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DNA – DOPC – GEMINI SURFACTANTS COMPLEXES: EFFECT OF IONIC STRENGTH

DNA-DOPC-GEMINI TENZIDY KOMPLEXY: VPLYV IÓNOVEJ SILY

Original research article

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Abstract The effect of ionic strength on DNA condensation by cationic liposomes prepared as a mixture of ethane-1,2-diylbis(dodecyldimethylammonium bromide) (C2GS12) and dioleoylphosphatidylcholine (DOPC) was studied using fluorescence spectroscopy. The DNA condensation followed by changes in emission intensity of ethidium bromide shows a strong dependence on the ionic strength of the solution. At physiologically relevant ionic strength (0.15 mol/l NaCl), the amount of DNA condensed between lipid bilayers is approximately 40% lower compared to 0.005 mol/l NaCl. The structure of formed complexes was studied using small angle X-ray diffraction (SAXD). DNA–C2GS12–DOPC complexes form a condensed lamellar phase organisation, which is partially disrupted by the increase of ionic strength. Both the lamellar repeat distance and DNA–DNA distance show dependence on C2GS12/DOPC molar ratio, temperature and also on ionic strength. We found that the method of preparation significantly affects both the quality of organisation and the structural parameters of complexes as discussed in the paper.

Slovak abstract Metódou fluorescenčnej spektroskopie sme študovali vplyv iónovej sily roztoku na DNA kondenzáciu v prítomnosti katiónových lipozómov pripravených zo zmesi etán-1,2-diylbis(dodecyldimetylamónium bromidu) (C2GS12) a dioleoylfosfatidylcholínu (DOPC). Kondenzácia DNA sledovaná prostredníctvom zmien v intenzite emisného žiarenia etídium bromidu vykazuje silnú závislosť na iónovej sile roztoku. Pri fyziologicky relevantnej iónovej sile (0,15 mol/l NaCl) je množstvo DNA kondenzovanej medzi lipidovými dvojvrstvami o viac než 40% nižšie než v prostredí 0,005mol/l NaCl. Štruktúra vzniknutých komplexov bola študovaná pomocou malouhlovej difrakcie RTG žiarenia (SAXD). DNA–C2GS12–DOPC komplexy vytvárajú kondenzovanú lamelárnu fázu, ktorej usporiadanie je čiastočne narušené pri zvýšenej iónovej sile. V závislosti od mólového pomeru C2GS12/DOPE, teploty a iónovej sily sme pozorovali zmeny periódy lamely ako aj vzdialenosti medzi DNA vláknami. Experimentálne výsledky sú diskutované vzhľadom na spôsob prípravy komplexov.

Keywords *DNA, Gemini surfactants, dioleoylphosphatidylcholine, Fluorescence spectroscopy, Small angle X-ray diffraction.*

Kľúčové slová: *DNA, Gemini tenzidy, dioleoylfosfatidylcholín, fluorescenčná spektroskopia, malouhlová RTG difrakcia*

INTRODUCTION

For the efficient and safe introduction of DNA into the cell nucleus (transfection) a suitable vector is needed. The most effective transfection agents are viruses (Robbins & Ghivizzani, 1998); however, there are serious safety concerns (Verma, 2000). As a result, the search for alternative non-viral vectors has intensified. One of the most promising groups of non-viral vectors are cationic liposomes. Since their first use by Felgner *et al.*, (1987) for gene delivery to somatic cells, a wide variety of different liposomal transfection vectors has been developed. Cationic liposomes consisting of cationic lipid (Caracciolo *et al.*, 2007; Mochizuki *et al.*, 2013; Wasungu & Hoekstra, 2006) or cationic surfactant (Badea *et al.*, 2005; Bombelli *et al.*, 2005; Donkuru *et al.*, 2012; Kirby *et al.*, 2003) with or without a helper lipid with neutral net charge has been widely studied as potential vectors for *in vitro* and *in vivo* gene delivery. Cationic lipids or surfactants form complexes with the DNA polyanion

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what results in DNA condensation. The condensed DNA is partially protected from degradation by enzymes (Rolland, 1998) and can be transferred through endocytosis more effectively (Kirby *et al.*, 2003). Helper lipid modifies the colloidal and structural properties of the complexes and supports their transport through cell membranes (Hirsch–Lerner *et al.*, 2005). Relations between the structure, physicochemical properties and transfection efficiency of liposomal vectors are still not fully understood.

Gemini surfactants (GS) were revealed as a promising group of cationic additive surfactants for gene delivery vectors by Kirby et al, (2003). GS consists of two hydrophobic chains and two ionic (polar) groups linked by a spacer (Menger & Keiper, 2000). GS as non-viral vectors for gene therapy have become a major focus of research because of the unique solution properties imparted by their molecular structure. Their critical micelle concentration is, in general, an order of magnitude or more lower and the surface activity is an order of magnitude greater than it is for comparable single-chain surfactants, but at comparable or lower levels of toxicity (Menger & Keiper, 2000; Wettig *et al.*, 2008). One of the most studied type of GS are alkane-α,ω-diyl-bis(alkyldimethylammonium bromide)s (CnGSm, where *n* is the number of spacer carbons and *m* is the number of carbons in the alkyl chains). CnGSm were found to increase the efficiency of DNA transfer into bacterial cells (Horniak *et al.*, 1989). In combination with helper lipid dioleoylphosphatidylethanolamine (DOPE) they have shown good transfection efficiency both *in vitro* (Cardoso *et al.*, 2014; Foldvari *et al.*, 2006; Muñoz-Úbeda *et al.*, 2012) and *in vivo* (Badea *et al.*, 2005). The physicochemical properties of GS–DNA complexes or without helper lipids were also intensively investigated (García *et al.*, 2014; Grueso *et al.*, 2013; Pietralik *et al.*, 2013; Uhríková *et al.*, 2005a).

Our group has studied systematically the structure and polymorphic behaviour of complexes DNA–CnGSm–neutral phospholipid (Pullmannová *et al.*, 2008, 2012a, 2012b; Uhríková *et al.*, 2002, 2005b, 2007). Our experiments showed that besides the composition of complexes their microstructure is influenced also by other factors such as the ionic strength of the aqueous medium or the method of preparation (Pullmannová *et al.*, 2012b).

This work extends our study focused on the effect of the ionic strength on DNA condensation and the structure of the complexes formed in respect to the used method of preparation. The complexes were prepared by direct mixing of dioleoylphosphatidylcholine (DOPC) unilamellar liposomes with a solution of C2GS12 and DNA in the aqueous medium at two concentrations of NaCl, 0.005 and 0.15 mol/l, respectively. Fluorescence spectroscopy was employed to follow DNA condensation by cationic liposomes. The structure of complexes was studied using small angle X-ray diffraction (SAXD). The results are compared and discussed with respect to our previous study (Pullmannová *et al.*, 2012b) where different methods of complexes preparation were used.

MATERIALS AND METHODS

Materials

Highly polymerised calf thymus DNA (sodium salt) Type I (average Mr of nucleotides = 308) was purchased from Sigma Chemicals, St. Louis, Missouri, USA; ethidium bromide (EtBr) was purchased from Merck, Germany and neutral phospholipid dioleoylphosphatidylcholine (DOPC) was purchased from Avanti Polar Lipids, Alabaster, Alabama, USA. Ethane-1,2-diyl-bis(dodecyldimethylammonium bromide), C2CS12, was prepared as described in (Imam *et al.*, 1983) and purified by manifold crystallisation from a mixture of acetone and methanol. The NaCl of analytical purity was purchased from Lachema, Brno, Czech Republic.

Preparation of DNA solutions

DNA was dissolved at concentration 5 mg/ml in 0.005 mol/l NaCl or 0.15 mol/l NaCl, respectively. The precise value of DNA concentration was determined spectrophotometrically (Hewlett Packard 8452A Diode array spectrophotometer), according to $c_{DNA} = A_{260} \cdot 47\times10^{-6}$ [g/ml], where A_{260} is the absorbance at wavelength λ = 260 nm. The concentration of DNA is referred as molar concentration of DNA bases. The purity of DNA was checked by measuring the absorbance A_λ at λ = 260 and 280 nm. We obtained the value of $A_{260}/A_{280} = 1.81$.

Preparation of cationic liposomes

Dispersions of DOPC multilamellar liposomes were prepared by hydration of dry lipid in 0.005 mol/l and 0.15 mol/l NaCl solutions and their homogenisation by vortexing. DOPC unilamellar liposomes were prepared by extrusion of the lipid dispersion through polycarbonate filters with pores of diameter 100 nm. The DOPC unilamellar liposomes were mixed with the solution of C2GS12 at various molar ratios C2GS12/DOPC and stored at 4°C for 24 hours.

Fluorescence experiments

The samples of DNA–C2GS12–DOPC complexes for fluorescence experiments were prepared in the range of C2GS12/DNA = 0–2 mol/base mol for both studied NaCl concentrations. DNA solutions were mixed with fluorescence probe EtBr at DNA/EtBr = 12 base mol/mol. After 5 minutes, mixtures of unilamellar liposomes at wished molar ratio C2GS12/DOPE were added into the samples and the volume completed to 3000 µl by appropriate NaCl solution. The florescence of samples was measured 30 minutes after the preparation using the Fluoromax-4 spectrofluorometer (Jobin Yvon, France). The emission fluorescence intensity of EtBr was measured at λ_{em} = 596 nm, using exciting wavelength λ_{ex} = 520 nm.

The emission intensity of each sample was corrected for the background fluorescence of EtBr in the absence of DNA and then normalised to the EtBr fluorescence of sample containing DNA without any C2GS12–DOPC liposomes (C2GS12/DNA = 0).

Small-angle X-ray diffraction experiments

The samples of DNA–C2GS12–DOPC complexes for SAXD experiments were prepared in 0.005 and 0.15 mol/l NaCl solutions by mixing the dispersions of C2GS12–DOPC unilamellar liposomes prepared at the range of molar ratios C2GS12/DOPC = 0.1-0.5 and the DNA solution. The samples were prepared at theoretical isoelectric point (DNA/C2GS12 = 2 base mol/mol).

SAXD experiments were performed at the soft condensed matter beamline A2 at HASYLAB at the Deutsches Elektronen Synchrotron (DESY) in Hamburg (Germany), using a monochromatic radiation of wavelength $\lambda = 0.15$ nm. The evacuated double-focusing camera was equipped with linear delay line readout detector. The samples were measured at 20°C and equilibrated at selected temperature 5 min before measurement. Temperature scans were performed at a scan rate 1°C/min and the diffractograms were recorded for 10 s every minute. The data were normalised against the incident beam intensity using the signal intensity measured in the ionisation chamber. The SAXD detector was calibrated using rat tail collagen (Roveri *et al.*, 1980). Each diffraction peak of SAXD region was fitted with a Lorentzian function above a linear background.

RESULTS

DNA condensation

DNA condensation was indicated by a decrease of emission intensity of fluorescence probe EtBr. The free molecules of EtBr in a solution follow a nonradiative decay pathway that involves donation of an amino group proton to 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 increase of fluorescence intensity about 20-fold (Izumrudov *et al.*, 2002). DNA interacts with cationic surfactants or cationic liposomes due to 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 of fluorescence intensity (Eastman *et al.*, 1997; Izumrudov *et al.*, 2002; Wiethoff *et al.*, 2003).

Fig. 1 shows the dependence of the emission intensity of EtBr on C2GS12/DNA molar ratio at two ionic strength of solutions, in 0.005 mol/l and 0.15 mol/l of NaCl, respectively. The concentrations of DNA (3 μmol/l), EtBr (0.25 μmol/l) and DOPC (12 μmol/l) were kept constant, while the concentration of C2GS12 varied depending on C2GS12/DNA molar ratio. The dependence of normalised fluorescence emission intensity of EtBr on the CnGS12/DNA molar ratio has a sigmoidal course and the minimal intensity reaches at C2GS12/DNA ≈ 1.2 mol/base mol in both used NaCl solutions. Above this molar ratio the decrease of emission intensity is insignificant. The main difference between the two used NaCl solutions is in the observed minimum of normalised emission intensity of EtBr. At low ionic strength (0.005 mol/l NaCl), the minimum is achieved at 21.89 ± 0.01%, while at high ionic strength (0.15 mol/l NaCl), we determined 62.71 ± 0.01% of total intensity.

The structure of DNA–C2GS12–DOPC complexes

We studied the structure of DNA–C2GS12–DOPC complexes hydrated by 0.005 mol/l and 0.15 mol/l NaCl solutions as a function of C2GS12/DOPC molar ratio. All samples were prepared at the theoretical isoelectric point based on nominal charges of each species, corresponding to the molar ratio C2GS12/DNA = 0.5 mol/base mol. Fully hydrated DOPC was measured as a control sample. At 20°C DOPC forms a liquid crystalline lamellar L_α phase (Wiener & White, 1992). The diffractogram of DOPC at 20°C (Fig. 2) shows two peaks, L(1) and L(2), related to the first and the second order of the lamellar phase. We determined the repeat distance $d = S_1 = 6.13 \pm 0.01$ nm, where s1 is the position of maximum of the first order

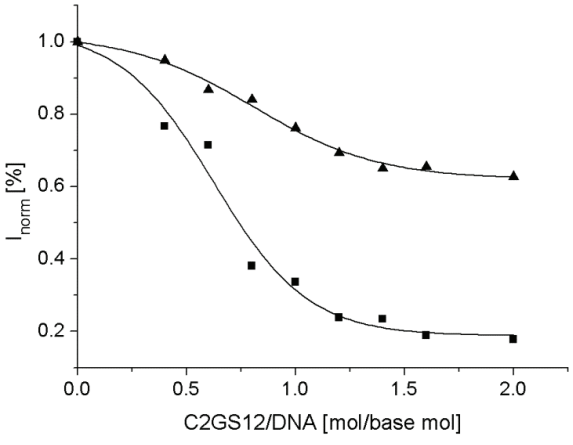

Fig. 1 Dependences of normalised fluorescence intensity I_{norm} of DNA–EtBr–C2GS12–DOPC complexes on C2GS12/DNA molar ratio at NaCl concentration 0.005 mol/l (■) and 0.15 mol/l (▲).

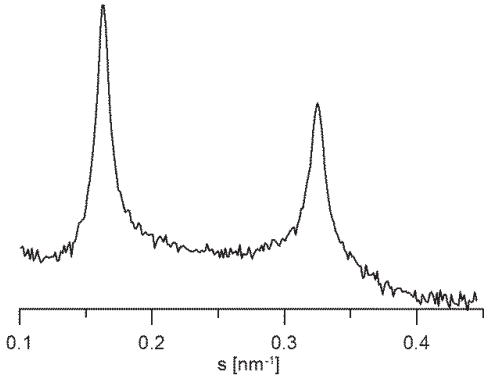

Fig. 2 Diffractogram of fully hydrated DOPC at 20°C. Intensity is in logarithmic scale.

peak. The repeat distance *d* includes the thickness of the phospholipid bilayer d_L and the water layer thickness, d_W, thus $d = d_L + d_W$. In the complexes *dw* contains a monolayer of hydrated DNA strands.

In our experimental protocol, we mixed DOPC unilamellar liposomes with a solution of C2GS12 to get the wished C2GS12/DOPC molar ratio. Due to the surfactant–lipid interaction, the hydrophobic alkyl substituents of C2GS12 molecules intercalate between the lipid acyl chains of DOPC bilayer. Polar headgroups of C2GS12 molecules create positively charged surface of the bilayer, and the C2GS12/DOPC molar ratio determines the surface charge density. Cationic C2GS12–DOPC liposomes interact with DNA and form complexes. Fig. 3 shows diffractograms of DNA–C2GS12–DOPC complexes prepared at molar ratios 0.1 ≤ C2GS12/DOPC ≤ 0.5 hydrated by 0.005 mol/l NaCl and measured at 20°C. Diffractograms are typical for a condensed lamellar phase (L_α^C) with DNA strands regularly ordered between the lipid bilayers (Lasic *et al.*, 1997; Rädler *et al.*, 1997). We observed two peaks characteristic for lipid bilayer stacking and a small broad peak related to a regular DNA packing.

The structural parameters, the repeat distance d_{LC} of L_α^C phase and DNA–DNA distance $d_{DNA} = 1/s_{DNA}$, of DNA–C2GS12–DOPC complexes hydrated by 0.005 mol/l NaCl are shown in Fig. 4. Incorporation of DNA between the lipid bilayers results in the increase *d* of L_α^C phase compared to the *d* of pure DOPC (6.13 nm). We observed a decrease of the repeat distance of L_α^C phase from $d_{LC} = 6.63 \pm 0.01$ nm at C2GS12/DOPC = 0.1 mol/mol to $d_{LC} = 6.10 \pm 0.01$ nm at C2GS12/DOPC = 0.5 mol/mol. This decrease of *d* is typical for a lamellar lipid system with incorporated amphiphilic molecules with shorter alkyl chains compared to the length of acyl chains of the lipid. This mismatch results in a higher incidence of *gauche*-conformation of the lipid chains, leads to a lateral expansion of phospholipid bilayer and the decrease in its thickness (Balgavý & Devínsky, 1996; King & Marsh, 1986). The C2GS12/DOPC molar ratio also influences the arrangement of the DNA strands. The higher amount of C2GS12 in the lipid mixture increases the surface charge density of liposomes. For complexes prepared at isoelectric point, the surface charge density is considered a key parameter influencing the d_{DNA} (Koltover *et al.*, 1999). The repeat distance d_{DNA} as a function of C2GS12/DOPC molar ratio shows a decrease from $d_{DNA} = 4.54 \pm 0.02$ nm (C2GS12/DOPC = 0.2 mol/mol) to $d_{DNA} = 3.46 \pm 0.03$ nm (C2GS12/DOPC = 0.4 mol/mol). For complexes formed at molar ratio C2GS12/DOPC = 0.1 and 0.5, respectively, the DNA peak is not observed due to its overlap with peaks of lamellar phase.

We have studied thermally induced changes of the structure of DNA–C2GS12–DOPC complexes at C2GS12/DOPE = 0.25 mol/mol in 0.005 mol/l NaCl. Fig. 5 shows the dependence of repeat distances *d* and d_{DNA} of the complexes in the range 20–60°C. For comparison, Fig. 5 shows also the temperature dependence of the DOPC repeat distance. With increasing temperature, we observe a small systematic decrease in *d* of DOPC due to thermally induced lateral expansion of the bilayer. Similarly to the pure lipid, we found the decrease of the repeat distance of L_α^C phase. d_{LC} decreases from 6.47 to 6.25 nm when the temperature increased gradually from 20 to

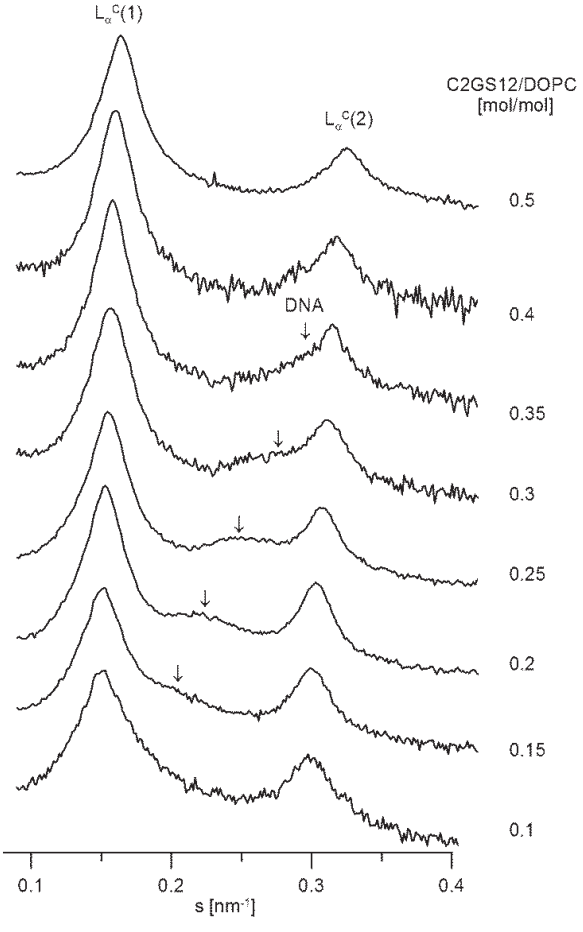

Fig. 3 Diffractograms of DNA–C2GS12–DOPC complexes in 0.005 mol/l NaCl at different C2GS12/DOPC molar ratios measured at 20°C. (Intensities are in logarithmic scale).

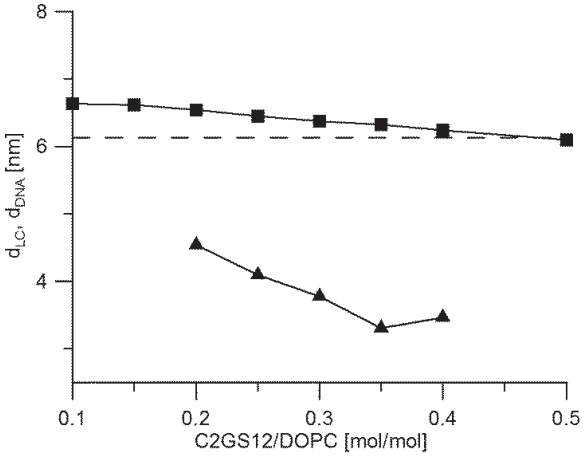

Fig. 4 Dependence of repeat distance d_{LC} (■) and DNA repeat distance d_{DNA} (▲) on C2GS12/DOPC molar ratio in 0.005 mol/l NaCl at 20°C. The dashed line represents the repeat distance of pure DOPC.

60°C. DNA follows thermally induced lateral expansion of the membrane. We determined the increase of d_{DNA} from 4.09 ± 0.03 nm at 20°C to 4.23 ± 0.03 nm at 60°C.

Diffractograms of complexes with the same composition, however, prepared in 0.15 mol/l of NaCl (Fig. 6) are different in comparison to those in Fig. 3. In addition to the first and second peaks of L_a^C phase, we observe smaller and broader peaks of another lamellar phase (L_2). The overlap of the peaks of L_a^C and L_2 phase indicates their close periodicities. The proportion of the L_a^C and L_2 phases change with the C2GS12/DOPC molar ratio: at C2GS12/DOPC = 0.1 mol/mol, the L_2 phase is only present structure. As the fraction of C2GS12 increases, the portion of L_2 phase decreases and the L_a^C phase becomes dominant. The difference in the shapes of the peaks indicates the smaller positional order of L_2 phase compared to L_a^C.

The higher ionic strength influences also the ordering of the DNA. The intensities of the DNA peak are lower compared to those observed in Fig. 3 for complexes prepared in 0.005 mol/l NaCl. This may indicate that at higher ionic strength, a lower fraction of the DNA is bound in the complexes or that the strands are less ordered.

Structural parameters of DNA–C2GS12–DOPC complexes prepared in 0.15 mol/l NaCl are plotted as a function of C2GS12/DOPC molar ratio in Fig. 7. The increase in C2GS12/DOPE molar ratio leads to similar changes in the structural parameters of L_a^C phase as observed for complexes prepared at lower ionic strength. We observe a decrease of d_{LC} from 6.75 ± 0.01 nm (at C2GS12/DOPC = 0.15 mol/mol) to d_{LC} = 6.27 ± 0.01 nm (at C2GS12/DOPC = 0.5 mol/mol) and the decrease of d_{DNA} 4.06 ± 0.04 nm (C2GS12/DOPC = 0.15 mol/mol) to 3.21 ± 0.02 (C2GS12/DOPC = 0.35 mol/mol). Thus at high ionic strength, the repeat distance of L_a^C phase increased slightly, while d_{DNA} decreased when compared to the structural parameters of complexes hydrated by 0.005 mol/l NaCl. The repeat distance of L_2 phase (d_2) is slightly lower compared to L_a^C phase. At C2GS12/DOPC = 0.1 mol/mol, the only observed phase is L_2 with, d_2 = 6.59 ± 0.01 nm. Changes in the C2GS12/DOPC molar ratio are reflected also in d_2, however, in less extensive manner compared to d_{LC}. As such, the values of d_{LC} and d_2 converge, and finally at C2GS12/DOPC = 0.5 mol/mol, they are almost equal ($d_{LC} \approx d_2$).

Repeat distances of L_a^C and L_2 phase of complexes prepared at molar ratio C2GS12/DOPC = 0.25 in 0.15 mol/l NaCl as a function of temperature are shown in Fig. 8. The repeat distances of both lamellar phases (L_a^C and L_2) consecutively decrease in the temperature range 20–42°C. Above 42°C, the values of lattice parameters of L_a^C and L_2 phases are similar and increase slightly with the temperature. Above 55°C, L_a^C phase was the only phase observed in the diffractograms.

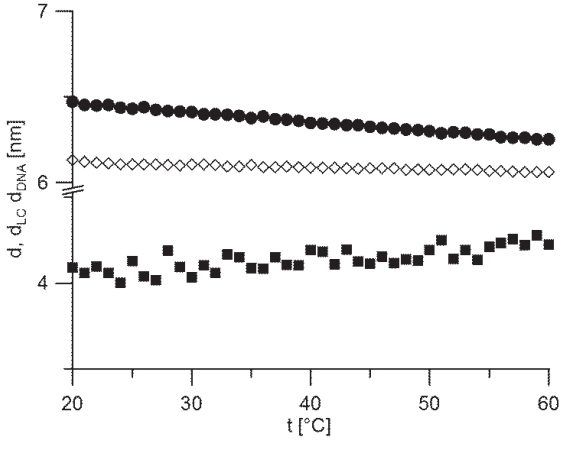

Fig. 5 Dependences of the repeat distance d_{LC} (●) of L_a^C phase and the DNA repeat distance d_{DNA} (■) on temperature at molar ratio C2GS12/DOPC = 0.25 and the repeat distance d *(◊) of pure DOPC in 0.005 mol/l NaCl.*

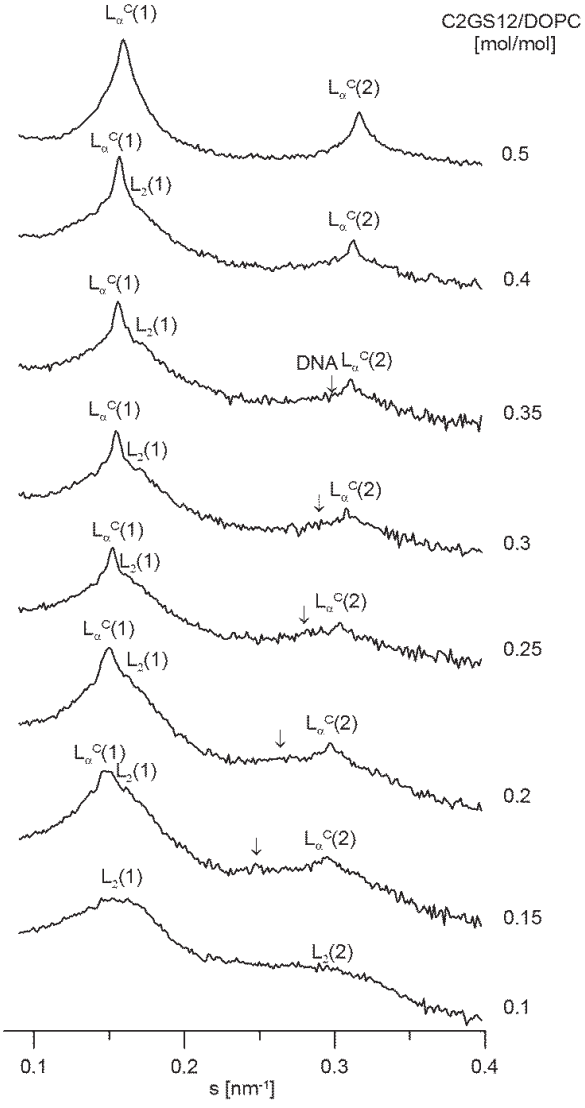

Fig. 6 Diffractograms of DNA–C2GS12–DOPC complexes at various molar ratios C2GS12/DOPC in 0.15 mol/l NaCl at 20°C (Intensities are in logarithmic scale).

DISCUSSION

Fluorescence spectroscopy experiments show clearly the influence of the ionic strength on the condensation of DNA by C2GS12–DOPC liposomes. While in 0.005 mol/l NaCl, the observed minimum of EtBr emission intensity is at approximately 22%, in 0.15 mol/l NaCl the minimum is at approximately 63% of the total emission intensity of DNA without any cationic liposomes. This minimum was achieved at the same C2GS12/DNA molar ratios (≈1.2 mol/base mol) for both studied ionic strengths. This suggests that regardless of the ionic strength of the solution, the formation of DNA–C2GS12–DOPC complexes ends at the same point. However, the experiments revealed a big difference in the minimum of EtBr emission intensity between the two ionic strengths used. This indicates a decrease in the efficiency of C2GS12/DOPC liposomes for DNA condensation at high ionic strength. The lower ability of cationic liposomes to condense DNA at higher ionic strength is caused by the screening effect of small ions present in solution on the electrostatic interaction between DNA polyanion and cationic liposomes (Jing *et al.*, 2004). Our results are in good agreement with the work of Eastman *et al.*, (1997) where it was observed a decrease in efficiency of DNA condensation by cationic lipid 1,2-dimyristyloxypropyl-3-dimethylhydroxyethyl ammonium bromide at higher ionic strength. Increase of the NaCl concentration to 1.5 mol/l lead to total suppression of DNA condensation by cationic liposomes. Eastman *et al.*, (1997) assumed that the high ionic strength leads to formation of complexes that even at the excess of cationic lipid contains uncompacted DNA without full compensation of its anionic charges. This partially uncompacted DNA would therefore still be accessible to EtBr even when it is bound in complexes with cationic liposomes.

SAXD measurements revealed that the ionic strength influences also the structure of DNA-C2GS12-DOPC complexes. In 0.005 mol/l NaCl, the complexes form typical condensed lamellar phase (L_a^C). When we increased concentration of NaCl to physiologically relevant values (0.15 mol/l), we observed a coexistence of L_a^C and a second lamellar phase L_2. The portion of both phases, L_2 and L_a^C, is dependent on the C2GS12/DOPC molar ratio and temperature. At low content of C2GS12, the dominating phase is L_2 while at C2GS12/DOPC = 0.5 mol/mol, the major structure is L_a^C phase. The closeness of lattice parameters of both phases, $d_{LC} \sim d_2$, suggests that the L_2 phase is most probably a condensed lamellar phase too, formed at slightly different C2GS12/DOPC molar ratios due to non-homogeneous mixing of the two solutions (DOPC and C2GS12) at the cationic liposomes preparation.

In our previous work Pullmannová *et al.* (2012b), the structure of DNA–CnGS12–DOPC complexes (n = 2-4) have shown differences when complexes were prepared by two different procedures: DOPC was mixed with CnGS12 in an organic solvent and then dried under a stream of gaseous nitrogen followed by vacuum. The dry lipid films were hydrated by NaCl solution at different concentration (0.005–0.200 mol/l), and multilamellar liposomes were prepared. DNA solution was added to the dispersion of liposomes by two different methods, either by drop-by-drop addition or by addition of all appropriate amount of DNA in one step. At low ionic strength, all complexes have shown L_a^C phase. At high ionic strength, the structure of complexes differed depending on the method used to DNA addition. While complexes prepared by one-step addition of DNA have shown L_a^C phase; in the complexes prepared by step-by-step addition of DNA, the phase separation was observed. Similar to our system, an additional lamellar phase has been detected, particularly at low C2GS12/DOPC molar ratios and high ionic strength of solution. However, contrary to our samples, SAXD has shown only a minor volume fraction of this lamellar phase through all CnGS12/DOPC molar ratios, and the L_a^C phase was the predominant structure. Generally, DNA–

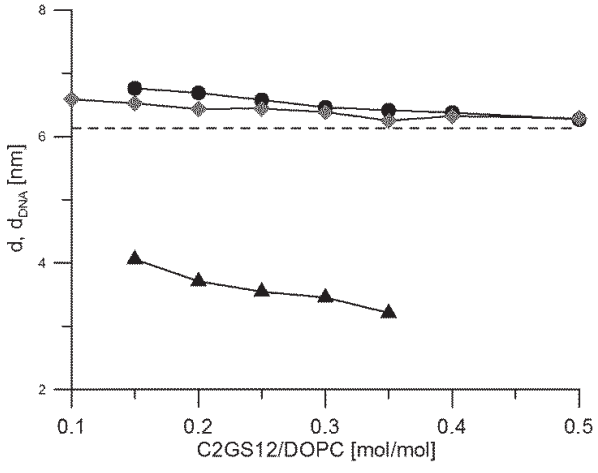

Fig. 7 Dependences of the repeat distance of L_a^C phase (d_{LC} (●)) and L_2 phase (d_2 (♦)) and DNA repeat distance d_{DNA} (▲) on C2GS12/ DOPC molar ratio in 0.15 mol/l NaCl at 20°C. The dashed line represents the repeat distance of pure DOPC.

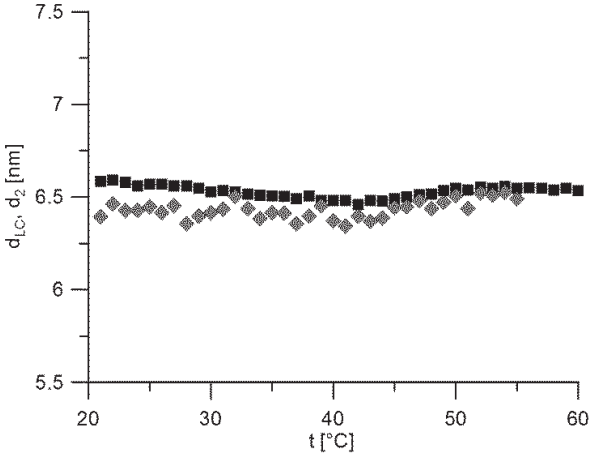

Fig. 8 Dependences of the repeat distances of L_a^C phase (d_{LC} (●)) and L_2 phase (d_2 (♦)) on temperature in 0.15 mol/l NaCl at molar ratio C2GS12/DOPC = 0.25.

CnGS12–DOPC complexes prepared by the method used in Pullmannová *et al.* (2012b) has shown better long-range ordered structure, manifested by higher intensities and sharper peaks on diffractograms. Thus preparation of complexes by direct mixing of components dispersed in aqueous solutions results in a formation of less organised structures. At high ionic strength, our method of the complex preparation also supports phase separation. We detected much higher portion of the L_2 phase compared to Pullmannová *et al.* (2012b). The revealed dissimilarities result from a different origin of the L_2 phase. In our samples, the L_2 phase was formed due to non-uniform incorporation of C2GS12 into DOPC bilayers during the components mixing. In the study of Pullmannová *et al.* (2012b), the phase separation is caused by drop-by-drop addition of DNA when in the first stages positively overcharged complexes were formed. In this stage of preparation, domains of lipid enriched with C2GS12 are formed (due to lateral diffusion of surfactants molecules in the lipid bilayer) on interaction with DNA. Both phases, the L_a^C and additional lamellar phase, are in coexistence within one structure and they cannot be separated macroscopically as proven by Pullmannová *et al.* (2012b). Similar microscopic phase separation induced by the high ionic strength was observed also in DNA complexes with mixture of cationic lipid dioleoyl trimethylammonium propane and DOPC (Koltover *et al.*, 1999), with mixture of C4GS12 and dilauroylphosphatidylcholine (Uhríková *et al.*, 2004) or with zwitterionic phospholipids in the presence of divalent cations (McManus *et al.*, 2003; Uhríková *et al.*, 2005a). Our observation of the thermally induced changes in the structure of the complexes also supports this assumption. With increasing temperature, the L_2 phase gradually merges with the L_a^C phase. This is enabled by a lateral mixing of domains in the lipid bilayer driven by the increased kinetic energy of heated molecules. Structural parameters of DNA–C2GS12–DOPC complexes reported by Pullmannová *et al.* (2012b) were determined for complexes prepared by step-by-step DNA addition at C2GS12/DOPC = 0.2 mol/mol through a range 0.005–0.200 mol/l of NaCl concentrations. Comparing structural parameters of complexes at the same composition but prepared by different methods, surprisingly, differences are small and more apparent at low ionic strength. In 0.005 mol/l NaCl, the d_{LC} of L_a^C phase is reduced by approximately 0.4 nm and the d_{DNA} by approximately 0.5 nm using our method of preparation, while in 0.15 mol/l NaCl, we detected differences smaller than 0.2 nm in d_{LC}, and ~ 0.3 nm in d_{DNA}.

The importance of the systematic study of changes in polymorphic behaviour of DNA–cationic liposomes complexes caused by the used preparation method is underlined by a recent work of Cardoso *et al.* (2014), which suggests that the way of preparation strongly affects also the transfection efficiency. Authors found that complexes DNA with C2GS12 or C2GS16 and mixture of DOPE and cholesterol has shown higher transfection efficiency when they were prepared by direct mixing of DNA, GS and helper lipid liposomes in the aqueous medium compared to the delivery vectors prepared by mixing of GS, DOPE and cholesterol in an organic solvent and, consecutively, DNA adding to GS/DOPE/cholesterol liposomes. Authors assumed that the difference in transfection efficiency could result from differences in structures of formed complexes. The structure of these complexes was not studied in the referred work.

CONCLUSIONS

We found that the ability of C2GS12-DOPC liposomes to condense DNA at physiologically relevant ionic strength is significantly reduced. While at low ionic strength (0.005 mol/l NaCl), the decrease in EtBr emission intensity indicates almost 80% of the total DNA condensed by the cationic lipid bilayer, less than 40% of DNA was bound in complexes prepared at 0.15 mol/l NaCl. Experiments revealed that the condensation process ends at the same ratio, C2GS12/DNA ≈ 1.2 mol/base mol, at both studied ionic strengths in spite of the difference in the condensation efficiency.

Our results confirmed that the method of preparation of DNA–cationic liposomes complexes affects their structure significantly. DNA–C2GS12–DOPC complexes prepared by direct mixing of DOPC unilamellar liposomes with a solution of C2GS12, and consecutively with DNA solution form a condensed lamellar phase showing a small shift in its structural parameters when compared to the complexes prepared by mixing the lipid components in an organic solvent and applying hydration method as used in Pullmannová *et al.*, (2012b). We revealed significant structural differences when complexes were formed at high ionic strength. The complexes hydrated by 0.15 mol/l NaCl have shown a lower degree of long-range order and two-phase coexistence in a large range of C2GS12–DOPC molar ratios and temperature.

The transfection efficiency of delivery vectors depends on their structure as indicate recently published experiments of Cardoso *et al.* (2014). During our many years of research in relation to the structure and methods of DNA–cationic liposomes complexes preparation, we observed that a condensed lamellar phase is formed when the lipid components are mixed in an organic solvent and dried under vacuum. Consecutively, the dry lipid film is hydrated by aqueous medium and mixed with a solution of DNA in one step. Other ways of the complexes preparation frequently result in a coexistence of two or more phases in their structure, particularly when complexes are prepared at low-surface charge density and in high-ionic strength of aqueous medium.

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REFERENCES

[1] Anderson WF. Prospects for human gene therapy. Science. 1984;226:401–409.
[2] Badea I, Verrall R, Baca-Estrada M, *et al. In vivo* cutaneous interferon-γ gene delivery using novel dicationic (gemini) surfactant–plasmid complexes. J Gene Med. 2005;7:1200–1214.
[3] Balgavý P, Devínsky F. Cut-off effects in biological activities of surfactants. Adv Colloid Interface Sci. 1996;66:23–63.
[4] Bombelli C, Faggioli F, Luciani P, Mancini G, Sacco MG. Efficient transfection of DNA by liposomes formulated with cationic gemini amphiphiles. J Med Chem. 2005;48:5378–5382.
[5] Caracciolo G, Pozzi D, Caminiti R, *et al.* Transfection efficiency boost by designer multicomponent lipoplexes. Biochim Biophys Acta. 2007;1768:2280–2292.
[6] Cardoso AM, Morais CM, Silva SG, *et al.* Bis-quaternary gemini surfactants as components of nonviral gene delivery systems: A comprehensive study from physicochemical properties to membrane interactions. Int J Pharm. 2014;474:57–69.
[7] Donkuru M, Wettig SD, Verrall RE, Badea I, Foldvari M. Designing pH-sensitive gemini nanoparticles for nonviral gene delivery into keratinocytes. J Mater Chem. 2012;22:6232–6244.
[8] Eastman S., Siegel C, Tousignant J, *et al.* Biophysical characterization of cationic lipid:DNA complexes. Biochim Biophys Acta. 1997;1325:41–62.
[9] Felgner PL, Gadek TR, Holm M, *et al.* Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Natl Acad Sci USA. 1987;84:7413–7417.
[10] Foldvari M, Badea I, Wettig S, Verrall R, Bagonluri M. Structural characterization of novel gemini non-viral DNA delivery systems for cutaneous gene therapy. J Exp Nanosci. 2006;1:165–176.
[11] García JP, Marrón E, Martín VI, Moyá ML, Lopez-Cornejo P. Conformational changes of DNA in the presence of 12-s-12 gemini surfactants (s=2 and 10). Role of the spacer's length in the interaction surfactant-polynucleotide. Colloids Surf B Biointerfaces. 2014;118:90–100.
[12] Grueso E, Kuliszewska E, Prado-Gotor R, Perez-Tejeda P, Roldan E. Improving the understanding of DNA-propanediyl-1,3-bis(dodecyldimethylammonium) dibromide interaction using thermodynamic, structural and kinetic approaches. Phys Chem Chem Phys. 2013;15:20064–20074.
[13] Hirsch-Lerner D, Zhang M, Eliyahu H, *et al.* Effect of "helper lipid" on lipoplex electrostatics. Biochim Biophys Acta. 2005;1714:71–84.
[14] Horniak L, Devínsky F, Balgavý P, Lacko I, Ebringer L, inventors; Commenius University in Bratislava, assignee. Quaternary ammonium halides for increased efficiency of bacterial transformation. Czechoslovakia, CS 269549; 1989.
[15] Imam T, Devínsky F, Lacko I, Mlynarčík D, Krasnec L. Preparation and antimicrobial activity of some new bisquaternary ammonium salts. Pharmazie. 1983;38:308–310.
[16] Izumrudov VA, Zhiryakova MV, Goulko AA. Ethidium Bromide as a Promising Probe for Studying DNA Interaction with Cationic Amphiphiles and Stability of the Resulting Complexes. Langmuir. 2002;18:10348–10356.
[17] Jing D, Zhang J, Ma L, Zhang G. Phase behavior of DNA in the presence of cetyltrimethylammonium bromide / alkyl polyglucoside surfactant mixture. Colloid Polym Sci. 2004;282:1089–1096.
[18] King MD, Marsh D. Free volume model for lipid lateral diffusion coefficients. Assessment of the temperature dependence in phosphatidylcholine and phosphatidylethanolamine bilayers. Biochim Biophys Acta. 1986;862:231–234.
[19] Kirby AJ, Camilleri P, Engberts JBFN, *et al.* Gemini Surfactants: New Synthetic Vectors for Gene Transfection. Angew Chem Int Ed. 2003;42:1448–1457.
[20] Koltover I, Salditt T, Safinya CR. Phase diagram, stability, and overcharging of lamellar cationic lipid-DNA self-assembled complexes. Biophys J. 1999;77:915–924.
[21] Lasic DD, Strey H, Stuart MCA, Podgornik R, Frederik PM. The structure of DNA-Liposome Complexes. J Am Chem Soc. 1997;119:832–833.
[22] McManus JJ, Rädler JO, Dawson KA. Phase Behavior of DPPC in a DNA–Calcium–Zwitterionic Lipid Complex Studied by Small-Angle X-ray Scattering. Langmuir. 2003;19:9630–9637.
[23] Menger, Keiper. Gemini Surfactants. Angew Chem Int Ed Engl. 2000;39:1906–1920.
[24] Mochizuki S, Kanegae N, Nishina K, *et al.* The role of the helper lipid dioleoylphosphatidylethanolamine (DOPE) for DNA transfection cooperating with a cationic lipid bearing ethylenediamine. Biochim Biophys Acta. 2013;1828:412–418.
[25] Muñoz-Úbeda M, Misra SK, Barrán-Berdón AL, *et al.* How Does the Spacer Length of Cationic Gemini Lipids Influence the Lipoplex Formation with Plasmid DNA? Physicochemical and Biochemical Characterizations and their Relevance in Gene Therapy. Biomacromolecules. 2012;13:3926–3937.
[26] Pietralik Z, Krzyszton R, Kida W, Andrzejewska W, Kozak M. Structure and Conformational Dynamics of DMPC/Dicationic Surfactant and DMPC/Dicationic Surfactant/DNA Systems. Int J Mol Sci. 2013;14:7642–7659.
[27] Pullmannová P, Uhríková D, Funari SS, *et al.* Polymorphic phase behavior of DNA – DOPE – GEMINI surfactant aggregates: a small angle x-ray diffraction. Acta Fac Pharm Univ Comenianae. 2008;55:170–182.
[28] Pullmannová P, Funari SS, Devínsky F, Uhríková D. The DNA-DNA spacing in gemini surfactants-DOPE-DNA complexes. Biochim Biophys Acta. 2012a;1818:2725–2731.

[29] Pullmannová P, Bastos M, Bai G, *et al.* The ionic strength effect on the DNA complexation by DOPC - gemini surfactants liposomes. Biophys Chem. 2012b;160:35–45.
[30] Rädler JO, Koltover I, Salditt T, Safinya CR. Structure of DNA-Cationic Liposome Complexes: DNA Intercalation in Multilamellar Membranes in Distinct Interhelical Packing Regimes. Science. 1997;275:810–814.
[31] Robbins PD, Ghivizzani SC. Viral Vectors for Gene Therapy. Pharmacol Ther. 1998;80:35–47.
[32] Rolland AP. From genes to gene medicines: recent advances in nonviral gene delivery. Crit Rev Ther Drug Carrier Syst. 1998;15:143–198.
[33] Roveri N, Bigi A, Castellani PP, *et al.* Study of rat tail tendon by x-ray diffraction and freeze-etching technics. Boll Soc Ital Biol Sper. 1980;56:953–959.
[34] Uhríková D, Rapp G, Balgavý P. Condensed lamellar phase in ternary DNA-DLPC-cationic gemini surfactant system: a small-angle synchrotron X-ray diffraction study. Bioelectrochemistry. 2002;58:87–95.
[35] Uhríková D, Hanulová M, Funari SS, *et al.* The structure of DNA-DLPC-cationic gemini surfactant aggregates: a small angle synchrotron X-ray diffraction study. Biophys Chem. 2004;111:197–204.
[36] Uhríková D, Hanulová M, Funari SS, *et al.* The structure of DNA-DOPC aggregates formed in presence of calcium and magnesium ions: a small-angle synchrotron X-ray diffraction study. Biochim Biophys Acta. 2005a;1713:15–28.
[37] Uhríková D, Zajac I, Dubnicková M, *et al.* Interaction of gemini surfactants butane-1,4-diylbis-(alkyldimethylammonium bromide) with DNA. Colloids Surf B Biointerfaces. 2005b;42:59–68.
[38] Uhríková D, Sabíková A, Hanulová M, *et al.* The microstructure of DNA-EYPC-gemini surfactant aggregates: A small angle X-ray diffraction study. Acta Fac Pharm Univ Comenianae. 2007;54:198–208.
[39] Verma IM. A tumultuous year for gene therapy. Mol Ther. 2000;2:415–416.
[40] Wasungu L, Hoekstra D. Cationic lipids, lipoplexes and intracellular delivery of genes. J Control Release. 2006;116:255–264.
[41] Wettig SD, Verrall RE, Foldvari M. Gemini Surfactants: A New Family of Building Blocks for Non-Viral Gene Delivery Systems. Curr Gene Ther. 2008;8:9–23.
[42] Wiener MC, White SH. Structure of a fluid dioleoylphosphatidylcholine bilayer determined by joint refinement of x-ray and neutron diffraction data. III. Complete structure. Biophys J. 1992;61:434–447.
[43] Wiethoff CM, Gill ML, Koe GS, Koe JG, Middaugh CR. A fluorescence study of the structure and accessibility of plasmid DNA condensed with cationic gene delivery vehicles. J Pharm Sci. 2003;92:1272–1285.