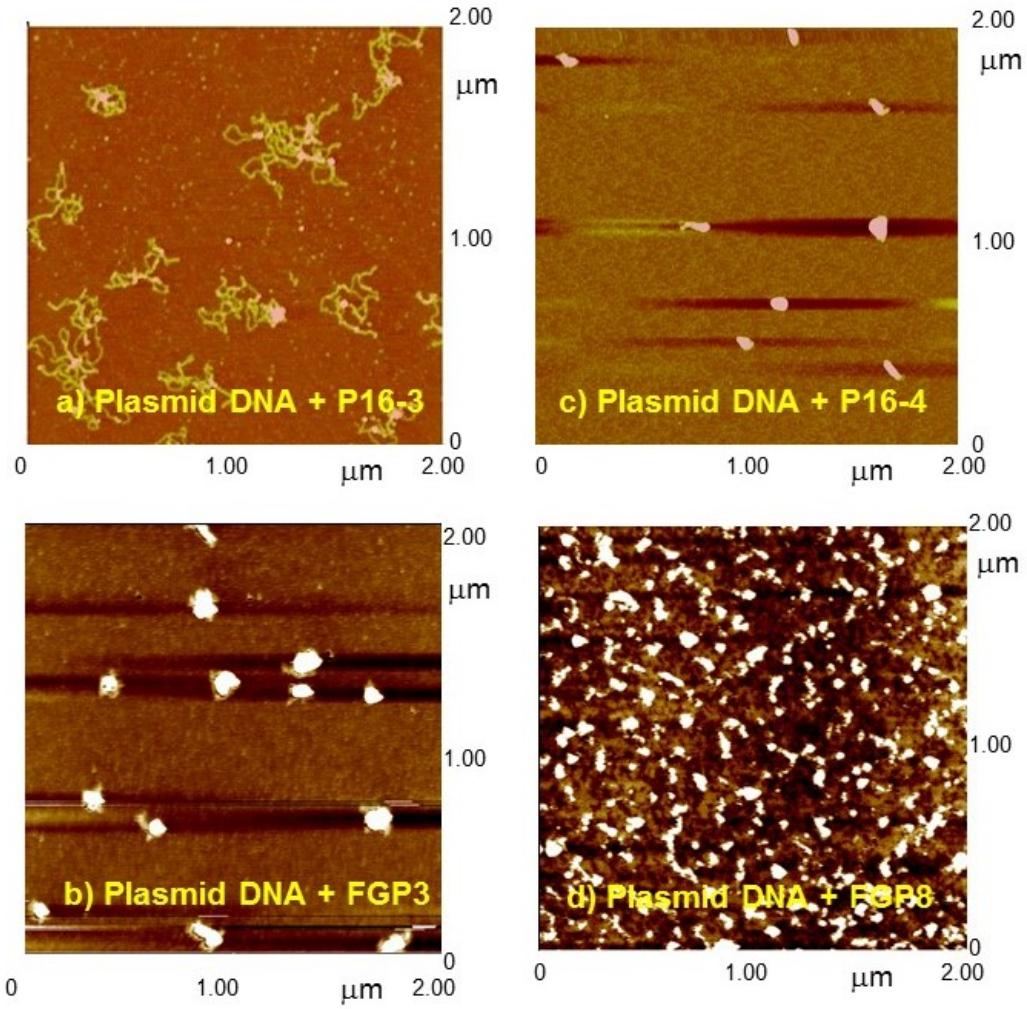

FIGURE 9. Comparison of AFM images of the objects obtained by the interaction of plasmid DNA with a) hydrogenated gemini P16-3 from ref. 30; b) fluorinated FGP3; c) hydrogenated gemini P16-4 from ref. 30; d) fluorinated FGP4.

In figure 9 c and d, are also compared the DNA nanoparticles formed by P16-4 and FGP8, this is to say the most active in gene delivery of the two classes of compounds: both are able to completely compact DNA, but the objects obtained by FGP8 are smaller and more uniform.

We have suggested from the results of the hydrogenated analogues that the compound with spacer formed by four carbon atoms is able to behave like molecular tongs able to grip basic group near each other, thus allowing the formation of compact and near spherical DNA particles.[30]

If the hydrophobic chain is modified by partial fluorination, a greater length of the spacer is needed for the molecular tongs formation, both for the greater steric hindrance and rigidity of the fluorinated moiety, and because the hydrogenated spacer cannot fold towards the lipophobic fluorinated tails, in perfect agreement with the trends of the curves of the apparent and partial molar enthalpies *vs*. *m*, shown in Figures 2 and 3.

If the molecule is folded up so that the pyridinium rings bringing the positive charge can look at each other, they can bind the neighbouring negatively charged groups of the DNA. Then, the interactions between the tails 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 interact with DNA sites far from each other so giving rise to bows not suited for gene delivery, as shown in Figure 7e. In FGP3, the charged nitrogens are still enough near to give rise to nanoparticles, but greater than with FGP8 and less efficient in transfection. FGP4, unlike of the corresponding hydrogenated, has still a spacer too short for an efficient conformational change, due to the presence of fluorinated moieties, and it gives rise to not enough compact structures.

CONCLUSIONS

The study of thermodynamic and biological properties of the homologous series of partially fluorinated gemini pyridinium surfactant here presented confirms that pyridinium *gemini* surfactants could be a valuable tool for gene delivery purposes, and that their performance is strictly related to their structure in solution, dependent on the spacer length, as for their hydrogenated analogues. Their transfection activity and the structure of the DNA nanoparticles, as studied by AFM, result strictly related to their thermodynamic properties in solution. All the compounds are unable to transfect RD-4 cells, if used alone for compacting DNA, but they are all able (except FGP12) to deliver a plasmid carrying an EGFP expression cassette, when coformulated with DOPE. The compound with spacer formed by eight carbon atoms (FGP8) gives rise to a gene delivery activity comparable to that of the commercial reagent, when formulated with DOPE, probably because it is

able to behave like molecular tongs able to grip basic groups near each other. This ability is due to a structure change in solution, suggested by the trends of apparent and partial molar enthalpies *vs. m*. For the hydrogenated analogues we have found the same behaviour when the spacer is 4 carbon atoms long. If the hydrophobic chain is modified by partial fluorination, a greater length of the spacer is needed for the folding of the molecule, both for the greater steric hindrance and rigidity of the fluorinated moiety, and because the hydrogenated spacer cannot fold towards the fluorinated lipophobic tails. The compound with the longest spacer (FGP12) 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, as for hydrogenated gemini surfactants. The comparison with the hydrogenated analogues of FGP3, FGP4 and FGP8, reveals a greater ability of the partially fluorinated compounds to compact DNA.

ACKNOWLEDGEMENTS

This work was supported by the Italian Ministry of Education, University and Research (MIUR), PRIN2011 "NANO Molecular tEchnologies for Drug delivery – NANOMED" and by the University of Torino (Ricerca Locale ex-60% Bando 2014). The authors thank the Interdepartmental Centre of Measurements (CIM) of the University of Parma for allowing the use of the AFM facilities.

Supporting Information Available: Dilution enthalpies, apparent and partial molar enthalpies *vs. m* for the compounds under investigation.

REFERENCES

1. Krafft, M. P.; Riess, J. G. Selected Physicochemical Aspects of Poly- and Perfluoroalkylated Substances Relevant to Performance, Environment and Sustainability-Part One. *Chemosphere* 2015, *129*, 4-19.
2. Krafft, M. P.; Riess, J. G. Perfluorocarbons: Life Science and Biomedical Uses. *J. Polym. Sci. Pol. Chem.* 2007, *45*, 1185-1198.
3. Menaa, F.; Menaa, B.; Sharts, O. N. Importance of fluorine and fluorocarbons in medicinal chemistry and oncology. *Journal of Molecular Pharmaceutics & Organic Process Research* 2013, *1(2),* 1000104/1-1000104/6, 6.
4. Riess, J. G. Fluorous Materials for Biomedical Uses. *Handbook of Fluorous Chemistry* Edited by Gladysz, J. A.; Curran, D. P.; Horvath, I. T. 2004, 521-573.
5. Kissa, E. *Fluorinated Surfactants*; Marcel Dekker, Inc.: New York, 1994.
6. Riess, J. G. Higly Fluorinated Amphiphilic Molecules and Self-Assemblies with Biomedical Potential. *Curr. Opin. Colloid In*. 2009, *14*, 294-304.
7. Raczka, E.; Kukowska-Latallo, J. F.; Rymaszewski, M.; Chen, C.; Baker, J. R. Jr. The effect of synthetic surfactant Exosurf on gene transfer in mouse lung in vivo. *Gene Therapy* 1998, *5*, 1333-1339.
8. Boulanger, C.; Di Giorgio, C.; Gaucheron, J.; Vierling, P. Transfection with fluorinated lipoplexes based on new fluorinated cationic lipids and in the presence of a bile salt surfactant. *Bioconjugate Chemistry* 2004, *15*, 901-908.
9. Gaucheron, J.; Santaella, C.; Vierling, P. Improved in Vitro Gene Transfer Mediated by Fluorinated Lipoplexes in the Presence of a Bile Salt Surfactants. *J. Gen. Med.* **2001**, *3*, 338-344.
10. Gaucheron, J.; Santaella, C.; Vierling, P. Highly Fluorinated Lipospermines for Gene Transfer: Synthesis and Evaluation of Their in Vitro Transfection Efficiency. *Bioconjugate Chemistry* **2001**, *12*, 114-128.
11. Courrier, H. M.; Pons, F.; Lessinger, J. M.; Frossard, N.; In Vivo Evaluation of a Reverse Water-in-fluorocarbon Emulsion Stabilized with a Semifluorinated Amphiphile as a Drug Delivery System through the Pulmonary Route. *International Journal of Pharmaceutics* **2004**, *282*, 131-140.
12. Li, X.; Turanek, J.; Knoetigova, P.; Kudlackova, H.; Masek, J.; Pennington, D. B.; Rankin, S. E.; Knutson, B. L.; Lehmler, H. J. Synthesis and Biocompatibility Evaluation of Fluorinated, Single-tailed Glucopyranoside Surfactants. *New Journal of Chemistry* **2008**, *32*, 2169-2179. Erratum in *New Journal of Chemistry* **2009**, *33*, 2491-2493.

13. Abe, M. Synthesis and applications of surfactants containing fluorine. *Current Opinion in Colloid & Interface Science* **1999**, *4*, 354-356.
14. Zana, R.; Xia, J. Gemini Surfactants, Interfacial and Solution Phase Behavior and Applications, Marcel Dekker, Inc., New York, **2004.**
15. Menger, F. M.; Keiper, J. S., Gemini Surfactants. *Angew. Chem. Int. Ed*. **2000**, *39*, 1906-1920.
16. Sharma, V. D.; Ilies, M. A. Heterocyclic Cationic Gemini Surfactants: A Comparative Overview of Their Synthesis, Self-assembling, Physicochemical, and Biological Properties. *Medicinal Research Reviews* **2014**, *34*, 1-44.
17. Yoshimura, T.; Ohno, A.; Esumi, K.; Equilibrium and Dynamic Surface Tension Properties of Partially Fluorinated Quaternary Ammonium Salt Gemini Surfactants. *Langmuir* **2006**, *22*, 4643–4648.
18. Kirby, A. J.; Camilleri, P.; Engberts, J. B. F. N.; Feiters, M. C.; Nolte, R. J. M. ; Söderman, O.; Bergsma, M.; Bell, P.C.; Fielden, M. L.; Garcia Rodriguez, C. L.; Guédat, P.; Kremer, A.; McGregor, C.; Perrin, C.; Ronsin, G.; van Eijk, M. C. P. Gemini surfactants: New synthetic vectors for gene transfection. *Angew. Chem. Int. Ed.* **2003**, *43*, 1448-1457.
19. Ahmed, T.; Kamel, A. O.; Wettig, S. D. Interactions Between DNA and Gemini Surfactant: Impact on Gene Therapy: Part I *Nanomedicine* **2016**, *11(3)*, 289-306.
20. Badea, I.; Shaterian, N.; Foldvari, M. Topical gene delivery in mice using gemini surfactant – lipid nanoparticles with and without tape electrode electroporation. *Drug Delivery Lett.* **2011**, *1*, 62-66.
21. Yang, P.; Singh, J.; Wettig, S.; Foldvari, M.; Verrall, R. E.; Badea, I. Enhanced gene expression in epithelian cells transfected with amino acid-substituted gemini nanoparticles. *Eur. J. Pharm. Biopharm.* **2010**, *75*, 311–320.
22. Fisicaro, E.; Compari, C.; Duce, E.; Donofrio, G.; Różycka-Roszak, B.; Wozniak, E. Biologically active bisquaternary ammonium chlorides: physicochemical properties of long chain amphiphiles and their evaluation as non-viral vectors for gene delivery. *Biochim. Biophys. Acta, Gen. Subj.* **2005**, *1722*, 224-233.
23. Sing, J.; Michel, D.; Chitanda, J. M.; Verral, R.; Badea, I. Evaluation of Cellular Uptake and Intracellular Trafficking as Determining Factors of Gene Expression for Amino Acid-Substituted Gemini Surfactant-Based DNA Nanoparticles. *J. Nanobiotech.* **2012**, *10*, 1-14.
24. Donkuru, M. D.; Badea, I.; Wettig, S.; Verrall, R. E.; Elsabahy, M.; Foldvari, M. Advancing nonviral gene delivery: lipid- and surfactant- based nanoparticle design strategies. *Nanomedicine* **2010**, *5*, 1103-1127.

25. Elsabahy, M.; Badea, I.; Verrall, R.; Donkuru, M.D.; Foldvari, M. Dicationic Gemini Nanoparticle Design for Gene Therapy. *Organic Nanomaterials* Edited by Torres, T.; Bottari, G. **2013**, 509-528,, 1 plate.
26. Sharma, V. D.; Lees, J.; Hoffman, N. E.; Brailoiu, E.; Madesh, M.; Wunder, S. L., Ilies, M. A. Modulation of pyridinium cationic lipid-DNA complex properties by pyridinium gemini surfactants and its impact on lipoplex transfection properties. *Mol. Pharmaceutics* **2014**, *11*, 545-559.
27. Quagliotto, P.; Viscardi, G. Barolo, C.; Barni, E.; Bellinvia, S.; Fisicaro, E.; Compari, C. Gemini Pyridinium Surfactants: Synthesis and Conductimetric Study of a Novel Class of Amphiphiles. *Journal of Organic Chemistry* **2003**, *68*, 7651-7660.
28. Fisicaro, E.; Compari, C.; Biemmi, M.; Duce, E.; Peroni, M.; Barbero, N. Viscardi, G.; Quagliotto, P. L. The Unusual Behaviour of the Aqueous Solutions of Gemini Bispyridinium Surfactants: Apparent and Partial Molar Enthalpies of the Dimethanesulfonates. *J. Phys. Chem. B* **2008**, *112*, 12312-12317.
29. Fisicaro, E.; Compari, C.; Bacciottini, F.; Barbero, N.; Viscardi, G.; Quagliotto, P. L. Is the Counterion Responsible for the Unusual Thermodynamic Behavior of the Aqueous Solutions of Gemini Bispyridinium Surfactants? *Colloids and Surfaces, A: Physicochemical and Engineering Aspects* **2014**, *443*, 249-254.
30. Fisicaro, E.; Compari, C.; Bacciottini, F.; Contardi, L.; Barbero, N.; Viscardi, G.; Quagliotto, P.; Donofrio, G.; Różycka-Roszak, B.; Misiak, P.; Woźniak, E.; Sansone, F. Nonviral Gene-Delivery: Gemini Bispyridinium Surfactant-Based DNA Nanoparticles. *J. Phys. Chem. B* **2014**, *118*, 13183-13191.
31. Barbero, N.; Magistris, C.; Quagliotto, P.; Bonandini, L.; Barolo, C.; Buscaino, R.; Compari, C.; Contardi, L.; Fisicaro, E.; Viscardi, G. Synthesis and Physico-Chemical Characterization of Long Alkyl Chain Gemini Pyridinium Surfactants for Gene Delivery. *ChemPlusChem* **2015**, *80*, 952-962.
32. Quagliotto, P.; Barolo, C.; Barbero, N.; Barni, E.; Compari, C.; Fisicaro, E.; Viscardi, G. Synthesis and Characterization of Highly Fluorinated Gemini Pyridinium Surfactants. *Eur. J. Org. Chem.* **2009**, 3167-3177.
33. Fisicaro, E.; Compari, C.; Duce, E.; Contestabili, C.; Quagliotto, P.; Viscardi, G. First evaluation of Thermodynamic Properties for Spheres to Elongated Micelles Transition of Some Propanediyl-α, ω-bis(dimethylalkylammonium bromide) Surfactants in Aqueous Solutions. *J. Phys. Chem. B* **2005**, *109,* 1744.

34. Fisicaro, E.; Ghiozzi, A.; Pelizzetti, E.; Viscardi, G.; Quagliotto, P. Effect of the Counterion on Thermodynamic Properties of Aqueous Micellar Solutions of 1-(3, 3, 4, 4, 5, 5, 6, 6, 6-nonafluorohexyl) Pyridinium Halides. Part I: Apparent and Partial Molar Enthalpies and Volumes at 298 K. *J. Colloid Interface Sci.* **1996**, *182,* 549-557.
35. Desnoyers, J. E.; Perron, G. Surfactant Solutions - New Methods of Investigation, Ed. R. Zana, Marcel Dekker, Inc., New York, **1987**, p.1 and references therein.
36. Fisicaro, E.; Biemmi, M.; Compari, C.; Duce, E.; Peroni M. Thermodynamics of Aqueous Solutions of Dodecyldimethylethylammonium Bromide. *Journal of Colloid and Interface Science* **2007**, *305*, 301–307.
37. Bustamante, C.; Rivetti, C. Visualizing Protein-Nucleic Acid Interactions on a Large Scale with the Scanning Force Microscope. *Annu. Rev. Biophys. Biomol. Struct*. **1996**, *25*, 395-429.
38. Hansma, H. G. Surface Biology of DNA by Atomic Force Microscopy. *Annu. Rev. Phys. Chem*. **2001**, *52*, 71-92.
39. Senapati, S.; Lindsay, S. Recent Progress in Molecular Recognition Imaging Using Atomic Force Microscopy. *Accounts of Chemical Research* **2016**, *49(3)*, 503-510.
40. Zou, Y.; Oupicky, D.; Mao, G. Atomic Force Microscopy Imaging of DNA Delivery Nanosystems. *NanoCellBiology* Edited by Jena, B. P.; Taatjes, D. J. **2014**, 321-340.
41. Volcke, C.; Pirotton, S.; Grandfils, C.; Humbert C.; Thiry, P. A.; Ydens, I.; Dubois, P.; Raes, M. Influence of DNA Condensation State on Transfection Efficiency in DNA/Polymer Complexes: an AFM and DLS Comparative Study. *M. J. Biotechnol*. **2006**, *125*, 11-21.
42. Ceci, P.; Cellai, S.; Falvo, E.; Rivetti, C.; Rossi, G. L.; Chiancone, E. DNA Condensation and Self-aggregation of Escherichia Coli Dps Are Coupled Phenomena Related to the Properties of the N-terminus. *Nucleic Acids Res.* **2004**, *32*, 5935-5944.
43. Mansouri, S.; Lavigne, P.; Corsi, K.; Benderdour, M.; Beaumont, E.; Fernandes, J. Chitosan-DNA Nanoparticles as Non-viral Vectors in Gene Therapy: Strategies to Improve Transfection Efficacy. *Eur. J. Pharm. Biopharm.* **2004**, *57*, 1-8.
44. Liu, F.; Yang, J.; Huang, L.; Liu, D. Effect of Non-ionic Surfactants on the Formation of DNA/Emulsion Complexes and Emulsion-mediated Gene Transfer. *Pharm Res*. **1996**, *13*, 1642–1646.
45. Hara, T.; Liu, F.; Liu, D.; Huang, L. Emulsion Formulations as a Vector for Gene Delivery in Vitro and Vivo. *Adv. Drug. Deliv. Rev*. **1997**, *24*, 265–271.
46. Chen, Y.; Lu, Y.; Tao, C.; Huang, J.; Zhang, H.; Yu, Y.; Zou, H.; Gao, J.; Zhong, Y. Complexes Containing Cationic and Anionic pH-Sensitive Liposomes: Comparative Study

of Factors Influencing Plasmid DNA Gene Delivery to Tumors. *Int. J. Nanomed.* **2013**, *8*, 1573-1593.

47. Balgavy, P.; Devinsky, F. Cut-off Effects in Biological Activities of Surfactants. *Adv. Colloid Interfac.* **1996**, *66*, 23-63.

48. Jennings, K. H.; Marshall, I. C. B.; Wilkinson, M. J.; Kremer, A.; Kirby, A. J.; Camilleri, P. Aggregation Properties of a Novel Class of Cationic Gemini Surfactants Correlate with Their Efficiency as Gene Transfection Agent. *Langmuir* **2002**, *18*, 2426-2429.