

DOPE–oleic acid–Ca²⁺ as DNA condensing agent

Original Paper

Galliková D.¹✉, Liskayová G.¹, Búcsi A.¹, Hubčík L.¹, Martínez J.C.², Uhríková D.¹

¹Comenius University in Bratislava,
 Faculty of Pharmacy, Department of Physical
 Chemistry of Drugs, Bratislava, Slovakia
²ALBA Synchrotron Light Source, Cerdanyola
 del Vallés, 08290, Barcelona, Spain

Received 21 June, 2017, accepted 22 November, 2017

Abstract Phospholipid-based non-viral carriers composed of neutral phospholipid dioleoylphosphatidylethanolamine (DOPE) and the binary mixture DOPE–oleic acid (OA) are examined as potential DNA delivery vectors. The process of DNA condensation in the presence of Ca²⁺ ions has been monitored through changes in emission intensity of fluorescent probe ethidium bromide. The decline in fluorescence intensity with increasing Ca²⁺ concentration at two different time intervals was correlated with the binding capacity of complexes and possible release of DNA from the complex. The microstructure of DOPE–OA mixtures at different OA/DOPE molar ratios and that of DOPE–OA–DNA–Ca²⁺ complexes were determined using synchrotron small angle X-ray diffraction (SAXD). We identified inverted hexagonal phase H_{II} as the dominant structure. OA affects the lattice parameter of H_{II} formed by DOPE. With the increasing OA/DOPE molar ratio, the lattice parameter decreases, which results in significantly lower fraction of DNA bound to the OA-enriched complexes.

Keywords DOPE – oleic acid – calcium – DNA – SAXD

INTRODUCTION

Gene therapy provides a unique approach to treat diseases by delivering a genetic material into the nucleus in order to correct the loss of function or to express the deficient gene product at physiological level (Glover et al. 2005). Owing to concerns about using viral gene transfer vehicles non-viral vectors represent promising alternative disposing multiple benefits such as biosafety, less immunotoxicity, low cost and ease of production (Ramamoorthi and Narvekar 2015). Chemical non-viral nucleic acid delivery systems are, amongst others, cationic lipid–DNA complexes called lipoplexes. For this purpose, hundreds of lipids have been developed for gene transfer; however, lipoplexes still face a lack of transfection efficiency. In our laboratory, lipidic carriers for DNA have been studied previously (Hubčík et al. 2014, 2015, Lengyel et al. 2011, Liskayová et al. 2017). The structure of lipid-based systems has been investigated and reviewed extensively for the past two decades (Uhríková et al. 2007, Uhríková et al. 2005, Pullmannová et al. 2012, Puri et al. 2009).

Dioleoylphosphatidylethanolamine (DOPE) was reported to stabilise lipoplexes and enhance *in vivo*

cationic lipid-dependent delivery of plasmid DNA to the mouse brain (Hassani et al. 2005). Free fatty acids (FFAs) have the effect on the organisation and local structures of membranes (Seddon et al. 1997, Templer et al. 1998). They also show the ability to modulate cell functions (Pérez et al. 1997). The effect of oleic acid (OA) on phosphatidylethanolamine (PE) membranes was studied previously (Funari et al. 2003, Prades et al. 2003, Gillams et al. 2014). It was found that OA induces important alterations in the supramolecular organisation of PE derivatives and also the thermal sensitivity of DOPE is altered by the presence of OA. OA modulates the membrane structure, inducing negative membrane curvature strain on DOPE caused probably by the lateral pressure of OA on fatty acids of DOPE.

In this study, we have prepared complexes made of OA–DOPE and studied their capability of DNA compaction in the presence of Ca²⁺ ions as one of the essential steps before DNA delivery. Fluorescence study provides valuable information about the binding capacity of lipid-based carriers for DNA (Geall and Blagbrough 2000). We have examined DNA-binding affinity and its

* E-mail: gallikova@fpharm.uniba.sk

condensation using ethidium bromide exclusion assay. We have used the small-angle X-ray diffraction (SAXD) to study the structure of lipoplexes as a function of their composition. Lipoplexes presented in this study have not been reported to be prepared and investigated yet and these new biophysical data may contribute to the knowledge of physico-chemical properties at designing non-viral lipid-based delivery vectors for the transfer of nucleic acids.

METHODS

Materials

Neutral phospholipid DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) was purchased from Avanti Polar Lipids, Inc., USA. Highly polymerised DNA (sodium salt) type XIV from herring testes (average Mr of nucleotide = 308) and OA (analytical standard) were purchased from Sigma Aldrich, USA. NaCl, NaOH and 35% HCl of analytical purity were obtained from Lachema, Brno, Czech Republic. Ethidium bromide (EtBr) and calcium chloride dihydrate for analysis were purchased from Merck, Germany. The chemicals were of the analytical grade and used without further purifications. The aqueous solutions were prepared with redistilled water.

Preparation of DNA solution/UV-VIS measurements

DNA was dissolved in 0.15 mol/l NaCl. The precise value of DNA concentration was estimated spectrophotometrically (Agilent 8453 Diode array spectrophotometer), according to $c_{\text{DNA}} = A_{260} \cdot 4.7 \times 10^{-5}$ [g/ml], where A_{260} is the absorbance at wavelength $\lambda = 260$ nm. The concentration of DNA is referred as the molar concentration of DNA bases. The purity of DNA was checked by measuring the absorbance A_{λ} at $\lambda = 260$ and 280 nm and evaluating A_{260}/A_{280} . UV-vis was used to determine the amount of DNA bound in the complexes of samples for SAXD experiments by applying the method described in Lengyel et al. 2011 and Rajnohova et al. 2010.

Preparation of complexes for fluorescence experiments

DOPE and OA-DOPE mixtures were dissolved in chloroform and mixed to obtain a mixture with the required OA/DOPE molar ratio. Lipid mixtures were dried under a stream of gaseous nitrogen and the residue of chloroform was removed under vacuum. Each sample contained 1.2 mg of DOPE and the corresponding amount of OA with respect to the OA/DOPE molar ratio. The dry mixtures were hydrated using the solution of

DNA in 150 mmol/l NaCl at the ratio of DOPE/DNA = 8 (mol/base) and the solution of CaCl₂·2H₂O to obtain the required molar concentration of calcium cations. The volume of samples was adjusted to 2.5 ml using 150 mmol/l NaCl. Afterwards, samples were homogenised (by vortexing and freezing-thawing). The pH of samples was measured before the fluorescence measurement. We recorded a slight decrease in pH with increasing calcium concentration. In the sample with 0 mmol/l calcium concentration, pH = 7.1, whereas at 40 mmol/l calcium concentration pH = 6.5. EtBr as fluorescence probe was added in the last step of sample preparation to obtain the EtBr/DNA molar ratio of 4 mol/base, and the final volume of the sample was 3 ml.

Preparation of complexes for X-ray diffraction experiments

OA-DOPE mixtures and DOPE-OA-DNA-Ca²⁺ complexes were prepared by hydrating the dry lipid film with the solution of DNA in 150 mmol/l NaCl at the DOPE/DNA ratio of 8 (mol/base) and the solution of CaCl₂·2H₂O to obtain the molar concentration of calcium cations of 15 mmol/l. The final volume of the sample was 1 ml. Homogenisation of the sample was carried out analogously as described in the previous section. A few minutes after the preparation, a sediment was formed. pH of samples was checked and was determined to be ~6 for both the binary mixtures and the DOPE-OA-DNA-Ca²⁺ complexes. At the OA/DOPE molar ratio of 1, the pH of samples was adjusted by adding 50 mmol/l HCl solution to slightly acidic (~4.8) or by adding 10 mmol/l NaOH solution to raise the pH to ~7.6. The supernatant was gently removed using a Pasteur pipette and the sediment was transferred into a capillary made of special glass with a diameter of 1.5 mm. Filled capillaries were centrifuged for 1 min at 500 RPM using Rotofix 32 A Hettich Centrifuge. The residue of the supernatant was removed using a syringe. Capillaries were sealed and prepared for X-ray diffraction experiments. The amount of DNA bound in the complexes was determined from the supernatant using UV-vis spectroscopy.

Fluorescence experiments

The fluorescence of samples was measured using the Fluoromax-4 spectrofluorometer (Jobin Yvon, France) at the laboratory temperature. The emission fluorescence intensity of EtBr was measured at $\lambda_{\text{em}} = 596$ nm using excitation wavelength $\lambda_{\text{ex}} = 510$ nm. The samples were measured 60 min after the complexes' preparation and repeatedly after 30 days. They were constantly stirred during the measurement. The emission intensity of each sample was corrected for the background fluorescence of EtBr in the absence of DNA and then normalised to

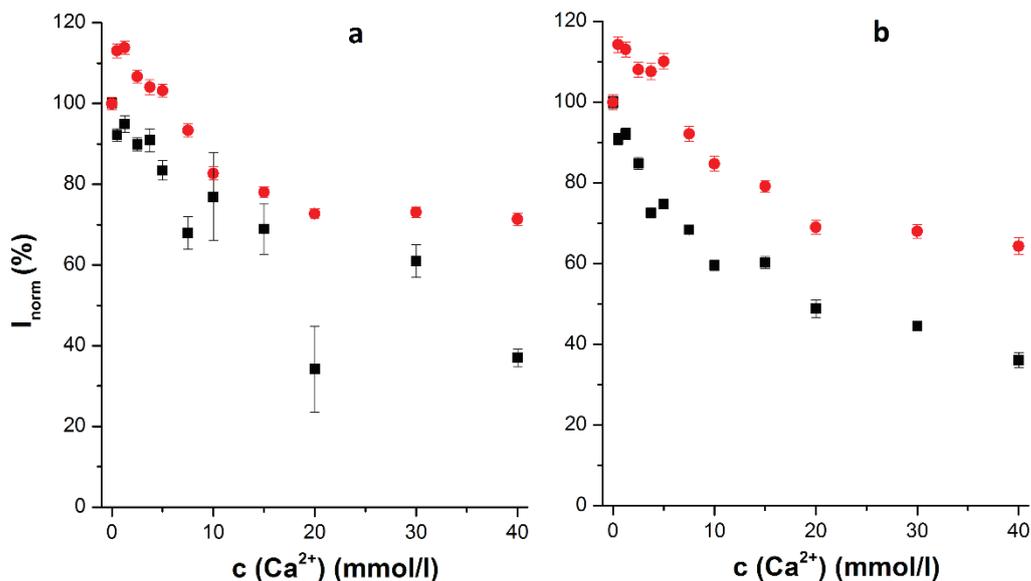


Figure 1. Dependence of normalised intensity I_{norm} of DNA–EtBr–DOPE–Ca²⁺ and DNA–EtBr–DOPE–OA–Ca²⁺ complexes on the presence of calcium cations without OA ■ and with OA at OA/DOPE molar ratio of 1 mol/mol · measured at 60 min (a) and 30 days after preparation (b)

the EtBr fluorescence of sample containing DNA without calcium cations according to equation 1:

$$I_{norm} = \frac{I_{DNA+DOPE+Ca^{2+}+EtBr} - I_{NaCl+EtBr}}{I_{DNA+DOPE+EtBr} - I_{NaCl+EtBr}} \quad (1)$$

Small-angle X-ray diffraction experiments (SAXD)

Small-angle synchrotron radiation diffraction experiments were performed at the NCD-BL11 beamline, ALBA Synchrotron, Barcelona (Spain) using radiation of wavelength $\lambda = 0.124$ nm. The sample in vertically placed capillary was equilibrated at the required temperature before being exposed to the radiation. CCD (Coupled-charged device) camera Quantum 210r was supplied by ADSC (Quantum 210r CCD) for SAXD detection. Raw data were normalised against the incident beam intensity. The SAXD detector was calibrated using silver behenate. Diffractograms were evaluated with the intensity of the incident beam following an exponential model. Diffraction peaks of SAXD region were fitted with a Lorentzian curve above a nonlinear background.

RESULTS

DNA condensation

DNA condensation was indicated by a decrease in the emission intensity of EtBr. Figure 1 shows the dependence of the emission intensity of EtBr on the concentration of

calcium cations at 25 °C in DNA–DOPE–Ca²⁺ and DNA–DOPE–OA–Ca²⁺ complexes at OA/DOPE molar ratios = 1 mol/mol. The concentration of DNA and EtBr was kept constant, whereas the concentration of calcium cations varied from 0 to 40 mmol/l. The normalised emission intensity of EtBr decreases with increasing concentration of calcium cations in both time intervals. In Figure 1, we can see that the I_{norm} decreases to ~37% ($\Delta I_{norm} = 63$ %) in the presence of DOPE–DNA–Ca²⁺ complexes but only to ~71% in the system with OA. After 30 days, ΔI_{norm} did not change significantly within the experimental error in DOPE–DNA–Ca²⁺ complexes. In case of DOPE–OA–DNA–Ca²⁺ complexes, ΔI_{norm} was increased by ~7% after 30 days as shown in Figure 1b.

X-ray scattering

We studied the structure of OA–DOPE mixtures and DNA–DOPE–OA–Ca²⁺ complexes as a function of OA content. Fully hydrated DOPE (Fig. 2A) forms at 20 °C an inverted hexagonal phase H_{II} showing diffraction peaks at reciprocal distances $q_{hk} = 4\pi(h^2 + k^2 - hk)^{1/2}/(a_H \cdot \sqrt{3})$ nm⁻¹, where h and k are Miller indices. The lattice parameter a_H ($a_H = 4\pi / q \cdot \sqrt{3}$ nm) is determined from the position of H_{II}(10) peak's maximum characterising distance between the axes of two adjacent cylinders forming a columnar hexagonal phase (see Fig. 3).

Figure 2a shows the diffractogram of fully hydrated DOPE in the environment of 150 mmol/l NaCl at 20 °C. We determined the lattice parameter a_H as 7.54 nm. The

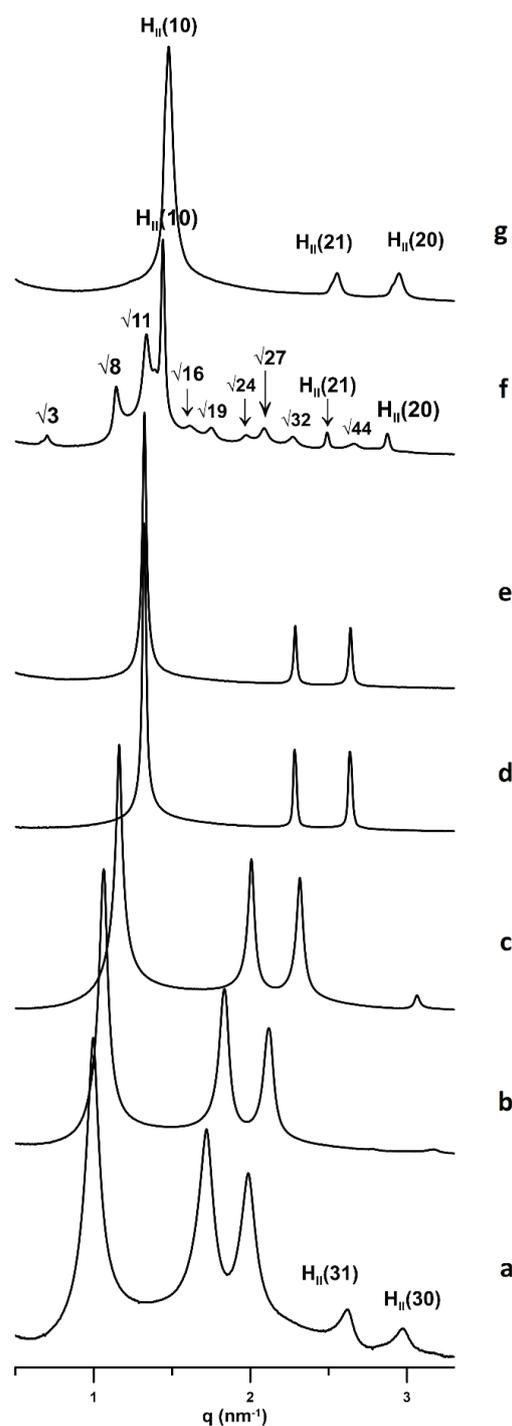


Figure 2. SAXD patterns of DOPE at 20 °C (a), OA-DOPE mixtures at 20 °C at OA/DOPE molar ratios of 0.2 (b), 0.4 (c) and 1 (d) (mol/mol), DOPE-OA-DNA-Ca²⁺ complexes at 20 °C (e), DOPE-OA-DNA-Ca²⁺ complexes at 80 °C at pH of ~4.8 (f) and ~7.6 (g). Cubic phase assignment follows $q_{hkl} = 2\pi(h^2+k^2+l^2)^{1/2}/a_c$, where h , k and l are Miller indices. All samples were prepared in the environment of 150 mmol/l NaCl at 15 mmol/l Ca²⁺ concentration.

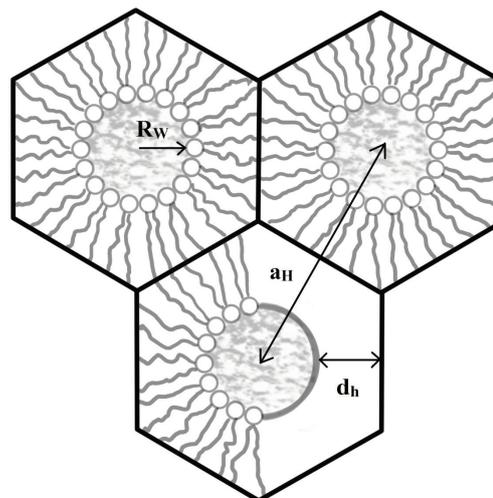


Figure 3. Illustration of the inverse hexagonal phase H_{II} in cross section. The shaded regions represent water. Tubes of water of radius R_w are arranged on a hexagonal lattice, surrounded by a lipid layer.

addition of OA resulted in the decrease of a_H value and was dependent on the OA/DOPE molar ratio. The greater the OA content, the smaller is the lattice parameters in the OA-DOPE systems. The diffractograms of OA-DOPE mixtures at individual molar ratios are shown in Fig. 2b-d. At the OA/DOPE molar ratio of 1, the a_H was reduced up to 5.50 nm. The obtained a_H values are summarised in Table 1.

The structure of DOPE-OA-DNA-Ca²⁺ complexes was studied at 15 mmol/l Ca²⁺ concentration prepared in the environment of 150 mmol/l NaCl at the OA/DOPE molar ratio of 1. The diffractogram of the complexes at 20 °C shows hexagonal arrangement of the mixture (Fig. 2e). The lattice parameter was maintained at the same values as in the OA-DOPE system at the OA/DOPE molar ratio of 1 (see Table 1).

OA/DOPE mixtures as well as DOPE-OA-DNA-Ca²⁺ complexes were heated in the temperature of 20–80 °C with 10 °C temperature increment. Heating sequences were recorded at two different pH values, ~4.8 and ~7.6. The lattice parameter of hexagonal phases for both the mixture OA-DOPE and the complexes DOPE-OA-DNA-Ca²⁺ decreased with rising temperature. The most significant structural polymorphism was recorded at OA/DOPE = 1 mol/mol. In addition to peaks related to hexagonal phase, diffractograms show new peaks corresponding to a cubic phase. Peaks assigned to positions $\sqrt{3}$, $\sqrt{8}$, $\sqrt{11}$, $\sqrt{16}$, $\sqrt{19}$, $\sqrt{24}$, $\sqrt{27}$, $\sqrt{32}$ and $\sqrt{44}$ fit well an inverse micellar cubic phase of the Fd3m space group. The lattice parameter of the Fd3m phase, $a_c = 16$ nm, was determined from the slope of the plot of the peak positions versus $\sqrt{(h^2 + k^2 + l^2)}$ passing by the

Table 1. Samples' composition and lattice parameters of hexagonal (a_H) and cubic phase (a_F) at 20 and 80 °C at different pH values

Composition	OA/DOPE (mol/mol)	t (°C)	pH	a_H (nm)	a_F (nm)
DOPE	0	20	6	7.54 ± 0.01	-
DOPE-OA	0.2	20	6	6.88 ± 0.01	-
DOPE-OA	0.4	20	6	6.27 ± 0.01	-
DOPE-OA	1	20	6	5.50 ± 0.01	-
DOPE-OA-DNA-Ca ²⁺	1	20	6	5.50 ± 0.02	-
DOPE-OA-DNA-Ca ²⁺	1	80	4.8	5.00 ± 0.01	15.70 ± 0.01
DOPE-OA-DNA-Ca ²⁺	1	80	7.6	4.93 ± 0.02	-

origin (0,0). For OA/DOPE, Fd3m phase appeared at ~60 °C at moderately acidic pH and was shifted to higher temperatures at pH of ~7. However, DOPE-OA-DNA-Ca²⁺ complexes prepared at neutral pH do not arrange into Fd3m phase at all, as hexagonal phase alone was identified (Fig. 2f and g). Lattice parameters of both phases at selected temperatures are summarised in Table 1.

DISCUSSION

Fatty acids (FAs) are important components of the plasma membrane, in which the core is formed by moieties of FAs as a part of phospholipid molecules. PE constitutes ~5–50% of total lipids in membranes (Cevc 1993), so that the study of FA-PE mixtures is of biological relevance. FAs affect membrane structure (Ibarguren et al. 2014), cell physiology (Lu et al. 1996) and their levels have also been associated with pathological states (O'Connor et al. 1999). The present study is investigating the physicochemical characteristics of potential non-viral nucleic acid vectors composed of the OA-DOPE mixture in the presence of Ca²⁺.

DNA itself can condense into polydispersed rod-like, spheroid and toroid structures in a first-order phase transition that is induced by multi-valent cations (Hud and Vilfan 2005). In the bulk solution, condensation cannot be used to shape DNA-based nanomaterials by design because of the lack of predetermined spatial organisation, the abruptness of the transition and the high stability of the condensed structures (Iwataki et al. 2004, Mel'nikov et al. 1995). However, DNA condensation can be controlled by layering DNA using cationic lipid membranes (Koltover et al. 2000). In our experiment the electrostatic interaction between the neutral phospholipid DOPE and polyanionic DNA is mediated by divalent calcium cations. Owing to the interaction, DNA can be packed into structures formed by the lipid. Fluorescence spectroscopy is a suitable method to follow DNA condensation by a decrease in the emission intensity of fluorescence probe EtBr. Free molecules of

EtBr in a solution follow a nonradiative decay pathway that involves donation of an amino group proton to the solvent. When EtBr is intercalated into DNA, the ethidium cation is isolated from the solvent and the proton transfer pathway between EtBr and the solvent is blocked. This leads to about 20-fold increase in the fluorescence intensity (Izumrudov et al., 2002) because of the electrostatic attraction between cationic agent and negatively charged phosphate groups of DNA. Neutralisation of the negative charge of DNA phosphate groups leads to compaction and condensation of the DNA molecules and their condensation (Eastman et al., 1997). The condensation of DNA leads to displacement of intercalated EtBr, that presents itself as the decrease in the fluorescence intensity. Eastman et al. (1997) suggested that the observed decrease in fluorescence is directly related to the amount of DNA bound in the formed aggregate. The greater the decrease in fluorescence intensity of EtBr, the more EtBr molecules have been displaced from the DNA base pairs because of the DNA condensation and thus the forming of DNA-phospholipid complexes. In the DOPE-DNA-Ca²⁺ complexes, ~63% of DNA was bound, but it was only 29% after the incorporation of OA to the complexes at the OA/DOPE molar ratio of 1. Complexes were also measured 30 days after the preparation to see if more DNA would encapsulate or, on the other hand, the DNA starts to release from the complex after one month. In the DOPE-DNA-Ca²⁺ system, the amount of DNA bound in the complexes did not change. The binding capacity of DOPE-OA-DNA-Ca²⁺ complexes was improved by ~7% after 30 days. Practically, no release of DNA was recorded in the system without OA. This was found in other cationic lipid-based system forming reverse hexagonal mesophase (Amar-Yuli et al. 2011). The presence of cationic substance leads to such a strong electrostatic confinement of DNA at the water-lipid interface that the release into the excess water is prevented.

OA is a molecule with an aliphatic chain and a carboxylic group whose ionisation is dependent on the solution pH.

Subsequently, intermolecular interactions depend on its ionisation state. The pKa of the carboxylic group is around 4.8 (Cistola and Small 1991). For medium- and long-chain fatty acids, this value increases to a higher apparent pKa (Cistola et al. 1988). The apparent pKa value of OA in the aggregated phase is somewhat ill-defined and difficult to estimate (Salentinig et al. 2010, Peterlin et al. 2009) and, depending on the used method, can vary between 7.5 at low concentrations inserted in a PC bilayer and up to 9.85 in pure monolayers. The pKa^{app} value of OA, determined by titration, was reported to be between 8.0 and 8.5 by Cistola et al. (1988) and 9.85 by Kanicky and Shah (2002). pH of samples in our EtBr accessibility experiment was ~6.5–7. We assume that the most part of OA present in the complexes is in its unionised form; however, the presence of the ionised form of OA in the system and subsequently the interaction of Ca²⁺ with negatively charged molecules of OA cannot be excluded. In Figure 1, one can see that the initial increase in the normalised EtBr fluorescence intensity takes the values of more than 100 at low calcium concentrations in the complexes with OA. In the DOPE-OA-DNA-EtBr system, small amounts of DNA can be captured in the tubules of the hexagonal structure even in the absence of calcium cations. Because of high volume fraction of OA (OA/DOPE = 1 mol/mol) in this experiment, we cannot exclude that Ca²⁺ may 'bridge' two molecules of OA. The formed salt of FA may result in the exclusion of DNA from the tubules, resulting in the increase in intensity as follows from the properties of EtBr used as a probe. We did not observe such behaviour at OA/DOPE < 1 mol/mol (not shown). With further increase in the calcium concentration, the complex formation is the predominant event and the emission intensity of EtBr decreases.

Fully hydrated DOPE at laboratory temperature arranges into nonlamellar, inverted hexagonal phase H_{II} with lattice parameter a_H of ~7.5–7.6 nm. The radius of water cylinders (R_w) inside the tubules was determined by Tate and Gruner (1989); they reported R_w = 2.2 nm at 20 °C, the thickness of the lipid layer d_h = 1.62–2.20 nm (as illustrated in Fig. 3). Inverse hexagonal phase H_{II}^c was also found in our OA-DOPE mixture at different OA/DOPE molar ratios, but with significantly lower lattice parameters. As we can see in Table 1, the addition of OA to DOPE remarkably decreases the lattice parameter. Note, at the OA/DOPE molar ratio of 0.2, we determined a_H = 6.88 nm, and the lattice parameter was reduced up to 5.50 nm at OA/DOPE = 1 mol/mol. Gillams et al. (2014) studied phase behaviour of OA/DOPE mixtures as a function of OA mole fraction. Pure water was used as hydrating medium. The authors report that OA increases the propensity of bilayer lipid membranes to curve, up to level of inverse micellar cubic phase formation. In their study, H_{II} phase was observed up to mole fraction of OA x_{OA} = 0.6. Similar to our finding, the authors observed the

decrease in the lattice parameter a_H up to 5 nm at x_{OA} = 0.6. Their analysis indicates that OA affects the elasticity of lipid/OA monolayers resulting in the reduction of the radius (R_w) of water cylinders. Whilst changes in the lipid layer thickness (d_h) can be considered of little importance, the variance in d_h with respect to R_w is less than 2% (Gillams et al. 2014 and references therein).

Interestingly, DOPE-OA-DNA-Ca²⁺ complexes prepared at OA/DOPE = 1 mol/mol in 15 mmol/l of CaCl₂ show the same value of a_H = 5.5 nm as determined for the OA-DOPE mixture itself. Let us analyse this finding: It was reported that DNA can be 'accommodated' in the water cylinders of inverted hexagonal phase formed by DOPE in the presence of divalent cations such as calcium or magnesium. For example, condensed inverted hexagonal phase H_{II}^c was observed at 20 °C in DOPE-DNA-Mg²⁺ complexes (Uhríková et al. 2006). The coexistence of both H_{II} and H_{II}^c phases was reported in the DOPE-DNA-Ca²⁺ system at 20 °C (Lengyel 2010). Lattice parameters (a_H) of H_{II}^c phase range were ~7.1 nm in both the experiments mentioned earlier. In these studies, 5 mmol/l NaCl was used as the hydrating medium. The difference between lattice parameters of H_{II} formed by DOPE and those H_{II}^c observed for DOPE+DNA+ion²⁺ is small (~0.5 nm). In this system, Ca²⁺ mediates DNA-phospholipid binding, and the tendency of DNA to be wrapped by the lipid results in R_w reduction, R_w = 1.7 nm. It is evident that hydrated DNA strands with diameter of 2.5 nm (Lieberman 2005) can arrange readily into water cylinders of H_{II}^c phase. As we see, the lattice parameters of DOPE+DNA+ion²⁺ are increased by ~1.6 nm in comparison to a_H value (a_H = 5.5 nm) found in our DOPE-OA-DNA-Ca²⁺ system. In view of discussion above and results of Tate and Gruner, if we assume the thickness of the lipid layer, d_h = 1.62 nm, one gets the radius of the water core R_w = 1.13 nm. Thus the structure shrinks, the water core with radius R_w = 1.13 nm in OA-enriched complexes can be seen as limit or even insufficient for DNA accommodation. However, our data from fluorescence spectroscopy indicate that up to ~29% of total DNA in the sample was bound in the complexes. Additional UV-VIS measurements on complexes prepared for SAXD experiments indicate that ~10–13% of total DNA bound four months after the complex preparation. In summary, both methods, fluorescence and UV-VIS spectroscopy measurements, confirmed that ~10–29% of DNA was bound in the complexes. Generally, it is supposed that at hexagonal arrangement of lipoplexes, in condensed inverted hexagonal phase H_{II}^c, DNA occupies interior of water-filled cylinders formed by lipid monolayers of inverted hexagonal phase. The symmetry of arrangement of DNA is dictated by symmetry of the lipid arrangement. As such, the difference between SAXD patterns of H_{II} and H_{II}^c is not obvious. The greater electron density of DNA with respect to water should lead to the relative suppression of the (21) and (20) Bragg

peak intensities compared with that in the lipid H_{II} phase (Koltover et al. 1998). Comparing patterns of OA–DOPE and DOPE–OA–DNA– Ca^{2+} (Fig. 2d and e, respectively), relative changes in the intensities of (21) and (20) are rather small. This fact together with closeness of the obtained lattice parameters raises the question: where is the detected DNA located in our complexes? From the physical point of view, our complexes are the so-called ‘polycrystalline’ samples with random orientation of microdomains (with internal order) of colloid mixture. As such, the lattice parameter derived from SAXD is an average value of lattice parameters of microdomains exposed to X-ray in the moment of an image collection. Our SAXD data show no difference between the lattice parameters of hexagonal phases formed by OA–DOPE and DOPE–OA–DNA– Ca^{2+} . However, we found up to ~29% of DNA bound. We suppose that a part of DNA can be localised in the interior of water cylinders of H_{II} phase; however, DNA strands can also be trapped by the structure itself at the lipid–water interface. Temperature measurements of the complexes support our assumption. High temperature stimulates the formation of micellar cubic phase Fd3m. In our studied system, Fd3m was observed at 60 °C first in OA–DOPE mixtures at both moderately acidic and neutral pH. After the addition of DNA to OA–DOPE mixture in the presence of Ca^{2+} at neutral pH, we did not detect any peak related to the cubic phase. DNA stabilises the inverse columnar hexagonal arrangement in the system DOPE–OA–DNA– Ca^{2+} , because the formation of H_{II} only was observed at pH of ~7.6 in the whole studied temperature range (see Fig. 2f). The disruption of hexagonally packed cylinders of H_{II} and their rearrangement into a phase of three-dimensional symmetry (Fd3m) depends strongly on external conditions, such as temperature and ionic strength. For example, Gillams et al. (2014) observed coexistence of H_{II} and Fd3m phases for OA/DOPE = 1 mol/mol already at 20 °C. Pure water was used as hydrating medium in this study. At the same composition of OA–DOPE mixture, we detected the presence of cubic phases at ~60 and ~70 °C in dependence on pH of the solution (pH of ~4.8 and 7.6, respectively). The high concentration of the salt (150 mmol/l NaCl) used in our experiment might be responsible for this difference. Recent knowledge documents no significant effect of NaCl on the bilayer structure for ion concentration up to 1 mol (Petrache et al. 2006, Pabst et al. 2007). Pabst et al. (2007) reported a decrease in bilayer elasticity and shift of main transition temperature in the presence of NaCl and

$CaCl_2$. Reduced elasticity of the bilayer because of a tight binding of sodium cations to the carbonyl oxygens of the phospholipid was also found elsewhere (Böckmann et al. 2003). This rigidification and ‘stabilisation’ of the structure because of high salt content is highly responsible for H_{II} phase as the only one observed in our system. Moreover, DNA trapped in DOPE–OA–DNA– Ca^{2+} complexes prepared at neutral pH stabilises hexagonal packing as well, up to high temperature as documented in our experimental data.

CONCLUSIONS

The study was aimed to design nucleic acid delivery vectors based on phospholipid. A mixture of zwitterionic DOPE and OA was used as lipid matrix. Fatty acids are natural components of biological membranes and are known as effective modulator of polymorphic behaviour of phospholipids. SAXD experiments revealed that OA induces structural changes in the inverted hexagonal phase H_{II} of DOPE. We have found that increasing the content of oleic acid, as additive, reduces the lattice parameter of H_{II} formed by DOPE. The effect is marked and recognised as crucial obstacle in efficient DNA binding into water cylinders that form the core of OA–DOPE tubules arranged in hexagonal symmetry. The amount of DNA bound in complexes (~10–29%) was determined using fluorescence and UV-vis spectroscopy. After 30 days, either the binding capacity of the complexes or the DNA release from the hexagonal phase into excess water was not affected. High content of OA and increase in temperature promote formation of an inverted micellar cubic phase of Fd3m space group. At neutral pH, DNA bound in complexes prevents disintegration of the structure and hexagonal phase was observed up to 80 °C. The findings obtained under this study contribute to knowledge about DNA complexation into supramolecular structure formed by lipid bilayer, what is the essential feature at gene delivery vectors designing.

ACKNOWLEDGMENTS

SAXD experiments were performed at BL11-NCD beamline at Alba Synchrotron with the collaboration of ALBA staff. The authors (D.G., A.B. and D.U.) thank Alba staff for hospitality and BioStruct-X/Calipso programme for the support. The research was supported by grants VEGA 1/0916/16, JINR project 04-4-1121-2015/2017 and UK/254/2017.

References

- [1] Amar-Yuli I., Adamcik J., Blau S., Aserin A., Garti N., Mezzenga R. 2011. "Controlled Embedment and Release of DNA from Lipidic Reverse Columnar Hexagonal Mesophases." *Soft Matter* 7 (18):8162–68. <https://doi.org/10.1039/C1SM05612C>.
- [2] Böckmann RA., Hac A., Heimburg T., Grubmüller H. 2003. "Effect of Sodium Chloride on a Lipid Bilayer." *Biophysical Journal* 85 (3):1647–55.
- [3] Cevc G. 1993. *Phospholipids Handbook*. New York: Marcel Dekker, Inc.
- [4] Cistola DP., Hamilton JA., Jackson D., Small DM. 1988. "Ionization and Phase Behavior of Fatty Acids in Water: Application of the Gibbs Phase Rule." *Biochemistry* 27 (6):1881–88.
- [5] Cistola DP., Small DM. 1991. "Fatty Acid Distribution in Systems Modeling the Normal and Diabetic Human Circulation. A ¹³C Nuclear Magnetic Resonance Study." *Journal of Clinical Investigation* 87 (4):1431–41.
- [6] Eastman SJ., Siegel C., Tousignant J., Smith AE., Cheng SH., Scheule RK. 1997. "Biophysical Characterization of Cationic Lipid: DNA Complexes." *Biochimica Et Biophysica Acta* 1325 (1):41–62.
- [7] Funari SS., Barceló F., Escribá PV. 2003. "Effects of Oleic Acid and Its Congeners, Elaidic and Stearic Acids, on the Structural Properties of Phosphatidylethanolamine Membranes." *Journal of Lipid Research* 44 (3):567–75. <https://doi.org/10.1194/jlr.M200356-JLR200>.
- [8] Geall AJ., Blagbrough IS. 2000. "Rapid and Sensitive Ethidium Bromide Fluorescence Quenching Assay of Polyamine Conjugate–DNA Interactions for the Analysis of Lipoplex Formation in Gene Therapy." *Journal of Pharmaceutical and Biomedical Analysis* 22 (5):849–59. [https://doi.org/10.1016/S0731-7085\(00\)00250-8](https://doi.org/10.1016/S0731-7085(00)00250-8).
- [9] Gillams RJ., Nylander T., Plivelic TS., Dymond MK., Attard GS. 2014. "Formation of Inverse Topology Lyotropic Phases in Dioleoylphosphatidylcholine/Oleic Acid and Dioleoylphosphatidylethanolamine/Oleic Acid Binary Mixtures." *Langmuir: The ACS Journal of Surfaces and Colloids* 30 (12):3337–44. <https://doi.org/10.1021/la404275u>.
- [10] Glover DJ., Lipps HJ., Jans DA. 2005. "Towards Safe, Non-Viral Therapeutic Gene Expression in Humans." *Nature Reviews. Genetics* 6 (4):299–310. <https://doi.org/10.1038/nrg1577>.
- [11] Hassani Z., Lemkine GF., Erbacher P., Palmier K., Alfama G., Giovannangeli C., Behr JP., Demeneix BA. 2005. "Lipid-Mediated siRNA Delivery down-Regulates Exogenous Gene Expression in the Mouse Brain at Picomolar Levels." *The Journal of Gene Medicine* 7 (2):198–207. <https://doi.org/10.1002/jgm.659>.
- [12] Hubčík L., Pullmannová P., Funari SS., Devínsky F., Uhríková D. 2014. "DNA – DOPC – Gemini Surfactants Complexes: Effect of Ionic Strength." *Acta Facultatis Pharmaceuticae Universitatis Comenianae* 61 (2):26–34. <https://doi.org/10.2478/afpuc-2014-0013>.
- [13] Hubčík L., Funari SS., Pullmannová P., Devínsky F., Uhríková D. 2015. "Stimuli Responsive Polymorphism of C12NO/DOPE/DNA Complexes: Effect of PH, Temperature and Composition." *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1848 (5):1127–38. <https://doi.org/10.1016/j.bbamem.2015.01.020>.
- [14] Hud NV., Vilfan ID. 2005. "Toroidal DNA Condensates: Unraveling the Fine Structure and the Role of Nucleation in Determining Size." *Annual Review of Biophysics and Biomolecular Structure* 34:295–318. <https://doi.org/10.1146/annurev.biophys.34.040204.144500>.
- [15] Ibarguren M., López DJ., Escribá PV. 2014. "The Effect of Natural and Synthetic Fatty Acids on Membrane Structure, Microdomain Organization, Cellular Functions and Human Health." *Biochimica et Biophysica Acta (BBA) - Biomembranes, Membrane Structure and Function: Relevance in the Cell's Physiology, Pathology and Therapy*, 1838 (6):1518–28. <https://doi.org/10.1016/j.bbamem.2013.12.021>.
- [16] Iwataki T., Kidoaki S., Sakaue T., Yoshikawa K., Abramchuk SS. 2004. "Competition between Compaction of Single Chains and Bundling of Multiple Chains in Giant DNA Molecules." *The Journal of Chemical Physics* 120 (8):4004–11. <https://doi.org/10.1063/1.1642610>.
- [17] Izumrudov VA., Zhiryakova MV., Goulko AA. 2002. "Ethidium Bromide as a Promising Probe for Studying DNA Interaction with Cationic Amphiphiles and Stability of the Resulting Complexes." *Langmuir* 18 (26):10348–56. <https://doi.org/10.1021/la020592u>.
- [18] Kanicky JR., Shah DO. 2002. "Effect of Degree, Type, and Position of Unsaturation on the PKa of Long-Chain Fatty Acids." *Journal of Colloid and Interface Science* 256 (1):201–7.
- [19] Koltover I., Salditt T., Rädler JO., Safinya CR. 1998. "An Inverted Hexagonal Phase of Cationic Liposome-DNA Complexes Related to DNA Release and Delivery." *Science (New York, N.Y.)* 281 (5373):78–81.
- [20] Koltover I., Wagner K., Safinya CR. 2000. "DNA Condensation in Two Dimensions." *Proceedings of the National Academy of Sciences of the United States of America* 97 (26):14046–51.
- [21] Lengyel A. 2010. Interkacia DNA s fosfolipidovými lipozómami v prítomnosti dvojmocných katiónov. PhD Thesis. Farmaceutická fakulta Univerzity Komenského. 2010
- [22] Lengyel A., Uhríková D., Klacsová M., Balgavý P. 2011. "DNA Condensation and Its Thermal Stability Influenced by Phospholipid Bilayer and Divalent Cations." *Colloids and Surfaces. B, Biointerfaces* 86 (1):212–17. <https://doi.org/10.1016/j.colsurfb.2011.04.001>.
- [23] Lieberman PM. 2005. *DNA Viruses: Methods and Protocols*. Springer Science & Business Media.
- [24] Liskayová G., Hubčík L., Šišková K., Paulíková I., Gallíková D., Devínsky F., Funari SS., Uhríková D. 2017. "PH-Sensitive N,N-(Dimethyl)-N-Alkanamine-N-Oxides as Gene Delivery Vectors." *Chemical Papers*, April, 1–10. <https://doi.org/10.1007/s11696-017-0171-2>.
- [25] Lu, G., Morinelli TA., Meier KE., Rosenzweig SA., Egan BM. 1996. "Oleic Acid-Induced Mitogenic Signaling in Vascular Smooth Muscle Cells. A Role for Protein Kinase C." *Circulation Research* 79 (3):611–18.
- [26] Mel'nikov SM., Sergeyev VG., Yoshikawa K. 1995. "Discrete Coil-Globule Transition of Large DNA Induced by Cationic Surfactant." *Journal of the American Chemical Society* 117 (9):2401–8. <https://doi.org/10.1021/ja00114a004>.

- [27] O'Connor LJ., Nicholas T., Levin RM. 1999. "Subcellular Distribution of Free Fatty Acids, Phospholipids, and Endogenous Lipase Activity of Rabbit Urinary Bladder Smooth Muscle and Mucosa." *Advances in Experimental Medicine and Biology* 462:265–73.
- [28] Pabst G., Hodzic A., Štrancar J., Danner S., Rappolt M., Laggner P. 2007. "Rigidification of Neutral Lipid Bilayers in the Presence of Salts." *Biophysical Journal* 93 (8):2688–96. <https://doi.org/10.1529/biophysj.107.112615>.
- [29] Pérez FR., Casabiell X., Camiña JP., Zugaza JL., Casanueva FF. 1997. "Cis-Unsaturated Free Fatty Acids Block Growth Hormone and Prolactin Secretion in Thyrotropin-Releasing Hormone-Stimulated GH3 Cells by Perturbing the Function of Plasma Membrane Integral Proteins." *Endocrinology* 138 (1):264–72. <https://doi.org/10.1210/endo.138.1.4888>.
- [30] Peterlin P., Arrigler V., Kogej K., Svetina S., Walde P. 2009. "Growth and Shape Transformations of Giant Phospholipid Vesicles upon Interaction with an Aqueous Oleic Acid Suspension." *Chemistry and Physics of Lipids* 159 (2):67–76. <https://doi.org/10.1016/j.chemphyslip.2009.03.005>.
- [31] Petrache HI., Tristram-Nagle S., Harries D., Kucerka N., Nagle JF., Parsegian VA. 2006. "Swelling of Phospholipids by Monovalent Salt." *Journal of Lipid Research* 47 (2):302–9. <https://doi.org/10.1194/jlr.M500401-JLR200>.
- [32] Prades J., Funari SS., Escribá PV., Barceló F. 2003. "Effects of Unsaturated Fatty Acids and Triacylglycerols on Phosphatidylethanolamine Membrane Structure." *Journal of Lipid Research* 44 (9):1720–27. <https://doi.org/10.1194/jlr.M300092-JLR200>.
- [33] Pullmannová P., Funari SS., Devínský F., Uhríková D. 2012. "The DNA–DNA Spacing in Gemini Surfactants–DOPE–DNA Complexes." *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1818 (11):2725–31. <https://doi.org/10.1016/j.bbamem.2012.05.021>.
- [34] Puri A., Loomis K., Smith B., Lee JH., Yavlovich A., Heldman E., Blumenthal R. 2009. "Lipid-Based Nanoparticles as Pharmaceutical Drug Carriers: From Concepts to Clinic." *Critical Reviews in Therapeutic Drug Carrier Systems* 26 (6):523–80.
- [35] Rajnohová Z., Lengyel A., Funari SS., Uhríková D. 2010. "The Structure and Binding Capacity of Lipoplexes." *Acta Faculty. Pharm. Univ. Comenianae* 57(2010), 57 (1).
- [36] Ramamoorth M., Narvekar A. 2015. "Non Viral Vectors in Gene Therapy- An Overview." *Journal of Clinical and Diagnostic Research: JCDR* 9 (1):GE01–GE06. <https://doi.org/10.7860/JCDR/2015/10443.5394>.
- [37] Salentinig S., Sagalowicz L., Glatter O. 2010. "Self-Assembled Structures and PKa Value of Oleic Acid in Systems of Biological Relevance." *Langmuir* 26 (14):11670–79. <https://doi.org/10.1021/la101012a>.
- [38] Seddon JM., Templer RH., Warrender NA., Huang Z., Cevc G., Marsh D. 1997. "Phosphatidylcholine–fatty Acid Membranes: Effects of Headgroup Hydration on the Phase Behaviour and Structural Parameters of the Gel and Inverse Hexagonal (HII) Phases." *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1327 (1):131–47. [https://doi.org/10.1016/S0005-2736\(97\)00047-3](https://doi.org/10.1016/S0005-2736(97)00047-3).
- [39] Templer RH., Seddon JM., Warrender NA., Syrykh A., Huang Z., Winter R., Erbes J. 1998. "Inverse Bicontinuous Cubic Phases in 2:1 Fatty Acid/Phosphatidylcholine Mixtures. The Effects of Chain Length, Hydration, and Temperature." *The Journal of Physical Chemistry B* 102 (37):7251–61. <https://doi.org/10.1021/jp972835a>.
- [40] Uhríková D., Hanulová M., Funari SS., Khusainova RS., Šeršeň F., Balgavý P. 2005. "The Structure of DNA–DOPC Aggregates Formed in Presence of Calcium and Magnesium Ions: A Small-Angle Synchrotron X-Ray Diffraction Study." *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1713 (1):15–28. <https://doi.org/10.1016/j.bbamem.2005.05.006>.
- [41] Uhríková D., Hanulová M., Funari SS., Balgavý P. 2005. The structure of aggregate DNA - dioleoylphosphatidylethanolamine - Mg²⁺. „HasyLab annual report 2005 Hamburg: HasyLab at Desy, 2006“. p. 977-978
- [42] Uhríková D., Lengyel A., Hanulová M., Funari SS., Balgavý P. 2007. "The Structural Diversity of DNA–neutral Phospholipids–divalent Metal Cations Aggregates: A Small-Angle Synchrotron X-Ray Diffraction Study." *European Biophysics Journal* 36 (4–5):363–75. <https://doi.org/10.1007/s00249-006-0086-2>.