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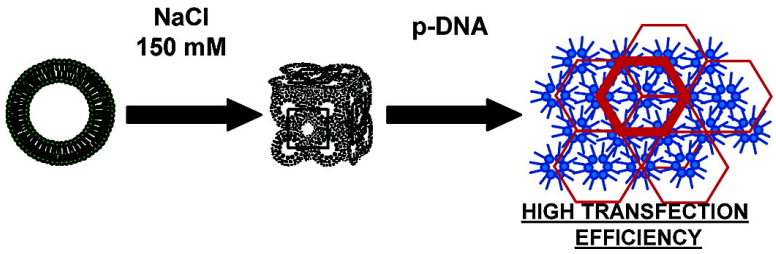

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Sunfish Cationic Amphiphiles: Toward an Adaptative Lipoplex Morphology

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Abstract: A detailed physicochemical study is presented on a new class of cationic amphiphiles, Sunfish amphiphiles, recently designed, synthesized, and tested for gene delivery. These materials have two hydrophobic tails, connected to the cationic pyridinium headgroup at the 1- and 4-positions. Two extreme morphologies can be visualized, i.e. one by back-folding involving association of both tails at one side of the pyridinium ring and one by independent unfolding of the tails, the two molecular geometries leading to considerable differences in the aggregate morphology. The behavior of six members of the Sunfish family in mixtures with DOPE, applying different conditions relevant for transfection, has been studied by a combination of techniques (DLS, DSC, NMR, SAXS, Cryo-TEM, fluorescence, etc.). The effects of structural parameters such as the presence of unsaturation in the tails and length of the alkyl chains on the properties of the aggregates have been assessed. A correlation of these structural data with cellular transfection efficiencies reveals that the highest transfection efficiency is obtained with those amphiphiles that are easily hydrated, form fluid aggregates, and undergo a transition to the inverted hexagonal phase in the presence of plasmid DNA (p-DNA) at physiological ionic strength.

Introduction

Cationic amphiphiles are considered promising alternatives for viral vectors in gene therapy due to the negligible immunogenicity, relative ease of large-scale production and amenability for structural modification. However, the relatively low gene transfer efficiency and low complex stability in the presence of serum as well as the often high toxicity thus far frustrate in vivo applications considerably.[1−10] As a consequence, research efforts are mainly directed toward the development of new formulations to obtain better defined morphological entities exerting an enhanced stability, reduced polydispersity as well as a reduced size of the amphiphile/helper-lipid/DNA complex (lipoplex), cumulating in improved DNA compaction and cell survival after internalization.[11]

To meet the criteria for efficient delivery, the cationic amphiphile system condenses the reporter-DNA by displacement of the positively charged (inorganic) counterions and, after binding, 'transports' the so-formed lipoplex to the cell surface. During these stages, DNA protection and preservation of the lipoplex morphology are important issues. After reaching the cell surface, interaction of the positively charged lipoplex with the negatively charged plasma membrane of the cell is facilitated, initiating the membrane transfer processes.[12,13] The subsequent passage across the cell membrane and escape of the DNA material into the cytoplasm finalize the first part of this trajectory. Endocytosis positively influences this part of the sequence, which involves the release of DNA from the endosomal compartment, facilitated by an as yet largely unresolved

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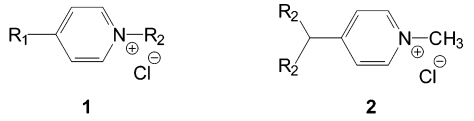
Scheme 1. Sunfish **1** and Saint **2** Amphiphiles[a]



1 **2**

R_1 = C_3, C_5, C_7, C_9, C_{11}, C_{13}, C_{14}, C_{15}, C_{17}, C_{19}
R_2= C_2, C_4, C_6, C_8, C_{10}, C_{12}, C_{14}, C_{16}, C_{18}

[a] The R_1 and R_2 tails can be saturated or unsaturated.

mechanism of disruption of the endosomal membrane.[14,15] However, it has become apparent that the different stages in the pathway clearly benefit from radically different types of morphological behavior of the lipoplex and the constituting lipids.

A potential method of improving the delivery capabilities of synthetic bilayer-forming amphiphiles is to induce a morphological change of the lipoplex from a relative stable lamellar phase (DNA condensing morphology) into an inverted hexagonal phase (H_{II}), recognized as the most effective morphology for in vitro transfection.[16] The hexagonal phase morphology does not necessarily facilitate the interaction with the cell membrane[15] but appears pivotal for the escape from the endosomal compartment.[17,18] Preferably, the morphological changes are only induced in the proximity of or after contact with the cell membrane or, alternatively, at the endosomal level.

This rational has been applied to the design of novel cytofectins, the so-called Sunfish amphiphiles[19] (Scheme 1), by attaching a flexible "antenna" to the cationic headgroup rather than a methyl moiety, as in the structurally related SAINT amphiphiles **2**.[20−22]

When the tails combine at the same interface by means of back-folding (Figure 1A), a lamellar bilayer vesicular morphology is attained (DNA condensing morphology), whereas partial unfolding leads to cone-shaped molecules which associate in the H_{II} phase (Figure 1B). The completely unfolded conformation of the Sunfish materials resembles the extended phospholipid conformation indicated by Holopainen and Kinnunen as intermediate in fusion and hemifusion processes.[23,24] In this conformation, the Sunfish antennae could potentially facilitate molecular interaction processes at the cell surface and the transport of DNA across the cell membrane.

The ability of Sunfish amphiphiles to attain a nonbilayer packing is a function of the interplay between the two alkyl

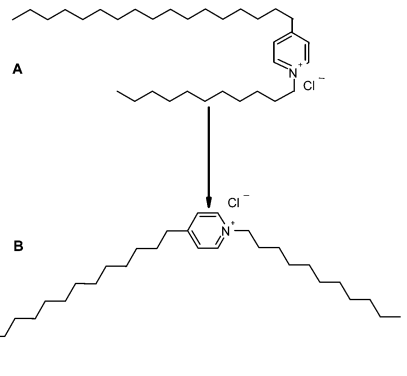
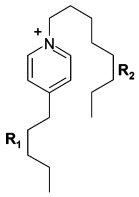


Figure 1. Sunfish concept: backfolding of the *N*-alkyl chain leads to a lamellar (vesicular, L_α) morphology (A) whereas unfolding (B) leads to an inverted hexagonal phase (H_{II}). See text for explanation.

Table 1. Sunfish Amphiphiles **1a**−**1f** Used in the Current Study



	R_1	R_2
1a	C13:0	C6:0
1b	C13:0	C18:0
1c	C13:0	C18:1
1d	C19:0	C18:1
1e	C19:1	C18:0
1f	C19:1	C18:1

chains, determined by their *length* and *presence of unsaturation(s)*. Therefore, modifications in length and unsaturation of the alkyl chains have been introduced and their influence on the transfection activity and toxicity has been evaluated (**1a**−**1f**, Table 1).[19]

The current study is focused on a detailed identification of the molecular features of the Sunfish amphiphiles and the corresponding complexes with DNA, in particular with regard to the preference for nonbilayer packing. The Sunfish amphiphiles were selected in such a way that the effect of small structural variations on the aggregation behavior could be identified by comparison.

Langmuir−Blodgett film balance, ^{31}P- and ^{2}H-NMR, cryo-TEM, SAXS, and light scattering studies have been performed applying conditions optimal for in vitro transfections. The morphological data obtained for the various lipid assemblies and DNA−lipid complexes were then correlated with the in vitro transfection capacity, using COS-7 cells.

Experimental Procedures

The Sunfish amphiphiles were prepared from 4-picoline and the appropriate alkyl iodides in a three-step procedure according to the methods published previously.[19] For the current study, an array of amphiphiles (**1a**−**1f**, Table 1) was chosen, differing in both tail length and unsaturation of the tails. DOPE was purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Nile Red was obtained from ACROS (Landsmeer, The Netherlands). A sample of 7.1-kilobase DNA was isolated from *Escherichia coli* using the Sigma-Aldrich GenElute HP Maxiprep kit (Sigma-Aldrich, Zwijndrecht, The Netherlands). The plasmid concentration was determined spectrophotometrically by measuring the absorption at $\lambda = 260$ nm using the relation 1.0 $A = 50$ μg/mL. Typically, the A_{260}/A_{280} values were ~1.85. All other chemicals were of the highest available grades.

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Sample Preparation. A solution of a Sunfish amphiphile in a 1:1 molar ratio with DOPE in dichloromethane was dried under a stream of nitrogen. Traces of residual solvent were removed under high vacuum. To obtain small unilamellar vesicles (SUVs), the lipid film was hydrated at room temperature (unless stated otherwise), vortexed and sonicated to clarity in a bath sonicator (G112SPIT, 600 V). For NMR measurements, the lipid films were hydrated with bidistilled water or HBS buffer (HEPES 15 mM, 150 mM NaCl, pH = 7.4) to a total lipid concentration of 0.1 M. The lipids were then vortexed and freeze−thawed five times to ensure homogeneous mixing.

Dynamic Light Scattering. Dynamic light scattering (DLS) measurements were performed at 25 °C on a Zetasizer 5000 instrument (Malvern Instruments) at $\lambda = 633$ nm. The intensity autocorrelation functions were all analyzed using CONTIN.[25]

^{31}P NMR Spectroscopy. ^{31}P NMR spectra were recorded on a Varian Unity-Plus 500 spectrometer operating at 202.653 MHz for the phosphorus channel. Data were acquired with single-pulse excitation applying high-power proton decoupling. The 90° pulse length was 28 μs, the recycle delay 1.3 s, the spectral width 40.588 kHz, and the data size 16.236 K. Typically 4000 transients were signal averaged and exponentially multiplied with 50 or 100 Hz line broadening functions. Spectra measured at different temperatures were allowed to equilibrate at each temperature for at least 30 min before data collection.

Small-Angle X-ray Scattering. SAXS measurements were performed at 25 °C on a NanoStar device (Bruker AXS and Anton Paar) with a ceramic fine-focus tube operated in a point focus mode. The tube was powered with a Kristalloflex K760 generator at 35 KV and 40 mA. The primary beam was collimated using cross-coupled Göbel mirrors and a 0.1-mm pinehole, providing a Cu Kα radiation beam with a wavelength of 0.154 nm and a full-width at half-maximum about 0.2 mm in diameter at the sample position. A sample detector distance of 0.24 m was chosen, because no new reflections were observed at longer distances. The use of a Hi-Star position-sensitive area detector (Siemens AXS) allowed the recording of the scattering intensity in the q range of 0.5−8.5 nm^{-1}. The scattering vector q is defined as $q = [(4\pi/\lambda) \sin(\theta/2)]$ where λ is the wavelength and θ is the scattering angle. The measurements of the samples, prepared by mixing Sunfish/DOPE liposomes (1:1, 750 nmol) and p-DNA solutions (50 μg, charge ratio ± 2.5), were performed in flame-sealed quartz capillaries with a diameter of 1 mm. After flame sealing, the samples were centrifuged at low speed and left for 2−3 days at room temperature to equilibrate. The measuring time was between 3 and 9 h.

Langmuir Isotherms. The spreading solutions were prepared by dissolving an appropriate amount of amphiphile in freshly distilled chloroform. Mixed solutions were prepared by mixing appropriate volumes of respective stock solutions (typical concentration 1 mg/mL). The solutions were stored in well-sealed flasks at 4 °C in a desiccator saturated with the solvent. $P-A$ isotherms were obtained with a computer controlled Lauda Filmbalance (FW2), with a total area of 927 cm^2. The compression velocity was about 25 cm^2/min. After spreading, the solutions were left for 5 min to ensure complete evaporation of the solvent before compression was initiated.

Gel Retardation Assay. Complex formation between Sunfish amphiphiles and p-DNA was determined using agarose gel electrophoresis. One microgram of plasmid DNA was incubated with Sunfish, either alone or in an equimolar mixture with DOPE (charge ratio = ± 2.5), for about 15 min in HBS buffer (pH = 7.4) at room temperature. Samples were loaded onto a 1% agarose gel containing 1.2 μM ethidium bromide. A voltage of 100 V was applied over the gel, immersed in a TBE buffer (0.045 M tris-borate, 1 mM EDTA, pH 8.3). The amount of DNA that migrated into the gel was visualized by ultraviolet (UV) illumination.

Cryo-Transmission Electron Microscopy. A drop of the lipid or lipoplex suspension was deposited on a glow-discharged holey carbon-coated grid. After blotting away the excess lipid, the grids were rapidly plunged into liquid ethane. The frozen specimen were mounted in a Gatan (model 626) cryo-stage and examined in a Philips CM 120 cryo-electron microscope operating at 120 KV. Micrographs were recorded under low-dose conditions.

Differential Scanning Calorimetry. DSC scans on SUVs were taken on a Nano II-DSC (Calorimetry Sciences Corp.) with a scan rate of 1 °C/min. Eight heating scans were performed between 1 and 90 °C and referenced against water. The samples were allowed to equilibrate for 30 min at 1 °C between the successive scans.

Fluorescence Spectroscopy. Nile Red fluorescence was measured at room temperature, on a SPF-500c spectrofluorometer (SLM Aminco), using an excitation wavelength of 550 nm. The signal was recorded between 560 and 700 nm with a 1-nm step size. The Nile Red emission maxima (λ_{max}) were deduced from the log-normal fittings of the emission bands.[26]

Results and Discussion

To determine the influence of the major structural features on the morphological behavior, a selection of Sunfish amphiphiles (**1a**−**1f**, Table 1) was prepared. The structural parameters examined include (i) the effect of the length of the saturated R_2 chain (**1a** vs **1b**), (ii) the effect of unsaturation in the R_2 alkyl chain (**1b** vs **1c**), (iii) the effect of prolongation of the R_1 alkyl chain in combination with an unsaturated alkyl chain R_2 (**1c** vs **1d**), (iv) the position of the unsaturation, either in the R_1 (**1e**) or in the R_2 alkyl chain (**1d**), and finally (v) the presence of unsaturation in both alkyl chains (**1f**).

Different techniques were employed in this comparative study. The Langmuir−Blodgett film balance technique provided information about the relative elasticity and stability of the monolayers formed by the Sunfish amphiphiles. From a combination of gel retardation electrophoresis, DSC, and DLS, further differences in the aggregation behavior and DNA binding ability were identified and rationalized. ^{31}P-NMR, SAXS, and cryo-TEM experiments were used to characterize the morphology of the aggregates, elucidating the effect of the structural variations on the phase behavior of the cationic amphiphiles in their mixtures with DOPE.

Sunfish Lipid Monolayers. Surface pressure (π) vs area (A) isotherms were measured to address the effect of the length of the tails and presence of unsaturations on the characteristics of the monolayers formed by the Sunfish amphiphiles at the air−water interface (Figure 2).

Compound **1a** does not form monolayers at the air−water interface and is the only micelle-forming amphiphile in the selected group. The CMC, 2.7 mM, has been measured by conductivity. The conductivity vs concentration plot (Figure 3) shows that micelle formation is a noncooperative process for this compound. Pre-micellar aggregates are probably formed also at very low concentrations, increasing the solubility of **1a** in water below the CMC. The CMC obtained by Nile Red fluorescence measurements (data not shown) is more than an order of magnitude smaller; the discrepancy between the values obtained with the two different techniques is another indication of the tendency of this amphiphile to form pre-micellar aggregates.

Monitoring the evolution of the surface area in time at a pressure of 20 mN/m (data not shown) revealed that the other selected Sunfish amphiphiles form thermodynamically unstable

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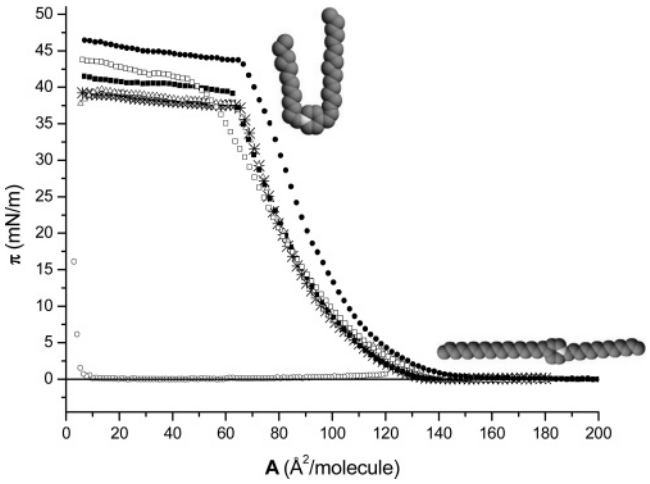

Figure 2. Surface-pressure vs area isotherms of monolayers of **1a** (○), **1b** (●), **1c** (□), **1d** (■), **1e** (△), **1f** (*), using water as the subphase.

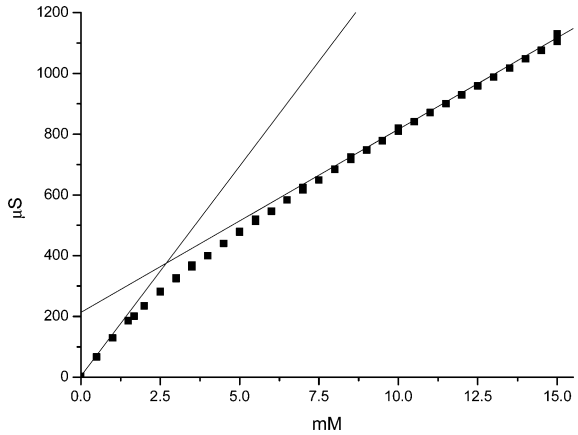

Figure 3. Determination of the CMC of compound **1a** in water by conductivity measurements.

monolayers. Nevertheless, the kinetic stability of these systems was sufficient to perform the isothermal compressions under well-defined and comparable conditions, as indicated by the reproducibility of the results and by the fact that decreasing the compression rate by a factor of 2, from 7.2 to 3.6 Å^2/(molecule) min^{-1}, did not change the outcome of the experiment. The observed differences in the isotherms recorded upon compression provide information about the tendency of the different amphiphiles to form more or less tightly packed structures when the available area per molecule is decreased in a comparable way.

All the compounds except **1a** show a similar offset at about 140 Å^2/molecule. These large values of *A* indicate a molecular conformation in which both alkyl chains lie flat at the surface and are similar to findings for ditetradecyldimethylammonium bromide and dihexadecyldimethylammonium bromide.[27] Also the collapse of the monolayer occurs at about the same surface area for the different amphiphiles (65 ± 5 Å^2/molecule). At the collapse pressure both alkyl chains point away from the water surface, as indicated by the fact that the expected value for the sum of the areas occupied by one vertical and one horizontal alkyl chain (about 80 Å^2/molecule) is significantly bigger than the measured surface area. The behavior at the air−water interface of the Sunfish surfactants with long chains and at least one unsaturation in either R_1 or R_2 (**1d**, **1e**, **1f**) is similar. The slope of the isotherm for compound **1b** is steeper, indicating a more condensed monolayer, as expected due to the presence of saturated tails. The collapse pressure of this monolayer is the highest. Brewster angle micrographs (data not shown) revealed another difference between compound **1b** and compound **1f**. While upon compression and expansion of **1f** monolayers the intensity of the reflected light increases and decreases homogeneously, during the expansion of **1b** monolayers "island" formation was observed, indicating the presence of strong(er) intermolecular interactions.

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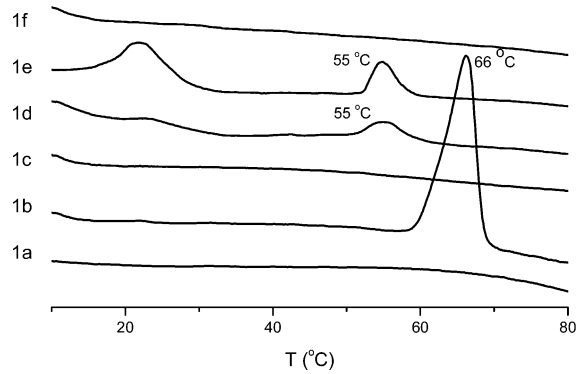

Figure 4. DSC up-scans of equimolar mixtures of Sunfish/DOPE in water (first scan).

Sunfish/DOPE−DNA Binding. To establish the ability of the Sunfish amphiphiles to bind DNA, the retardation on 1% agarose gel after complexation of p-DNA with lipid **1** or an equimolar mixture of lipid **1** and DOPE was determined by gel electrophoresis (data not shown). In this assay, free or partially bound DNA migrates into the gel under the effect of the applied electric field and is detected as a fluorescent labeled band (ethidium bromide, included in the gel, intercalates into the free DNA). Pure p-DNA gives rise to three fluorescent bands which can be attributed, respectively, to aggregated DNA that remains in the slot, open circular DNA and supercoiled DNA. The negative charge of the DNA is neutralized upon cationic amphiphile binding, and the lipoplex does not migrate on the gel. Moreover, if upon binding the DNA becomes inaccessible to the ethydium bromide, the DNA cannot be visualized under UV illumination. On the basis of the results obtained for the lipoplex with a charge ratio ± 2.5, conditions found optimal for the in vitro transfection,[19] the Sunfish materials were divided into three groups: (1) Sunfish amphiphiles which bind DNA completely without additional DOPE (e.g. **1a**, **1c**, **1f**), (2) Sunfish amphiphiles that are DOPE-dependent for attaining complete DNA binding (e.g. **1d**, **1e**) and (3) **1b**, that was not able to bind DNA completely even in the presence of DOPE. Interestingly, the binding of **1b**/DOPE and DNA could be significantly improved by hydration and sonication at 60 °C. In the lipoplexes in which the helper lipid was present, ethidium bromide intercalated into the DNA even upon complete binding. These data indicate a looser binding of the amphiphilic carriers to the DNA when the helper lipid is present with respect to the complexation to the pure cationic amphiphile, which could be beneficial for the escape of the DNA in the cytosol.

The observed differences can be rationalized in terms of the solubility of the surfactants. The DSC spectra recorded for **1b**, **1d**, and **1e** in water (Figure 4) present a high-temperature peak

Table 2. Sizes of the Aggregates Formed by Sunfish/DOPE Equimolar Mixtures in Water[a,b,c]

	size of the aggregates in nm	
Sunfish	vesicles	lipoplexes
1a	100 ± 10	450 ± 80
1b	170 ± 10*	1400 ± 200
1b (60 °C)	80−190	400 ± 80
1c	140 ± 4	1250 ± 40
1d	460 ± 30*	1250 ± 40
1d (60 °C)	80−200	600 ± 60
1f	160 ± 10	600 ± 80

[a] After hydration and sonication (vesicles). [b] Asterisks indicate the presence of a second aggregate population in the micrometer range. [c] Sizes of the corresponding DNA complexes prepared in HBS buffer and diluted in culture medium at room temperature after 10 min of equilibration (lipoplexes).

at 55 °C for the lipids with one unsaturation in the tails and at 66 °C for the saturated-tail amphiphile. As expected and confirmed by fluorescence depolarization experiments using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a probe (data not shown), these peaks do not correspond to the main phase transitions but arise from the solubilization of the amphiphiles.

The complete solubilization of **1a**, **1c**, and **1f** at room temperature is consistent with the less efficient packing (higher dynamic character) of these lipids in the crystalline state, determined by the presence of short chains (**1a**) or a short chain in combination with an unsaturation (**1c**) or by unsaturation in both chains (**1f**). The comparison between **1c** and **1d** (or **1e**) indicates that the prolongation of the alkyl chain is sufficient to stabilize the crystal packing even in the presence of an unsaturation. The fluidizing effect of DOPE is anyway sufficient to obtain complete vesicle formation at room temperature with materials containing unsaturated alkyl tails (**1d** and **1f**).

The extreme case of **1b**, which bears a long saturated alkyl chain ($C_{18:0}$) in combination with a saturated shorter chain ($C_{13:0}$), leading to the formation of the most rigid morphological entity, needs both the helper-lipid DOPE and higher temperature (about 60 °C, data not shown) to establish complete DNA binding. The improvement of DNA binding by the parallel application of both conditions also leads to higher transfection efficiencies (vide infra).

Aggregation Behavior of the Lipids. The aggregation behavior of cationic lipids generally affects the DNA binding. Therefore, the aggregation behavior of Sunfish/DOPE (1:1) mixtures was studied using light scattering techniques. The results of the measurements performed with hydrated and sonicated Sunfish/DOPE films are summarized in Table 2.

In the series of amphiphiles with saturated R_2 alkyl chains, only for **1a**, with the shortest R_1 ($C_{13:0}$) and R_2 ($C_{6:0}$) chains, the formation of a homogeneous single vesicle population with a size of approximately 100 nm was observed. Elongation of R_2 to C_{12}, C_{16}, or C_{18} (**1b**, R_1 is $C_{13:0}$) leads to incomplete vesicle formation, and large aggregates in the micrometer range were also observed.

With regard to R_1, a chain length shorter than C_{19} is necessary to ensure proper vesicle formation; **1d** (R_1 is $C_{19:0}$ and R_2 is $C_{18:1}$) does not form a homogeneous vesicle population despite the presence of the favorable unsaturation in the R_2 chain. The suggestion that the mobility of the alkyl chains plays a key role in the formation of vesicular states is further supported by the observation that sonication at 60 °C resulted in the formation of smaller vesicles (80−200 nm), and now aggregates in the micrometer range were no longer observed. Similarly, sonication of a heated **1b**/DOPE mixture resulted in complete vesicle formation with an average size between 80 and 190 nm.

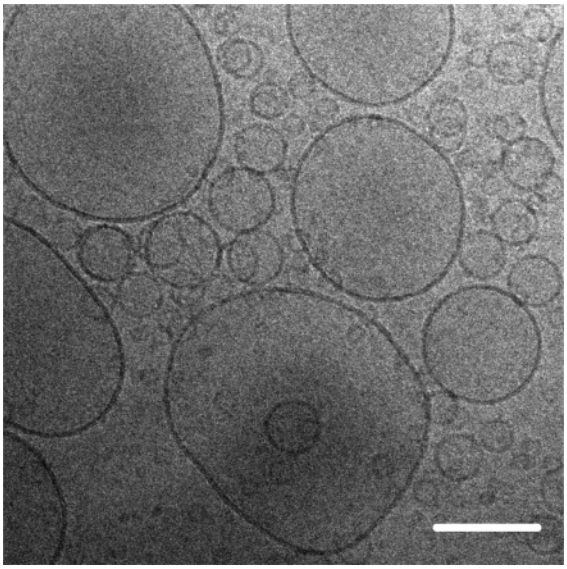

Figure 5. Cryo-TEM picture of the vesicular structures formed by the equimolar mixture of **1f**/DOPE in water. Bar indicates 100 nm.

Cryo-TEM measurements on equimolar **1f**/DOPE preparations (Figure 5) confirmed the vesicular nature of the aggregates studied by DLS; the stability of these aggregates was checked by means of turbidity measurements run for over a week (data not shown), during which period no significant changes were observed.

Aggregation Behavior of Lipid/DNA Complexes. The increased particle size of Sunfish/DOPE−DNA lipoplexes in comparison to the DNA-void morphologies, suggests the enhanced aggregation of the lipid membranes in the presence of DNA. Addition of plasmid to a Sunfish/DOPE (1:1) mixture at conditions used for the in vitro transfection (vide infra) generated large aggregates with the ability to grow quickly to sizes above 1 μm. In general, if the starting lipid formed single-vesicle populations in the DNA-free situation, the complexes with DNA were smaller compared to Sunfish amphiphiles for which also aggregates in the micrometer range were observed. These data show how the optimal amount of lipid cannot be recruited by the DNA from the rigid assemblies present in the polydisperse suspensions. Under these condition, the DNA seems to promote aggregation in large particles, characterized by low transfection abilities (vide infra).

Phase Behavior of Mixtures of Sunfish and DOPE in Water. The phase behavior of the Sunfish/DOPE mixtures has been studied both in water and in HBS.

The lipoplexes used for in vitro transfection are prepared by adding DNA to a vesicular suspension of the cationic amphiphiles in equimolar mixtures with DOPE in water. Since the driving force for complex formation is mainly electrostatic, the ionic strength is raised to physiological values (necessary for cell survival) by dilution with a saline buffer only after 10 min of incubation.

The line-shapes of the ^{31}P NMR spectra recorded in pure water (Figure 6), indicate the formation of stable bilayer structures. In every sample, the L_α phase is predominant over the entire range of temperatures examined (5−65 °C). A sharp

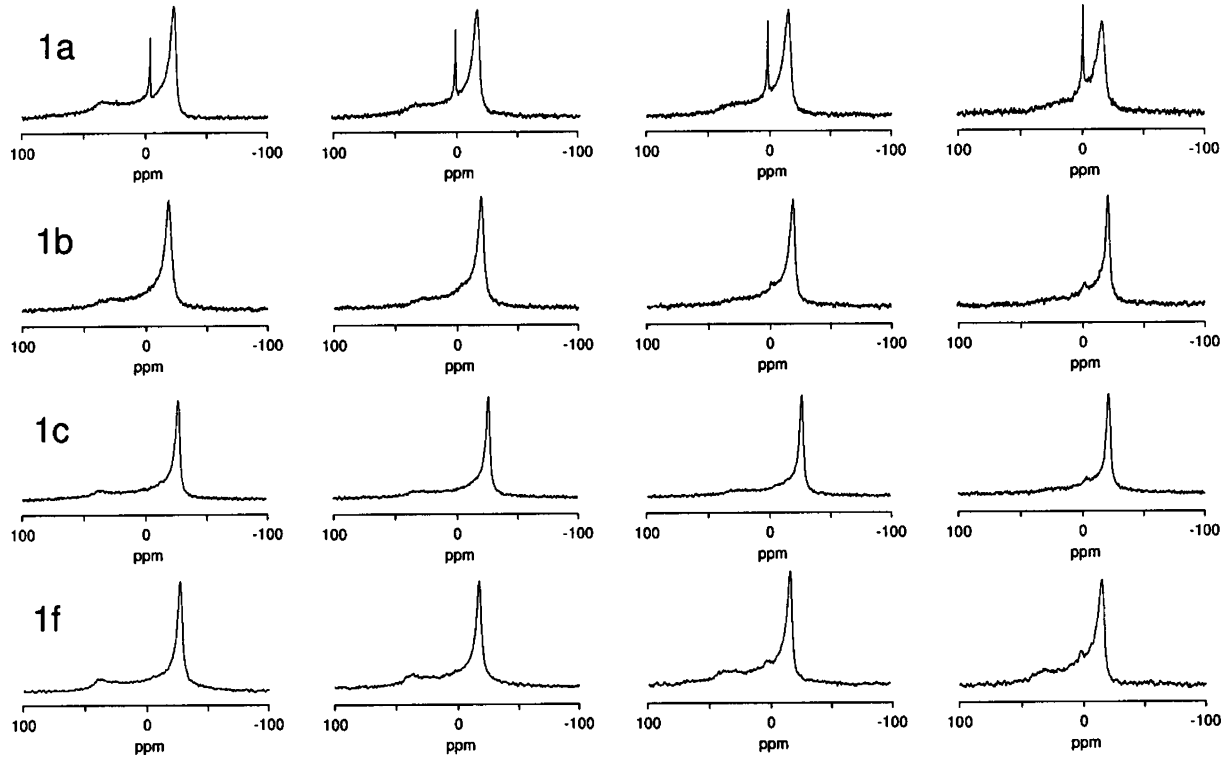

Figure 6. ^{31}P NMR spectra of Sunfish in equimolar mixtures with DOPE in water at different temperatures: from left to right, the respective temperatures are 5, 25, 35 and 65 °C. The spectra recorded on **1d** and **1e** mixtures with DOPE are comparable with the ones recorded for **1f**.

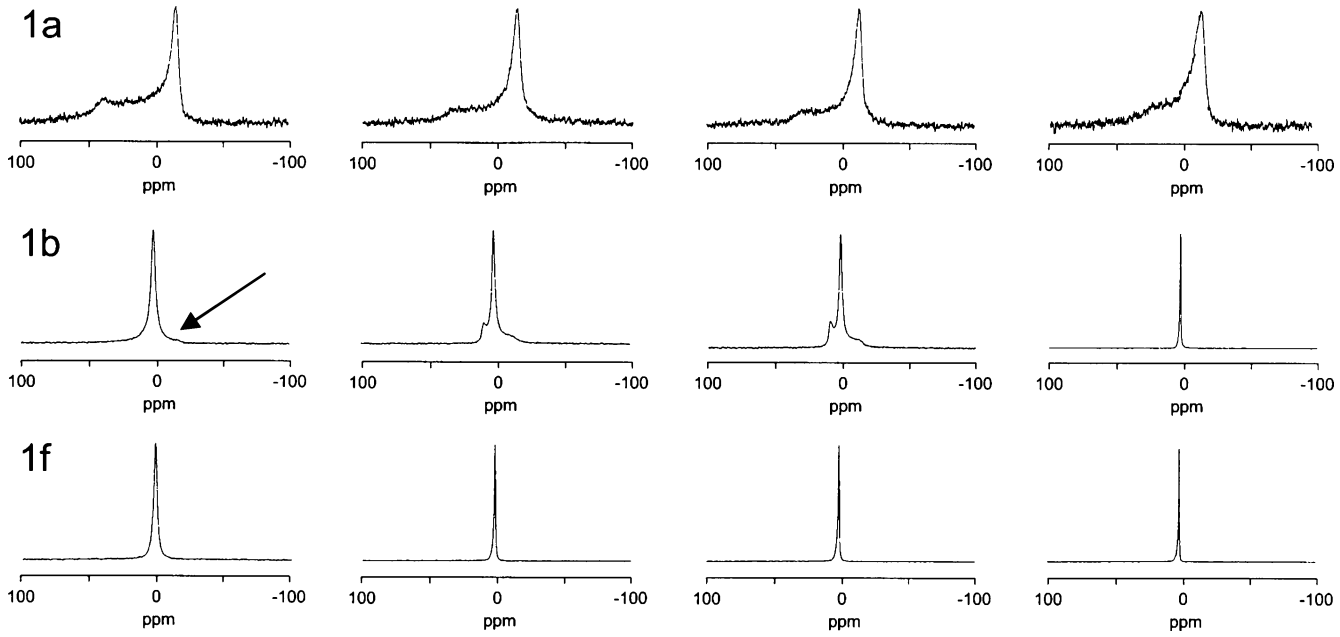

Figure 7. ^{31}P NMR spectra of Sunfish in equimolar mixtures with DOPE in HBS at different temperatures: from left to right, the respective temperatures are 5, 25, 35, and 65 °C. The arrow indicates a weak bilayer signal. The experiments performed on **1c**, **1d**, **1e** gave results analogous to those for **1f**.

isotropic peak of which the contribution increases with temperature is present in the spectra of **1a** and is due to the co-presence of small vesicles, which were also observed by means of cryo-TEM at these high concentrations (data not shown).

The spectra indicate, moreover, complete mixing of all Sunfish amphiphiles with DOPE, since pure DOPE would be present in the hexagonal phase at temperatures exceeding 10 °C.

Phase Behavior of Mixtures of Sunfish and DOPE in HBS. The ^{31}P NMR experiments performed on analogous samples hydrated with HBS (Figure 7) clearly show that the increase in ionic strength has a dramatic effect on the morphology of the aggregates.

The only surfactant of the selected group that maintains the lamellar morphology is **1a**. The predominant feature of the spectra recorded for mixtures of the other surfactants is an isotropic signal, indicating the presence of cubic phases.[28]

In Figure 8A, the SAXS spectra recorded on the equimolar mixture **1f**/DOPE in HBS are shown, confirming the presence

(28) Cullis, P. R.; De Kruyff, B. *Biochim. Biophys. Acta* **1979**, *559*, 399−420.

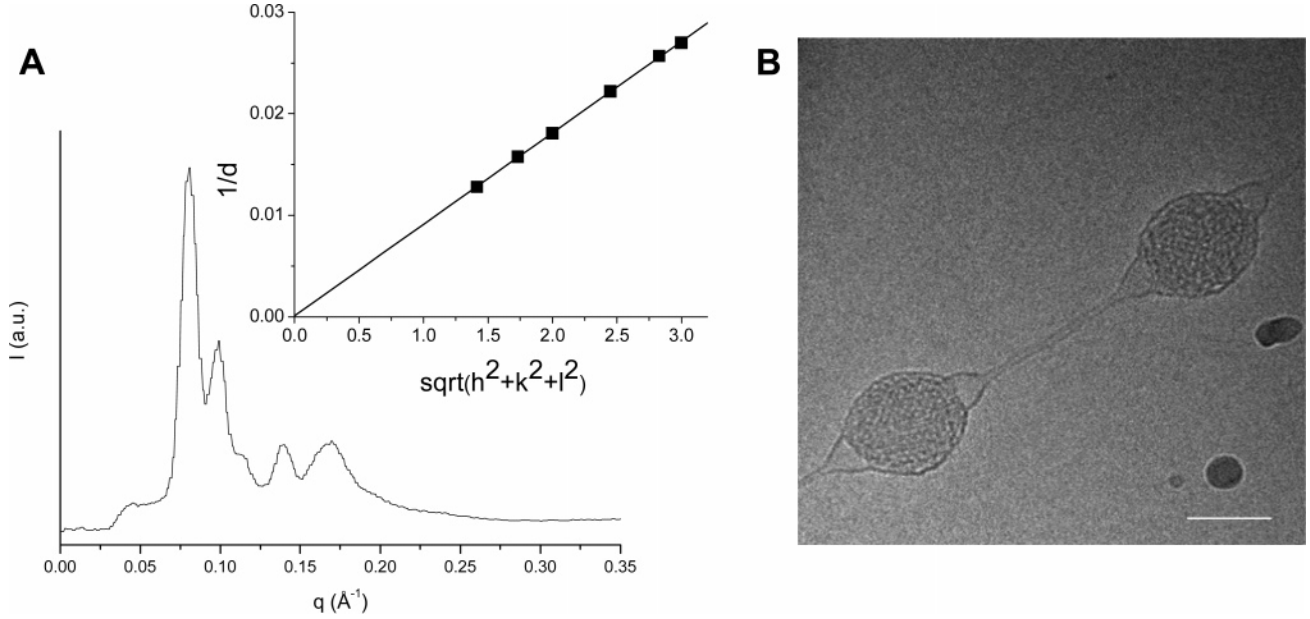

Figure 8. Cubic phase of the equimolar mixture of **1f**/DOPE in HBS. (A) SAXS spectrum obtained on a 100 mM total lipid concentration, also used for ^{31}P NMR experiments; in the insert, plot of the inverse spacing $1/d$ against $\sqrt{h^2+k^2+l^2}$ (see text for explanations). (B) Cryo-TEM pictures of the cubosomes obtained by diluting a vesicular aqueous suspension of Sunfish/DOPE to a final 150 mM ionic strength and 2.5 mM total lipid concentration. Bar indicates 100 nm.

of a cubic phase. The space group ratios of the reflections (1, $\sqrt{3/2}$, $\sqrt{2}$, $\sqrt{3}$, 2, $3/\sqrt{2}$,) are consistent with a cubic phase of the *Pn*3*m* space group, the diamond-type cubic phase. In the insert, the plot of the inverse spacing ($1/d$, Å^{-1}) against $\sqrt{h^2+k^2+l^2}$, where *h, k, l* are the Bragg parameters, is given; the data can be fitted with a straight line passing through the origin, which supports the tentative assignment. The unit cell length calculated from the line slope is 11.1 nm.

Cryo-TEM experiments (Figure 8B) showed that a dispersed cubic phase (cubosomes)[29] is obtained starting from vesicles of **1f**/DOPE in water and diluting the suspension with HBS to 150 mM ionic strength. The dimensions of the particles varied, and grain boundaries are clearly recognizable within the aggregates. The Fourier transform of a homogeneous part of the texture reveals a repeat distance of 63 Å, which corresponds to the expected spacing between the (111) planes, $d = 111$ Å/$\sqrt{3}$. The correspondence between the SAXS and cryo-TEM data recorded on the samples obtained via different preparations, suggests that the observed phase is determined by the final ionic strength and not by the history of the sample, indicating the thermodynamic stability of the observed phase. The only surfactant of the selected Sunfish group that maintains the lamellar morphology is **1a**, consistent with its smaller packing parameter (vide supra).

The presence of the characteristic line-shape of the H_{II} phase in the spectra of **1b** in mixtures with DOPE, if recorded at 25 and 35 °C, and the absence of a comparable signal in lipids with higher packing parameters, suggests the incomplete mixing of the two lipids in the temperature range up to 35 °C. The presence of a weak bilayer signal at 5 °C, indicated by the arrow in Figure 7, is in agreement with this suggestion. This finding is consistent with the strong intermolecular interactions of **1b**, already deduced from the monolayer experiments and the DSC results. Inhomogeneous mixing with DOPE has been previously found for a different pyridinium amphiphile with long saturated tails.[30] The ^{31}P NMR experiments on **1b**/DOPE samples hydrated with water clearly show that it is possible to obtain mixed systems, stable for several weeks, by freeze and thaw of equimolar quantities of cationic and helper lipid. The demixing, observed in an analogous sample preparation in HBS, is consistent with the decrease of the repulsive interactions between the positively charged headgroups of the cationic amphiphiles as a consequence of the increase in ionic strength. Upon increasing the temperature, the favorable entropic contribution to the Gibbs energy of mixing leads to a $\Delta G_m < 0$ only above 35 °C. Equilibration of this sample at 65 °C leads to a mixed cubic phase that is stable at room temperature for several hours.

Phase Behavior of Lipoplexes of Sunfish and DOPE in HBS. The effect of the complexation with DNA on the phase behavior of the equimolar mixtures of Sunfish/DOPE, at charge ratio ± 2.5, was determined by means of SAXS measurements (Figure 9).

The lamellar morphology of the mixture **1a**/DOPE in HBS is maintained after complexation with DNA. The calculated spacing between two bilayers ($d = 2\pi/q_{001}$) is 6.29 nm.

The diffraction pattern obtained for the analogous lipoplex of compound **1b** shows the coexistence of different phases. The high(er) value of the unit cell length, $a = 4\pi/(\sqrt{3}q_{10}) = 6.86$ nm, with respect to the analogous preparations of the other Sunfish amphiphiles under investigation (vide infra), indicates the inhomogeneous mixing of this cationic amphiphile with the helper lipid. As already mentioned for the analogous preparations in the absence of DNA, this finding is consistent with the Langmuir−Blodgett film balance experiments and the DSC results, which show the strength of **1b** intermolecular interaction

(29) Andersson, S.; Jacob, M.; Lidin, S.; Larsson, K. *Z. Kristallogr.* **1995**, *210*, 315−318.

(30) Scarzello, M.; Chupin, V.; Wagenaar, A.; Stuart, M. C. A.; Engberts, J. B. F. N.; Hulst, R. *Biophys. J.* **2005**, *88*, 2104−2113.

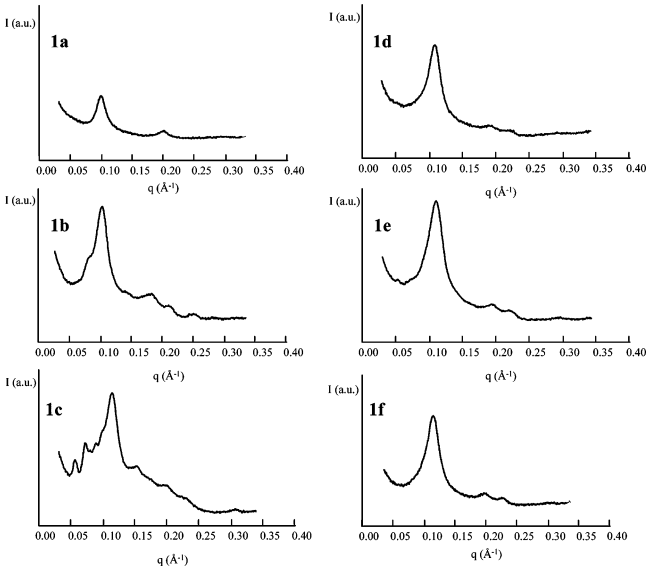

Figure 9. SAXS spectra of lipoplexes of Sunfish in equimolar mixtures with DOPE.

even in pure water, and with the ^{31}P NMR experiments in HBS, indicating the presence of unmixed DOPE domains.

The introduction of an unsaturation in the R_2 tail (**1c**) leads again to a polymorphic system, most likely coexisting cubic and hexagonal phases ($a = 6.43$ nm). The small value of the unit cell length of the H_{II} phase indicates the presence of a mixed system.

The lipoplexes formed by the amphiphiles with longer tails and at least one unsaturation (**1d**, **1e**, **1f**) all produce similar diffraction patterns, characteristic of the presence of an H_{II} phase only. The calculated unit cell lengths, respectively 6.60, 6.54 and 6.46 nm for **1d**, **1e**, and **1f**, are comparable as expected.

On the basis of these results, it appears that the ability of the Sunfish amphiphiles to form nonlamellar phases in their complexes with DNA increased in the order: short R_1 and R_2 alkyl chain (**1a**) < unsaturated R_2 alkyl chain in combination with a short R_1 alkyl chain (**1c**) < unsaturated R_1 or R_2 in combination with a long saturated alkyl chain < both R_1 and R_2 unsaturated (**1d**, **1e**, **1f).** The counter-intuitive presence of a higher content of H_{II} phase in the lipoplex formed by **1b** can be explained by the presence of unmixed DOPE in the first sample.

The aggregate morphologies of the Sunfish/DOPE mixtures as a function of different solution conditions were confirmed by Nile Red fluorescence experiments (Figure 10). The use of this solvatochromic probe in the determination of the phase behavior of lipid aqueous suspensions has been recently described.[31] In the biophysical characterization of the actual transfection systems, the low concentrations used when operating on cells (in the nanomolar range) constitute a significant experimental difficulty. Even if the outcome of the Nile Red fluorescence experiments is less compelling than the diffraction data, the comparable results obtained on analogous systems, prepared via different procedures, indicate that the information on the phase behavior of the amphiphiles can be extended to the actual transfection systems.

(31) Stuart, M. C. A.; van de Pas, J. C.; Engberts, J. B. F. N. *J. Phys. Org. Chem.* **2005**, in press.

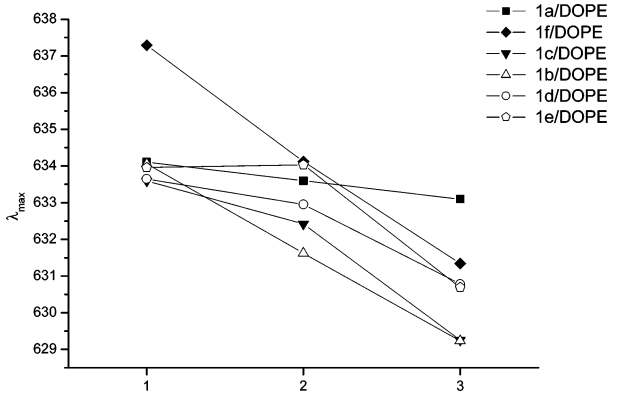

Figure 10. Emission maxima obtained after excitation of the fluorescent probe Nile Red in Sunfish/DOPE mixtures in different conditions: (1) in water, (2) in HBS, (3) in HBS in the presence of p-DNA at charge ratio ±2.5.

Table 3. Variations ($\Delta\tilde{\nu}$) of the Nile Red Emission Wave Numbers ($1/\lambda$) in Sunfish/DOPE Mixtures[a]

	$\Delta\tilde{\nu}$ (cm^{-1}) from H_2O to HBS	$\Delta\tilde{\nu}$ (cm^{-1}) from H_2O to HBS + DNA
1a/DOPE	13	13
1b/DOPE	126	−136
1b/DOPE *	61	60
1c/DOPE	29	80
1d/DOPE	−8	83
1d/DOPE *	−2	84
1e/DOPE	72	−61
1e/DOPE *	18	54
1f/DOPE	78	69

[a] Asterisks indicate that the sonication was performed at 60 °C.

The maximum emission wavelength of Nile Red, bound to an aggregate in solution, undergoes a hypsochromic shift for a decrease in the aggregate curvature. The value of the emission wavelength associated with a particular phase depends on the polarity of the environment at the binding sites and varies for different amphiphiles. The variations in the energy (expressed as wavenumbers) of this optical transition as a consequence of the increase in ionic strength and p-DNA complexation are reported in Table 3.

The only amphiphile which maintains the lamellar morphology at physiological ionic strength and in the presence of p-DNA according to SAXS measurements, **1a**, shows variations in the wavenumber of the emitted radiation of 13 cm^{-1}. The small shifts observed are probably associated with the change in the hydration of the bilayers as conditions are changed. The variations associated with the expected transition to the H_{II} phase after addition of DNA are 54 cm^{-1} or more. The experiments performed on the mixtures of **1b**, **1d**, and **1e**, tip-sonicated at room temperature (data not shown) indicate, at every solution condition, a remarkable shift of the fluorescence maximum toward shorter wavelength; this evidence confirms that part of the lipids are not fully hydrated, as also deduced from the DSC results. The lower quality of the fits of the emission data for these samples with respect to the fully hydrated systems indicates their nonhomogeneity.

SAXS and Nile Red data clearly show that DNA is effective in promoting H_{II} phase formation in Sunfish amphiphiles with long alkyl chains and an unsaturation in at least one of the tails. For analogous SAINT amphiphiles, in which both tails branch from a methyne group attached to the 4-position of the pyridine

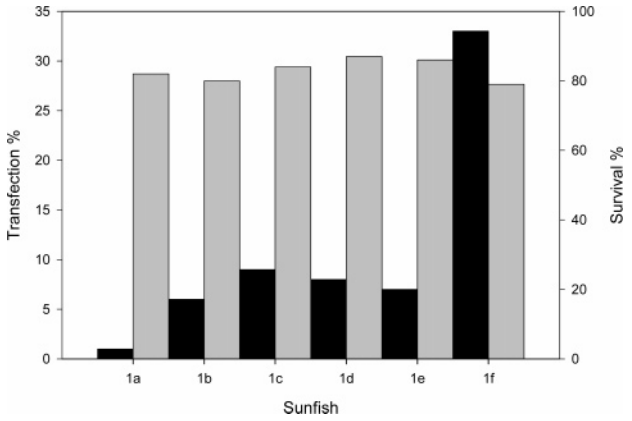

Figure 11. Transfection efficiency (black bars) and cell survival (gray bars) of COS-7 cells transfected by equimolar mixtures of the selected Sunfish materials with DOPE.[19] See text for explanation.

ring, the increase in ionic strength to physiological values is sufficient to promote H_{II} phase formation and the presence of DNA does not further affect the morphology.[17,30] The comparison with the SAINT materials shows how attaching a long tail to the 1-position of the pyridine ring leads to a surfactant with a higher packing parameter. The Sunfish amphiphiles with long tails and at least one unsaturation in the alkyl chains form cubic phases at physiological ionic strength and they undergo a transition toward the H_{II} phase only after DNA complexation. While DNA complexation is known to generally affect the main phase transition temperature of the carrier mixture and the mixing with the helper lipid,[32] a clear effect on the aggregate morphology has been rarely observed.[33]

Correlation with the Transfection Efficiencies. The back-folding and unfolding ability of the Sunfish amphiphiles was first confirmed by 2D-NOESY NMR techniques,[19] indicating that within a series of model compounds with increasing R_2 chain lengths from C_1 to C_7, R_2 should have a minimal chain length of 6 carbon equivalents (C_6 or more) to allow back-folding to occur and to back-pass the pyridinium moiety. As mentioned above, conditions promoting the unfolding of the alkyl chains of the lipid also promote the formation of the H_{II} phase, provided that the cone-shaped geometry of the lipid molecules with a broadened hydrophobic domain is recognized as a partially unfolded state.

The excellent transfection ability of several Sunfish amphiphiles, using different cell lines and with different helper lipids, has already been described.[19] The transfection results of equimolar mixtures of the selected materials with DOPE on the COS-7 cell line are summarized in Figure 11.

To avoid over-interpretation of the transfection results, we divide the Sunfish amphiphiles in three categories: (1) very good transfectant (**1f**); (2) moderate transfectants (**1b**−**1d**); (3) bad transfectant (**1a**). The highest transfection efficiency is achieved by **1f**, the lipid with both alkyl chains unsaturated. When only saturated alkyl chains are present the transfection efficiency is strongly retarded. The presence of relatively long tails appears to be beneficial (**1b**−**1f** vs **1a**). Using Sunfish amphiphiles only differing in their hydrophobic domain, the preference for the hexagonal (nonbilayer) morphology is a function of the size of the hydrophobic region covered by both alkyl chains. Spectroscopic and diffraction studies showed that by manipulating the Sunfish lipid tails, we were able to prepare Sunfish/DOPE−DNA lipoplexes with nonbilayer morphology. The dependence of the aggregate morphology on the molecular shape of an amphiphile is expressed by the dimensionless packing parameter, $P = V/a \cdot l$, which is proportional to the hydrophobic chain volume (V) and inversely proportional to the optimal cross-sectional headgroup area (a) and to the length of the hydrophobic tails (l). When $^1/_2 < P \leq 1$, a lamellar organization is favored, when $P > 1$ inverted structures are preferred. Cubic phases are often intermediates in the transition between L_α and H_{II} phases.[34]

The expected variations in the Sunfish packing parameters, as a consequence of the structural modifications and of the different solution conditions, are consistent with the observed phase transitions. A lamellar to hexagonal phase transition of the Sunfish amphiphiles was promoted by the elongation of the R_2 alkyl chains from $C_{6:0}$ (**1a**/DOPE) to $C_{18:0}$ (**1b**/DOPE). Elongation of the R_1 alkyl chain to $C_{19:0}$ (**1d**/DOPE) led to an improvement in hexagonal packing compared to **1c**/DOPE (R_1 is $C_{13:0}$), which revealed highly disordered hexagonal packing.

As expected, the presence of an unsaturation in the alkyl chains clearly promoted lamellar to hexagonal phase transitions. In **1c**/DOPE the positive effect of the *cis*-double bond in R_2 was counter-balanced by the intermediate length of the R_1 alkyl chain, resulting in a "sub-optimal" structural transition from the lamellar to the hexagonal phase.

Unexpectedly, variations in the position of the unsaturation (in either R_1 or R_2) did not lead to any morphological difference (compare **1d**/DOPE, **1e**/DOPE, and **1f**/DOPE). In the rationalization of the differences in the transfection efficiencies observed for these three isomorphic transfection cocktails and for the other selected amphiphiles, the stability of the crystal packing has a profound influence. The mobility of the relatively long and less-adaptative alkyl chains of **1b**, **1d**, and **1e** was limited to such an extent that a complete dissolution of the crystals could not be achieved at room temperature, not even after sonication. The remarkable stability of the crystal packing results in incomplete vesicle formation and DNA binding. Warming to 60 °C increased the mobility of the lipid chains, which had consequences for the vesicle formation and the binding with DNA, as evidenced in particular by **1b**/DOPE. The presence of the *cis*-double bond in **1d**, **1e**, and **1f** resulted, similarly to the temperature effect on the **1b**/DOPE bilayer, in, a more "fluid" bilayer and improved vesicle formation as well as accommodation of the DNA.

The suboptimal vesicle formation of **1b**, **1d**, **1e** lowers their transfection efficiencies. Moreover, the tendency of the Sunfish amphiphiles to readily undergo a transition from a lamellar to nonlamellar packing is correlated with the ability to promote cationic lipid-mediated transfection. In fact, the absence of a propensity for transition to the H_{II} phase of **1a** and **1c** resulted in a lower transfection potential compared to the complex that exhibited a higher-order hexagonal morphology (**1f**).

Besides parameters such as length and the presence of unsaturations in the alkyl chains, the position(s) of the unsaturation(s), although not varied in the present study, might prove

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(33) Bell, P. C.; Bergsma, M.; Dolbnya, I. P.; Bras, W.; Stuart, M. C. A.; Rowan, A. E.; Feiters, M. C.; Engberts, J. B. F. N. *J. Am. Chem. Soc.* **2003**, *125*, 1551−1558.
(34) Seddon, J. M. *Biochim. Biophys. Acta* **1990**, *1031*, 1−69.

to be of significant importance for the morphological behavior. With SAINT amphiphiles, the configuration of the double bonds as such (85% cis, 100% cis, or 100% trans) has a dramatic influence on the transfection outcome.[35]

Conclusions

In this study the aggregation behavior of six structurally related 1,4-dialkylpyridinium amphiphiles (Sunfish) has been investigated, using conditions mimicking key steps of the transfection process. The amphiphile with short saturated tails (**1a**) forms micellar aggregates in water. The addition of the helper lipid DOPE to **1a** increases the average packing parameter and leads to the formation of mixed bilayers. The L_α phase is stable in the range of temperatures explored (up to 65 °C) upon an increase in ionic strength to physiological values (150 mM) and upon p-DNA complexation. Quite consistently, an inability of the lipoplex to undergo a transition to the hexagonal phase is associated with low transfection efficiency. Optimal packing of the alkyl chains is obtained by elongating the R_2 alkyl chain to $C_{18:0}$ (**1b**). The low propensity of **1b** for hydration and mixing with DOPE at room temperature is suggestive for the strength of the attractive intermolecular interaction, despite the obvious electrostatic repulsion between the charged headgroups. The introduction of a double bond ($R_2 = C_{18:1}$, **1c**) leads to a less compact crystal packing and, consequently, to easier dispersal in water. The increase in the volume of the alkyl chains also favors H_{II} phase formation. In the presence of the helper lipid and at high ionic strength only a cubic phase is formed, but lipoplexes at charge ratio $\pm$ 2.5 show the coexistence of cubic and hexagonal phases. As a result of a further increase in the length of the alkyl chains (**1d**, **1e**), the lipoplex assumes a well-defined H_{II} morphology, but the high(er) tendency of these surfactants to crystallize seems to affect the transfection negatively. The similar behavior of the amphiphiles which differ in the position of the unsaturations in either R_1 or R_2 (**1d** and **1e**) indicates a certain degree of equivalence of the alkyl chains. This finding is unexpected since the weak delocalization of the positive charge in the pyridinium ring is likely to influence the orientation of the headgroup at the interface, making the 1- and 4-positions of the ring nonequivalent. The best transfectant (**1f**) is obtained upon introduction of a double bond in both the alkyl chains. This compound can be easily dispersed in water, and it forms stable vesicles in mixtures with DOPE. Upon an increase in ionic strength, these mixtures aggregate in a relatively stable, dispersed cubic phase and finally undergo a quantitative transition to the H_{II} phase upon p-DNA complexation.

In sum, the aggregate morphology and DNA binding characteristics of several Sunfish amphiphiles were identified and correlated with the transfection efficiency for COS-7 cell line. Features that the most active Sunfish amphiphiles have in common were the ability to form a "soft bilayer" which is reflected by adequate vesicle formation and complete DNA binding and the tendency to form lipoplexes with sizes in the micrometer range. In case of a comparable DNA binding capacity, Sunfish amphiphiles with preferences for highly ordered hexagonal H_{II} packing are likely to overcome the critical barrier in the transfection process, the endosomal escape, and to deliver the genetic payload in vitro more efficiently.

On the basis of the ability of some of these materials to form dispersed cubic phases, the possibility of a wider use of these molecules for drug delivery is suggested.

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