Interactions of Cationic Lipids with DNA: A Structural Approach

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ABSTRACT: Colloidal nucleic acid carrier systems based on cationic lipids are a promising pharmaceutical tool in the implementation of gene therapeutic strategies. This study demonstrates the complex behavior of DNA at the lipid–solvent interface facilitating structural changes of the lyotropic liquid-crystalline phases. For this study, the structural properties of six malonic acid based cationic lipids were determined using small- and wide-angle X-ray scattering (SAXS and WAXS) as well as differential scanning calorimetry (DSC). Selected lipids (lipid 3 and lipid 6) with high nucleic acid transfer activity have been investigated in detail because of the strong influence of the zwitterionic helper lipid 1,2-di(9Z-octadecenyl)-sn-glycero-3-phosphoethanolamine (DOPE) on the structural properties as well as of the complex formation of lipid–DNA complexes (lipoplexes). In the case of lipid 3, DNA stabilizes a metastable cubic mesophase with *Im3m* symmetry and an *Im3m* Q_{α}^c lipoplex is formed, which is rarely described for DNA lipoplexes in literature. In the case of lipid 6, a cubic mesophase with *Im3m* symmetry turns into a fluid lamellar phase while mixing with DOPE and complexing DNA.

1. INTRODUCTION

Cationic lipid–DNA complexes (lipoplexes) are used as vectors for efficient gene transfer into cells.^{1–3} To obtain outstanding systems, novel lipids are constantly synthesized and new formulations are persistently tested.^{4–8} To increase transfection rates, the cationic lipid structure is varied, the ratio with uncharged helper lipids is changed, different amounts of DNA are added, and lipoplexes are tested in systematical studies (e.g., Heinze et al.⁹ or Wölk et al.¹⁰).¹¹ Usually, only a few lipoplexes turn out to be successful gene delivery systems. In this case, it is important to investigate the physical–chemical properties of the lipids in order to understand the correlations to gene transfer rates. On the basis of complete characterizations of lipids and vectors, successful lipoplexes can be designed more easily in the future.

Especially important is the supramolecular assembly of lipids and lipoplexes in bulk. This topic is usually approached by X-ray scattering experiments. The first pioneering work was done by Rädler et al. with the finding of lamellar lipid–DNA complexes (L_{α}^c) as early as 1997.¹² One year later, the existence of inverted hexagonal structures (H_{II}^c) was reported,¹³ followed by the description of hexagonal structured lipoplexes (H_I^c).¹⁴ It is believed that by dependence on these structures different transfection rates can be achieved.² Lately, the discussion focuses also on complex structures, e.g., cubic lipid–DNA complexes and mixed phases.^{15–18} Recently, a breakthrough was achieved by Koynova et al. with a review

concerning over 30 cationic phospholipids.¹ The authors were able to show that not only the simple adaption of the H_{II}^c structure results in an efficient gene transfection^{13,19} but also the pathway into the cell and structural changes on this path seem to be important. For example, lamellar lipoplexes work very well if upon contact with (model) cell membranes a lamellar to nonlamellar phase transition occurs.¹ In summary, structural properties of lipids and lipoplexes cannot be related to transfection rates in a straightforward manner for all cases. Moreover, it is important to understand and explain each single case in order to identify different promising structures and mechanisms.

This work focuses on the physical–chemical characterization of six lipids designed for gene transfection (Figure 1). Synthesis and transfection results are already described.¹⁰ Lipids 1–3 and 4–6 possess different headgroups. The lipid chains are either saturated or unsaturated. Lipids 1 and 2 are not able to incorporate colipids and show no transfection activity.¹⁰ Lipids 3 and 6 are the most promising gene transfer vehicles in complexes with noncharged colipids and DNA. The mixtures of lipid 6 with DOPE 2:1 (n/n) and lipid 3 with DOPE 1:2 (n/n) exhibit transfection efficiencies higher than

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Figure 1. Chemical structures of lipids 1–6.

those of commercially available transfection reagents in cell culture models even in the presence of serum.¹⁰ The lipids 4 and 5 showed moderate transfection efficiency.¹⁰ The article by Wölk et al. also presents some preliminary data about the phase behavior of these lipids.¹⁰ However, the data required more detailed investigations to reveal structural characteristics which determine the degree of transfection efficiency, and this is the topic of the present work. In this work, supramolecular structures and thermodynamic properties of lipids 1–6 in bulk are investigated by X-ray scattering and DSC. The phase structures of the pure lipids as well as of the selected lipid mixtures, which are especially promising gene transfer systems, have been determined. The physical–chemical properties have been connected with the transfection results described earlier.¹⁰ The lipoplex formation between the lipid mixtures and DNA changes the supramolecular structures, demonstrating the complex interaction behavior between DNA and cationic lipids at the interface.

2. EXPERIMENTAL SECTION

2.1. Materials. For all measurements and sample preparations, Milli-Q Millipore water with a specific resistance of 18.2 MΩ·cm was used. DNA from calf thymus (product number D1501) was purchased from Sigma-Aldrich. The helper lipid 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) was purchased from Avanti Polar Lipids. The synthesis and purification of the cationic lipids were described earlier.¹⁰ All other chemicals were of analytical grade and used without further purification.

2.2. Differential Scanning Calorimetry (DSC). Lipids were dispersed in Milli-Q water to a concentration of 1 mg·mL^{−1}. After hydration, the aqueous dispersions were vortexed, sonicated, and heated (usually above the phase transition temperature of the corresponding lipid) several times. DSC measurements were performed on a microcalorimetry system (MCS DSC, MicroCal Inc., Northampton, MA). The sample and the reference were degassed for at least 20 min by stirring under a vacuum. After filling, the DSC cells were kept under 2 bar of nitrogen pressure to prevent evaporation at higher temperatures. The heating rate was 60 K·h^{−1}. Samples were scanned at least 4× up to ≈85 °C after being cooled to 10 °C to prove reproducibility.

For data treatment, the first heating scan was neglected. The main transition temperature from gel to liquid-crystalline state T_m was determined as the maximum of the corresponding heat flow curves. Transition enthalpies ΔH (main phase transition enthalpy ΔH_m and pretransition enthalpy ΔH_p) were determined by numerical

integration after baseline correction. An estimated error of about 10% has to be taken into account for transition enthalpies.

2.3. X-ray Scattering. Lipids and lipid mixtures were prepared as described in section 2.2. Lipid concentrations of 200 mg·mL^{−1} in Milli-Q water were used. Lipid–DNA complexes were prepared by adding a 2 mg·mL^{−1} DNA solution to the solid lipid mixture in the corresponding N/P ratio (ratio of moles of primary amine groups of cationic lipid to phosphate groups of DNA). The immediately formed complex was vortexed and sonicated for at least 15 min. If necessary, samples were centrifuged. Finally, the supernatant was separated from the lipoplex with a micropipette. Afterward, samples containing DNA were heated (2 K·min^{−1}) once up to 60 °C.

All samples were transferred into open glass capillaries (inner diameter 1.5 mm, GLAS). A flexible tube was used to connect a syringe to one open end of the capillary. The dispersions were sucked in, and the capillary was sealed on both sides with a Microtorch device. Samples were stored for 7–10 days at 4 °C before measurements.

Synchrotron small-angle (SAXS) and wide-angle X-ray scattering (WAXS) experiments were carried out at the Soft Condensed Matter beamline A2, HASYLAB, DESY (Hamburg, Germany). The standard procedures that were used have been described elsewhere.^{20–23} SAXS and WAXS data were taken simultaneously by a MAR CCD detector (Evanston, Illinois, USA) and a linear detector with delay line readout, respectively. The incoming beam had a wavelength of 0.15 nm, and the exposure time was between 20 and 300 s. The temperature was fixed during the measurements. After each heating step (2 K·min^{−1}), a waiting time of 5 min was used to ensure that the sample was in thermal equilibrium. Dry rat tail collagen (SAXS) and polyethylene terephthalate (WAXS) were used for calibration. Positions of the peaks were converted into reciprocal spacings $s = 1/d$, with d being the real space repeat distance of the lattice planes. In order to determine peak maxima and the full-width at half-maximum (fwhm) Δs , the experimental points were fitted with a Lorentzian function. Overlaying diffraction peaks were fitted by a superposition of Lorentzians (including diffuse scattering). Correlation lengths ξ were calculated using $\xi = 1/\Delta s$ of the first-order diffraction peak.

Lamellar phases can be identified by equidistant peaks in the SAXS patterns. In this case, the lamellar repeat distance d (lipid bilayer + one adjacent layer of water) can be calculated using $d = 1/s$. The relative peak positions of hexagonal and inverted hexagonal phases are given by $\sqrt{h^2 + k^2 - hk}$ (1, $\sqrt{3}$, $\sqrt{4}$, $\sqrt{7}$, $\sqrt{9}$, ...), with h and k being the Miller indices that characterize a set of lattice planes. The lattice parameter a for the hexagonal unit cell can be calculated using

$$a = \frac{2\sqrt{h^2 + k^2 - hk}}{\sqrt{3}s_{hk}}.$$

Cubic liquid-crystalline lattices show spacings in the ratio of $\sqrt{h^2 + k^2 + l^2}$ (1, $\sqrt{2}$, $\sqrt{3}$, $\sqrt{4}$, $\sqrt{5}$, $\sqrt{6}$, $\sqrt{9}$, ...), with h , k , and l being the Miller indices that characterize a set of lattice planes. The absence of peaks determines the lattice symmetry. Typical phases are $Ia3d$, $Pn3m$, $Im3m$, and $Pm3n$ (see Lindblom et al.²⁴ for details). To identify cubic phases, the experimentally obtained peak positions s are plotted as a function of the sum of the Miller indices to which the peaks are indexed. A linear function with an (0, 0) intercept is generated. The average lattice parameter a equals the inverse slope of this function.

3. RESULTS AND DISCUSSION

3.1. DSC with Pure Lipids. Figure 2 shows the thermograms of lipids 1–6. The transition temperatures and enthalpies are given in Table 1. The DSC curves of lipids 1 and 6 have already been presented in previous work¹⁰ but are shown here again for comparison.

Lipids 3, 4, and 6 show no phase transitions in the accessible temperature range. Lipid 5 shows a transition with a small ΔH_m at low temperatures. Lipid 2 reveals a slightly higher T_m with a similar value for ΔH_m . In contrast, the thermogram of lipid 1 shows a pretransition and a main phase transition at

Figure 2. DSC thermograms (heat flow dQ/dT as a function of T) of lipids 1–6. The red circles indicate the temperatures where X-ray data were taken. The dotted line represents an approximated separation of the two phase transitions of lipid 1 and is only to guide the eye. The dashed line defines the area that was integrated to obtain the total ΔH_m of lipid 1. Curves are shifted vertically for clarity. The DSC curves of lipid 1 and lipid 6 are taken from the literature.¹⁰

Table 1. Main Transition Temperatures T_m , Main Transition Enthalpies ΔH_m , Pretransition Temperatures T_m , and Pretransition Enthalpy ΔH_p of Lipids 1, 2, and 5^a

lipid	T_m [°C]	ΔH_m [kJ·mol ^{−1}]
lipid 1	$T_p = 29.4$	$\Delta H_p = 0.9$
	49.7, 56.5	50.6
lipid 2	31.4	5.1
lipid 5	14.9	~4.4

^aThe values are determined from the DSC curves presented in Figure 2. Transitions outside the investigated temperature window might be possible but cannot be determined from the presented experiments.

high temperatures. The latter can be divided into a broad peak and a sharp transition peak. ΔH_m of the complete transition is 50.6 kJ·mol^{−1} which is high compared to those of well-known lipids with similar chain lengths as dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylethanolamine (DPPE) that exhibit main phase transition enthalpies between 35 and 40 kJ·mol^{−1}.^{25,26} It is reasonable to assume that the high ΔH_m is not only a result of the chain melting in that region. Lipids with similar structures exhibit similar high transition enthalpies.²⁷ Contributions made by the breaking of hydrogen bonds between headgroups are discussed. Hydration of lipid heads might need additional energy as well and is discussed in sections 3.2 and 3.3.

Lipids 1–3 have the same headgroup but different chain patterns. The decrease of T_m and ΔH_m is clearly connected with the insertion of double bonds into the lipid chains (lipid 1, C₁₆ and C₁₆; lipid 2, C_{18:1} and C₁₆; lipid 3, C_{18:1} and C_{18:1}). The extension of the saturated chains would lead to an increase of the gel to liquid-crystalline transition temperature and enthalpy as observed, e.g., for phosphatidylcholines.²⁸ In the present case, the chain length increase is overcompensated by the introduction of the double bond which needs more space due to steric hindrance. Therefore, van der Waals interactions are reduced and lipids with more double bonds are found to have much lower phase transition temperatures, allowing lipid 3 to be in the liquid-crystalline state already at room temperature.

In the case of lipids 4–6 (the headgroup is more bulky than in the case of lipids 1–3), the tendency is not so straightforward. The insertion of one double bond leads to

an apparent phase transition in the case of lipid 5 (C_{18:1}, C₁₆). In contrast, lipid 4 (C₁₆, C₁₆) and lipid 6 (C_{18:1}, C_{18:1}) show no phase transition in the accessible temperature range. The large headgroup of this series seems to be the key factor governing the phase state. The mismatch in the area requirement of the chains and of the headgroup of lipid 4 disables favorable van der Waals interactions between the neighboring chains. Maybe the combination of one oleyl and one hexadecyl chain in mixed-chain lipids with large headgroups leads to increased chain–chain interactions due to more efficient packing with fewer defects, thereby enhancing the van der Waals contacts as compared to those of the analogues with two hexadecyl or two oleyl chains, and the phase transition of lipid 5 is in the accessible temperature range. Finally, the strong disorder induced by two double bonds leads again to the observed decrease of the transition temperature.

The correlation of DSC experiments with transfection results suggests that especially membrane fluidity plays an important role. While lipids 1 and 2 are still in the gel phase at room temperature, the other four lipids are in the liquid-crystalline state. This statement was mentioned in previous work¹⁰ but is proved in the present article by DSC and WAXS experiments (this section and section 3.2). The inability of lipids 1 and 2 to mix with noncharged helper lipids which are in the liquid-crystalline state seems to be connected with the immobility of the lipid chains in the gel state.^{52,53} According to Sackmann and Demus, two compounds never mix completely if they form different phase structures.⁵⁴ An important role can be, therefore, ascribed to the degree of saturation which is closely related to the phase state and seems to be connected with the efficiency data published earlier.¹⁰ This work confirms former studies that were also able to show that especially C_{18:1} chains are more suitable for gene transfection.^{4,7,27,29} Also the headgroup affects the efficiency. A certain increase in gene transfer activity has even been observed for lipids with two saturated chains by increasing the size of the headgroup (lipid 4 → weak transfection compared to lipid 1 → no transfection).¹⁰ Obviously the increase in the space requirement of the headgroup reduces the chain–chain interactions. Nevertheless, the introduction of oleyl chains has more pronounced positive effects on the transfection efficiency. The delicate interplay between hydrophobic and hydrophilic parts is obviously very important for successful transfection. In any case, the most promising gene transfer results were obtained with lipids 3 and 6 with high degrees of unsaturation and, therefore, high fluidities.

3.2. WAXS Patterns of Pure Lipids. Figure 3 shows WAXS patterns of all lipids at selected temperatures according to the DSC thermograms. If no phase transition was observed, only one experiment was performed at 20 °C to identify the phase state. The scattering curves of lipid 1 at 20 and 60 °C as well as the curve of lipid 6 have been already published¹⁰ but shown here again for comparison. The chain lattice parameters are given in Table 2.

A broad halo in the WAXS region at 20 °C indicates that the lipids 3, 4, 5, and 6 are in the liquid-crystalline state (L_a) (see section 3.1) and proves the assumptions that were made earlier.¹⁰ The small remaining peak of lipid 4 is discussed in section 3.3. For lipid 5, the gel state can be reached at temperatures ≤10 °C. At 20 °C, the corresponding gel peak with weak intensity is still present, showing that the phase transition is not yet completed. An orthorhombic lattice with

Figure 3. WAXS patterns of lipids 1–6 at selected temperatures. The arrows indicate a Bragg peak that is not arising from the chain lattice of lipid 1. Numbers indicate the relative intensities (normalized after integration to the intensity observed at 20 °C) of the hydrogen bond peak of lipid 1 after baseline correction. Curves are shifted vertically for clarity. The scattering curves of lipid 1 at 20 and 60 °C as well as of lipid 6 are taken from literature.¹⁰

Table 2. Peak Positions s and Cross-Sectional Areas A of Lipids 1–6 at Different Temperatures T Derived from the WAXS Data

lipid	T [°C]	s_{HB}^a [nm ⁻¹]	s_{11} [nm ⁻¹]	s_{20} [nm ⁻¹]	s_{HG}^b [nm ⁻¹]	A [Å ²]
lipid 1	20	2.10	2.38	2.59	2.80	19.3
	26	2.10	2.38	2.58	2.81	19.4
	31	2.10	2.38	2.58	2.80	19.4
	40	2.10	2.38	2.57	2.79	19.4
	50	2.10	2.37	2.57		19.6
	60		liquid like			
lipid 2	20	2.09	2.36	2.51	2.82 ^c	20.0
	40		liquid like			
lipid 3	20		liquid like			
lipid 4	20		liquid like			
lipid 5	4		2.35	2.49		20.2
lipid 6	20		liquid like			

^a s_{HB} represents the hydrogen bond peak. ^b s_{HG} represents the headgroup lattice peak. ^cLow intensity.

of lipid 1 is very tight and suggests a subgel phase (L_c). A unit cell of $5.0 \times 7.7 \text{ Å}^2$ at 20 °C can be compared to the well-known herringbone arrangement of hydrocarbon chains in organic crystals ($5.0 \times 7.5 \text{ Å}^2$).³⁴ Another peak is found at higher s values (arrows in Figure 3), which cannot be assigned to the chain lattice. Its origin is based on an independently formed headgroup lattice (s_{HG} peak). Unfortunately, this peak was the only one available in the range of the detector. A headgroup lattice structure can, therefore, not be derived. It should be mentioned that a similar structure is obtained by grazing incidence X-ray diffraction experiments at the air–water interface.³³ The accessible s -range is much larger, allowing the observation of a number of scattering peaks which allowed identification of the lattice structure.

The lattice structure of lipid 1 does not change upon increasing the temperature to 40 °C. Thus, the pretransition found in the DSC thermogram cannot be assigned to the rearrangement of the chain lattice. At 50 °C, s_{HG} and the other peaks decrease strongly in their intensities. The chains start to melt at temperatures above 40 °C and not only during the second transition (sharp DSC peak at 56.5 °C). Because of the tight chain packing of lipid 1 and the additional headgroup interactions, water molecules cannot interact easily with the lipid heads at low temperatures. With increasing temperature, the s_{HB} peak decreases continuously in intensity (see relative intensities in Figure 3), showing increasing disorder in the headgroup region. The loosening of the hydrogen bonds in the headgroup region favors the penetration of water molecules. Therefore, a certain hydration heat is constantly produced, helping to break the hydrogen bonds over a large temperature range. The chains of already fully hydrated lipids (region of the broad DSC transition) start to melt first; the less hydrated lipids follow at higher temperatures. At 60 °C, the chain lattice of lipid 1 disappears completely. The transition from the L_c state to a liquid-crystalline state seems to occur without any intermediate phase state. The chains of lipid 2 have already melted at 40 °C. It is important to note that the oleylamine from Sigma-Aldrich used for the synthesis of the lipids (oleylamine is bound via an amide bond as R_1 ; see Figure 1) contains only 70% of the $C_{18:1}$ chain and different saturated chains but is a primary amine to 98%. The synthesis and purification lead to a slightly better purity ($C_{18:1}$ 81 mol %, $C_{18:0}$ 6 mol %, $C_{16:0}$ 10 mol %, and $C_{14:0}$ 3 mol %).³³ This oleylamine is used because of the idea of a cheap large scale production of such lipids which are successfully used in transfection experiments. Knowing that, one would expect broader transition ranges for the lipids containing the unsaturated chains.

The WAXS data gives an explanation for the preparation of lipid dispersions (in general mixtures with uncharged helper lipids) used in transfection experiments. Lipid 1 shows strong self-association on the basis of interactions between chains and headgroups. The tight packing and the L_c state are disadvantageous for the formation of lipid mixtures which have a charge density suitable for transfection. The helper lipids cannot easily be incorporated into the bilayers of this lipid, and the complexes are, therefore, unstable. Lipid 2 is found to be in the gel state but with fewer headgroup interactions due to the larger area required by the chains. However, the inflexibility of the lipid chains seems to be enough to prevent effective incorporation of helper lipids. In contrast, lipids 3–6 which are in the liquid-crystalline state at room temperature exhibit a

270 tilted chains (L_B) and a cross-sectional area typical of gel
271 phases can be found at 4 °C.

272 The WAXS patterns of lipids 1 and 2 show more details than
273 those of the other lipids. At 50 °C (below the sharp phase
274 transition), a Bragg peak at $s_{HB} \sim 2.10 \text{ nm}^{-1}$ ($d = 0.476 \text{ nm}$) is
275 an indication of hydrogen bonds between the lipid headgroups.
276 This peak was first assigned to hydrogen bonds between N—
277 H...O=C groups of β -sheets of proteins.^{30–32} Lately, this has
278 been discussed with regard to headgroup interactions of lipids
279 with similar structures as well.^{27,33} The lattice structure of lipid
280 2 below T_m is gel-like (L_B) whereas the packing of the chains

Table 3. Structure, Peak Positions s , and Calculated Interlamellar Repeat Distance d of Lipids 1–6 at Different Temperatures T Obtained from the X-ray Data

lipid	T [°C]	structure	s_{001} [nm ⁻¹]	s_{002} [nm ⁻¹]	s_{003} [nm ⁻¹]	s_{004} [nm ⁻¹]	d [nm]
lipid 1	20	lamellar, correlated + uncorrelated (brick structure, vesicles)		0.108	0.158	0.210	18.8
	26			0.109	0.158	0.211	18.7
	31			0.110	0.162	0.211	18.5
	40			0.116	0.170	0.222	17.6
	50			0.119	0.176	0.232	17.0
	60		0.062 ^a	0.127	0.192		15.7
lipid 2	10	lamellar, correlated + uncorrelated (brick structure, vesicles)		0.112	0.164	0.210	18.3
	20			0.114	0.168	0.217	17.9
	30			0.115	0.172	0.224	17.6
	40			0.107	0.161	0.215	18.6
lipid 3	20	lamellar	0.154	0.307			6.50
lipid 4	20	uncorrelated (vesicles) + lamellar	0.099	0.198	0.299		10.08
lipid 5	4	lamellar correlated + uncorrelated	0.183	0.366			5.46
lipid 6	20	cubic					

^a $a^b = 13.42$ nm

^aNo Lorentzian fit, the peak is found as a small shoulder on direct beam. ^bLattice parameter for the unit cubic cell.

better ability to incorporate helper lipids and to build mixtures with a charge density suitable for effective transfection.³

3.3. SAXS Patterns of Pure Lipids. SAXS patterns reveal different structural assemblies of lipids 1–6. In Table 3, the structural parameters derived from the X-ray data are summarized. The X-ray patterns for lipids 1 and 2 are shown in Figure 4.

Figure 4. SAXS patterns of lipids 1 and 2 at different temperatures. Curves are vertically shifted for clarity.

The profiles show distinct Bragg reflexes which indicate a liquid-crystalline phase in the presence of uncorrelated bilayers,³⁵ which result in diffuse scattering (broad background). The reciprocal spacings of the quasi Bragg peaks are found to be roughly in a ratio of 1:1.5:2 for all diffraction patterns (see Table 3). An equidistant spacing (1:2:3:...) characteristic for lamellar phases can only be achieved if it is assumed that the first-order scattering peak is located at very low s values which are out of the detector range. This is, in fact, the case and can be supported by a small shoulder observed at $s = 0.062$ nm⁻¹ for lipid 1 at 60 °C. This shoulder appears in the intensity profile of the direct beam (not shown).

From the small s values, unusually large d values are calculated (between ≈ 16 and ≈ 19 nm, see Table 3). Because of tight packing in the case of lipid 1 (see section 3.2), it can be expected that chain and headgroups are almost completely stretched. Therefore, the membrane thickness is estimated to be between 7 and 8 nm. The interlamellar repeat distances, d , calculated for both lipids are much larger than estimated. The cause can be either that there is a unit cell that does not contain one lipid bilayer but a double bilayer or, what is more plausible, that the large interspace is filled with water and results from strong repulsive forces between the positively charged lipid bilayers. The hypothesis of a unit cell with two lipid bilayers is described in the literature for different systems of mixed lipids.^{36,37} However, one condition is that both bilayers showed different properties: e.g., each one consisted of a different amphiphile. In the present case, structural differences in single component systems are not reasonable whereas the hypothesis of an extended water layer between the lipid bilayers is more favored. Therefore, we consider that the bilayers of both lipids are separated by ~ 10 nm of water.

In order to obtain the electron density profiles (Figure 5), the X-ray patterns of lipids 1 and 2 at 20 °C were fitted exemplarily according to Pabst.^{35,38,39} The software that was used (Global Analysis Program v. 1.3) allowed the fitting of data that consisted of (quasi) Bragg peaks and diffuse scattering. The fits are in good agreement with the experimental data points and prove that the data can originate from one bilayer with a huge interlayer spacing filled only with water. The membrane thickness (taken at the fwhm of the headgroup Gaussians) is 71 Å for lipid 1 and 64 Å for lipid 2. This difference is in good agreement with the WAXS data. Due to the tight packing of lipid 1 molecules, the headgroups are more stretched than the ones of lipid 2 (reduced packing density of the chains). Additionally, the chains of lipid 2 have the possibility to interdigitate partially due to the asymmetric chain pattern.

The water-containing interlayer amounts to 122 Å for lipid 1 and 114 Å for lipid 2. We asked why a correlation over such large distances exists so that clear Bragg reflexes appear (without such a correlation only a very broad intensity distribution would be expected) and which driving force is behind it. The fitting parameters suggest that the average

Figure 5. X-ray diffraction patterns (○) and corresponding electron density profiles of lipids 1 and 2 at 20 °C. The solid red lines represent the fits. A model that is able to explain the large d values is shown on the bottom.

Figure 6. SAXS pattern of lipid 3 at 20 °C.

Upon increasing temperature, d of lipid 1 decreases constantly until the broad phase transition range starts at 40 °C. The decrease is much more drastic in the transition range between 40 and 60 °C measured by DSC (Table 3). The first process can be explained by hydration of headgroups already discussed in the WAXS section (see section 3.2). The higher the temperature the greater is the number of water molecules that penetrate into the headgroup region. The following drastic decrease of d at higher temperature can be assigned to the main phase transition (see section 3.1). The melting of the chains leads to the shrinkage of the bilayers. A similar decrease of d can be observed at increasing temperature for lipid 2 (Table 3). However, after the phase transition, d increases suddenly. Such behavior could be explained by structural rearrangement of the lipids. A more plausible explanation is at this point difficult to find.

The structurally similar lipids 1–3 show that a certain balance in the area requirement of headgroups and tails is necessary to form certain structures. While lipids 1 and 2 form aggregate-like brick structures, the introduction of one double bond in each chain of lipid 3 changes the curvature of the bilayer. Thus, a simple lamellar structure is established.

The SAXS patterns of lipids 4 and 5 are presented in Figure 7. Lipid 4 shows mainly diffuse scattering of uncorrelated bilayers (vesicular structures). The occurring Bragg peaks on top are in equidistant spacing and indicate a lamellar phase (Table 3). They cannot be compared to the ones of lipids 1 and 2. The d spacing is much smaller and might result from a

Figure 7. SAXS pattern of lipids 4 and 5 at different temperatures.

number of bilayers per scattering domain is ~ 2.5 for both lipids and the percentage of diffuse scattering is high. However, there are still some bilayers that are correlated over this distance. One explanation might be a brick-like structure of the membranes. Due to strong interactions of head and tail groups, the membranes are very rigid and pack, possibly, as illustrated in Figure 5 without breaking apart. The “bricks” are built due to the large area requirement of the headgroups, unlike the chains, which prohibits an extended lamellar arrangement and leads to the shown structures (no continuous bilayers). Such a model also means that the lipid forms into multilamellar stacks rather than vesicles. Lyotropic phases with large interlamellar water layers have been described (“iridescent phases”).^{55,56} It is believed that electrostatic forces between charged surfactants are necessary. However, they are too small to explain the stability of the systems. Therefore, it is assumed that the bilayers are strongly fluctuating and are stabilized by steric repulsion.

This assumption is supported by the supramolecular structure of lipid 3 at 20 °C. Due to the introduction of a double bond in each chain, a “normal” lamellar phase forms at 20 °C (Figure 6). Chains and headgroup now need similar amounts of space. The sharp Bragg peaks with an equidistant spacing of $s_{001} = 0.154$ and $s_{002} = 0.307$ nm^{−1} indicate a continuous bilayer system with a correlation length that is ~ 10 times higher ($\xi = 147$ nm) than those of lipids 1 and 2. The lamellar repeat distance is 6.5 nm and is reasonable for a lipid of that size with chains in L_α -state.

single monolayer. An attempt to fit the data, as performed for lipids 1 and 2, failed. Most likely this structure appears only in high concentrated bulk systems where some lipid molecules cannot be fully hydrated. It was shown in section 3.2 that a WAXS signal of weak intensity is evidence for the presence of an ordered chain lattice. It is, therefore, believed that this inhomogeneity is a lamellar phase which is in the gel state. However, no phase transition could be detected in the highly diluted system in the DSC experiment.

Lipid 5 shows a lamellar phase with equidistant spacing of the Bragg peaks at 4 °C. Upon heating to 20 °C, the system exhibits pure diffuse scattering. The interbilayer positional correlations are lost. This so-called unbinding transition has been observed before and usually coincides with the $L_{\beta'} \rightarrow L_{\alpha}$ transition (as in the present case).⁴⁰ It is explained by a drastic reduction of the bending rigidity upon entering the L_{α} -phase. The stronger steric repulsion leads to the unbinding of the stacks.

By the comparison of lipids 4 and 5 with the first ones, similar structural relationships can be derived. Vesicular structures are found for lipid 4 due to differently sized hydrophilic and hydrophobic parts. The membranes will bend to a high degree, and continuous bilayers cannot be formed. Only with the introduction of one double bond into lipid 5 are the chains able to sterically compensate for the size of the headgroup and a lamellar phase is established.

A completely different structure is found for lipid 6 at 20 °C (Figure 8). The peaks can be indexed in the order $\sqrt{2}$, $\sqrt{4}$,

Figure 8. SAXS pattern of lipid 6 at 20 °C. The inlay shows the linear fit of the peak positions s as a function of $(h^2 + k^2 + l^2)^{1/2}$.

$\sqrt{6}$, $\sqrt{8}$, $\sqrt{10}$, $\sqrt{12}$, $\sqrt{16}$, and $\sqrt{18}$. This pattern is typical for a body-centered cubic (bcc) lattice (=Im3m symmetry).²⁴ The inlay of Figure 8 shows the linear fit of the Miller indices as a function of s with a correlation coefficient of $r = 0.9992$. It should be mentioned that the reflection for $\sqrt{14}$ was not found (intensity already too low). The lattice parameter a obtained from the linear fit is 13.42 nm.

Finally, the structural assemblies of lipids 1–6 shall be discussed with respect to transfection efficiencies. It is eye-catching that lipids 1 and 2 show very similar structures with very large d values. The unusual, brick-like structures seem to be disadvantageous for mixtures with colipids. In contrast, lipids 3–6 form completely different structures. All of them were able to establish colloidal systems with particle sizes below 200 nm in a mixture with helper lipids (DOPE or cholesterol) and later on in complexes with DNA.¹⁰ Never-

theless, only the lipoplexes of lipid 6, a lipid which forms cubic mesophases, and lipid 3, a lipid which prefers lamellar assembling, are highly effective.¹⁰ For a better understanding, we focus now on these two lipids.

3.4. SAXS Patterns of Lipid Mixtures and Complexes with DNA. In order to correlate transfection experiments with supramolecular structures of lipoplexes, closely related model systems are investigated. Lipid mixtures with colipids and complexes with DNA are chosen exemplarily according to Wölk et al.¹⁰ All systems under investigation show outstanding transfection results in in vitro cell culture models even in comparison with commercially available reagents.¹⁰ In Table 4, structures and lattice parameters are presented.

Table 4. Structure and Interlamellar Repeat Distance d (if Lamellar) or Lattice Parameter a of the Unit Cell (if Hexagonal or Cubic) of Lipid Mixtures with and without DNA at 20 °C Obtained from the X-ray Data^a

sample	structure	d/a [nm]
lipid 3: DOPE 1:2	L_{α}	5.92
	H_{II}	7.33
lipid 3: DOPE 1:2 (after heating to 80 °C)	L_{α}	5.87
	H_{II}	7.34
	$Im3m$	16.5
lipid 3: DOPE 1:2 + DNA	L_{α} (DNA)	7.21
	$Im3m$ (DNA)	20.6
lipid 6: DOPE 2:1	L_{α}	6.41
lipid 6: DOPE 2:1 + DNA	L_{α}	6.45
	L_{α} (DNA)	7.41

^a L_{α} represents the lamellar, liquid-crystalline phase and H_{II} represents the inverted hexagonal phase.

Figure 9 shows the mixture of lipid 3 with DOPE in a ratio of 1:2 (n/n) directly after 7 days of storage at 4 °C (=thermal equilibrium) and 5 min after heating once to 80 °C and cooling to 20 °C with a cooling rate of 2 K min^{−1}. In both cases, a complex SAXS pattern is found. In thermal

Figure 9. SAXS patterns of lipid 3 in 1:2 ratio (n/n) with DOPE before (a) and after (b) heating to 80 °C (2 Kmin^{−1}), cooling (2 Kmin^{−1}), and a waiting period of 5 min at 20 °C. The lamellar phase of lipid 3 and DOPE is indexed with L_{α} . The peaks of the inverted hexagonal phase of pure DOPE are marked with H_{II} . The additional cubic phase with $Im3m$ symmetry is indexed using square roots. Curves are shifted vertically and the intensity of (b) was increased by a factor of 2 for clarity. The inlay shows the linear fit of the peak positions s as a function of $(h^2 + k^2 + l^2)^{1/2}$ resulting from pattern (b).

equilibrium, two coexisting phases are present. A lamellar L_α -phase (halo in WAXS region) with equidistant spacings of the peaks can be seen. The interlamellar repeat distance is ~ 0.6 nm smaller than that found for the pure lipid 3. This can be assigned to the interplay of lipid 3 with DOPE. The chains of both lipids are in a liquid-like state; therefore, the thickness of the hydrophobic part does not change so dramatically. However, interactions between the headgroups can reduce the effective extension of the hydrophilic region. A change in the interlayer water thickness is also possible. The SAXS patterns show additional peaks with low intensities which are typical for the H_{II} -phase of pure DOPE in water.⁴¹ It can be concluded that a maximum miscibility of DOPE is reached in the lamellar phase of lipid 3. Above a certain DOPE ratio, the H_{II} -phase of phase-separated DOPE is formed additionally. If the lipid mixture is heated to 80 °C and immediately cooled to 20 °C, the same two phases can be found after 5 min (Figure 9). However, the peaks representing the L_α -phase are strongly diminished in intensity and a third phase appears. This phase can be indexed as a cubic phase with $Im3m$ symmetry (see inset of Figure 9). Only six peaks were found, but their positions are in almost perfect correlation with the linear fit ($r = 0.99995$). Increasing temperature leads to a change of miscibility of DOPE with lipid 3. The fast cooling process allows the conservation of this state (some phase-separated DOPE is still present), and the cubic phase can be seen. The $Im3m$ phase becomes especially interesting if complexes of the lipid 3:DOPE mixture (1:2 n/n) and DNA (N/P 4:1) are considered. This model system represents a real transfection system that turned out to be promising in the presence and absence of serum.¹⁰ Figure 10 shows the corresponding SAXS pattern.

Figure 10. SAXS pattern of lipid 3 in 1:2 ratio (n/n) with DOPE complexed with DNA (N/P 4:1) at 20 °C. The lamellar L_α -phase built by lipid 3, DOPE, and DNA is indexed with L_α (DNA). The coexisting cubic phase with $Im3m$ symmetry complexed with DNA is indexed using square roots. The inset shows the linear fit of the peak positions s as a function of $(h^2 + k^2 + l^2)^{1/2}$ of the cubic phase.

Again two coexisting phases are found: (i) A lamellar L_α -phase (halo in WAXS region) represented by a peak pattern of equidistant spacing. The interlamellar repeat distance d is increased by 1.3 nm if compared to that of the system without DNA (Table 4). (ii) A cubic phase with $Im3m$ symmetry. It was possible to assign 10 peaks and fit s versus the Miller indices linearly ($r = 0.99991$). The lattice parameter of the unit cell a is increased by 4.1 nm (Table 4). It can be concluded

that the expansion is caused in both cases by the integration of DNA strands. In the case of lamellar phases, as usual, d does not increase enough to accommodate DNA with a diameter of ~ 2 nm (B-form).^{12,42} The additionally needed space is gained in a different way (e.g., the squeezing out of water between the bilayers, changing the tilting of the lipid chains, or disordering of the chains). The DNA-bearing cubic phase with $Im3m$ symmetry is a special lipoplex structure which needs additional attention. Cubic lipoplexes with different symmetries are rarely described after interaction of small siRNA with cationic lipid mixtures.^{43–45} That long DNA molecules induce the formation of cubic lipoplexes is a rather new observation: Bilalov et al. described a DNA-bearing cubic phase with $Ia3d$ symmetry.¹⁵ McLoughlin et al. described a cubic lipoplex with $Pm3m$ symmetry for cationic surfactant/DNA mixtures which exhibit low effectivity and are highly toxic in mammalian cells, comparable to the systems described by Zhou et al.^{17,18} Cubic phases are intermediates in membrane fusion processes.⁴³ In literature, it is proposed that cubic phases have efficient fusogenic properties which are independent of the membrane charge density and consequently trigger endosomal escape.^{44,45,49} However, a generalized connection between cubic lipoplex structures and high transfection efficiency does not exist. Koynova et al. described that several lipoplex structures undergo phase transitions toward cubic phases when interacting with model biomembranes.⁴⁶ Our findings suggest that combined lamellar and cubic lipoplexes, which are formed before the contact of the lipoplexes with cellular lipids, can also trigger transfection efficiency. Leal et al. were also able to connect siRNA-loaded cubic lipoplex structures with an efficient nucleic acid delivery.⁵⁰ Nevertheless, recently, we demonstrated that a lipoplex formulation with a lamellar superstructure was more efficient than a lipoplex with a cubic structure.⁵¹ Obviously, more factors (for instance lipoplex stability, lipoplex charge, and binding strength between lipids and DNA) than the fusogenic potential of cubic phases affect the transfection efficiency.

The coexistence of both phases might be decisive considering the transfection results with and without serum. It is shown that L_α -phases are effective gene vectors for applications with serum and in vivo studies.^{2,47,48} It is proposed that the tight packing of the lamellar structures prevents the dissociation of lipoplexes enforced by molecules in biological fluids. In contrast, in the absence of serum, usually H_{II} or cubic phases are more effective gene vectors.^{1,2} Therefore, coexistence of two phases could be the reason for effective gene transfer with and without serum. In each case, one phase will be more effective. One possible scenario for the combination of both structures is a cubic structure embedded in a lamellar one (see Figure 12).

Lipid 6 shows especially high transfection results in a 2:1 mixture (n/n) with DOPE complexed with DNA (N/P 1.67:1).¹⁰ The SAXS patterns of the model system with and without DNA are shown in Figure 11.

In the case of the lipid mixture, an equidistant peak pattern indicates that a lamellar L_α -phase is formed (halo in WAXS region). The addition of DOPE to lipid 6 transforms the lattice structure from cubic to lamellar. The same SAXS peak pattern is present in the complex with DNA. Furthermore, a second set of peaks with high scattering intensities appears and indicates the integration of DNA between bilayers. The interlamellar repeat distance is increased by 1.0 nm compared to that of the pure lipid mixture. It can be concluded that a small amount of

Figure 11. SAXS patterns of lipid 6 in 2:1 ratio (n/n) with DOPE at 20 °C (a) and in complex with DNA (N/P 1.67:1) (b) at 20 °C. Lamellar phases of the lipids without DNA are indexed with L_α . The lamellar phase containing DNA is indexed with $L_\alpha(\text{DNA})$. The broad scattering peak marked with d_{DNA} represents the one-dimensional repeat distance of aligned DNA strands.

gene transfer activity of lipid 3 with DOPE, in the presence and absence of serum, might be based on the coexistence of these two phases.

Figure 12. Schematic illustration of the alteration of the supramolecular self-assembling structures of lipid 3 and lipid 6 after addition of DOPE and interaction with DNA.

4. CONCLUSIONS

The physical–chemical properties of six new lipids designed for gene transfection were investigated in bulk using DSC and SAXS/WAXS. The lipids can be categorized into two groups with different headgroup structure: (i) lipids 1–3 and (ii) lipids 4–6. In these groups, the saturation degree of the chains is varied.

In (i), lipids 1 (saturated) and 2 (one double bond) are strongly self-associating. Interactions between lipid chains and between headgroups are found. Lipid 1 is in the subgel state, and lipid 2 is in the gel state at room temperature with strongly reduced mobility compared to liquid-crystalline phases. Both lipids arrange in brick-like structures. This disadvantageous behavior explains the low affinity to helper lipids which were not incorporated. Therefore, both lipids are not suited for gene transfer.

Due to insertion of one double bond in each chain, the phase transition temperature (from gel to liquid-crystalline state) of lipid 3 is decreased below room temperature and a lamellar phase is formed. Increased fluidity in the liquid-crystalline state leads to a possible incorporation of zwitterionic helper lipids. In mixtures with DOPE, the lamellar structure is preserved and only upon heating are changes to coexisting lamellar and cubic structures observed. The cubic one is, therefore, a metastable mesophase. The addition of DNA leads to similar coexisting phases with larger lattice parameters due to the incorporated DNA. The often described L_α^c lipoplex structure and a rarely observed $Im3m$ Q_α^c lipoplex structure occur. Because the coexistence is found without heating, DNA has a stabilizing effect on the assembly in cubic structures. The alteration of the self-assembled supramolecular structure of lipid 3 with the addition of DOPE and after interaction with DNA is summarized in Figure 12. The high

In (ii), interactions of chains are disrupted by larger headgroups which need more space. Therefore, all lipids are in the liquid-crystalline state at room temperature. The fluidity and mobility of the lipids is increased, and the incorporation of noncharged or zwitterionic helper lipids is possible. Interestingly, lipid 6 with two double bonds shows again the highest transfection rates. The pure substance forms a cubic phase with $Im3m$ symmetry, while in a mixture with DOPE and DNA, lamellar phases are found. Therefore, the results of Koynova et al.¹ showing that lamellar phases can be efficient gene carriers, like H_{II} phases, are supported. In contrast to lipid 3 ($L_\alpha \rightarrow L_\alpha/Q_\alpha$ (metastable) $\rightarrow L_\alpha^c/Q_\alpha^c$ $Im3m$), the self-assembling behavior of lipid 6 changes after adding DOPE and complexing DNA in the following way: Q_α $Im3m \rightarrow L_\alpha \rightarrow L_\alpha^c$ (see Figure 12).

It can be concluded that double bonds are crucial for the phase structures of the presented lipids and for transfection efficiency. Saturated lipids especially show disadvantageous rigid molecular assemblies. With increasing degree of unsaturation, significant structural changes occur together with increasing gene transfer activity. Furthermore, DNA can change the supramolecular self-assembly in an unpredictable manner.

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Notes

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