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The structural diversity of DNA - neutral phospholipids - divalent metal cations aggregates: a small-angle synchrotron X-ray diffraction study[#]

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[#] Dedicated to Prof. Dr. Klaus Arnold on the occasion of his 65th birthday

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Summary

We investigate the structure of aggregates formed due to DNA interaction with saturated neutral phosphatidylcholines (DPPC and DMPC) in presence of Ca^{2+} and Mg^{2+} cations using simultaneous synchrotron small- and wide-angle X-ray diffraction. For DPPC:DNA=3:1 mol/base and in the range of 1-50 mM Ca^{2+}, the diffractograms show structural heterogeneity of aggregates. We observe the coexistence of two lamellar phases in aggregates prepared at 1 mM Ca^{2+}: L^x phase with the DNA strands (of unknown organization) intercalated in water layers between adjacent lipid bilayers, and L_{DPPC} phase of DPPC bilayers without any divalent cations and DNA strands. Aggregates prepared in the range 2-50 mM Ca^{2+} show a condensed gel lamellar phase L_g^c with the lipid bilayer periodicity d≈8.0 nm, and the DNA-DNA interhelical distance d_{DNA}≈5.1 nm. The increase of temperature induces the decrease in the intensity and the increase in the width of the DNA related peak. In the fluid state, the condensed lamellar phase L_α^c gradually converts into L^x phase. The aggregates do not exhibit rippled P_β phase. The thermal behaviour of aggregates was investigated in the range 20-80^{o}C. Applying heating-cooling cycles, the aggregates converted into energetically more favorable structure: a condensed lamellar phase L^c (or L^x) is preserved or we observe lateral segregation of the DNA strands and metal cations (L^x phase) in coexistence with L_{PC} phase of pure phospholipids.

Keywords: DNA, dipalmitoylphosphatidylcholine, dimyristoylphosphatidylcholine, Ca^{2+}, Mg^{2+}, X-ray diffraction

Introduction

The interaction of DNA with liposomes positively charged by cationic amphiphiles results in three different types of microscopic structures: (i) spaghetti-like structure, in which DNA is covered by a cylindrical lipid bilayer (Sternberg et al., 1994), (ii) condensed lamellar L^c phase with DNA molecules intercalated between lipid bilayers (sandwich-like structure) (Radler et al., 1997), and (iii) honeycomb-like condensed columnar inverted hexagonal phase H_{II}^c with DNA molecules surrounded by lipid monolayers forming inverted cylindrical micelles arranged in a hexagonal lattice (Koltover et al., 1998).

Neutral phospholipid bilayers in presence of small metal cations (Ca^{2+}, Mg^{2+}, etc.) spontaneously form vesicles with a positive surface charge (Akashi et al., 1998; Inoko et al., 1975). The formation of large light scattering aggregates due to the interaction of polynucleotides with phosphatidylcholine vesicles in presence of Mg^{2+} ions was documented more than two decades ago (Budker et al., 1978). Earlier microcalorimetric and ESR studies performed in our laboratory indicated the formation of a new phase due to DNA interaction with multilamellar and unilamellar dipalmitoylphosphatidylcholine (DPPC) vesicles in the presence of Mg^{2+} cations (Vojčíková et al., 1989; Vojčíková, 1990). Electron freeze fracture micrographs of aggregates of DNA formed with natural egg yolk phosphatidylcholine (EYPC) and synthetic saturated DPPC in presence of Ca^{2+} cations suggested structures with long-range organization. The honeycomb-like and spaghetti-like structures were observed in EYPC+DNA+Ca^{2+} aggregates (Tarahovsky et al., 1996), while structures formed in presence of DPPC were identified either as sandwich or spaghetti-like, depending on the DNA base:DPPC molar ratio (Khusainova et al., 1999). Small-angle X-ray diffractograms show two possible structural organizations of DPPC+DNA+divalent cation aggregates: We identified a sandwich structure in the aggregate DPPC:DNA:Mg^{2+}=1:1:5 mol/base/mol in the temperature range 20-60°C, with a lipid bilayer repeat distance d~8.0-7.6 nm and interhelical DNA - DNA distance d_{DNA}~6.4-6.0 nm (Uhríková et al., 2001). For DPPC:DNA=8:1 mol/base aggregates formed in presence of 1-100 mM $CaCl_2$, the coexistence of two lamellar phases was reported (McManus et al., 2003a, b), one with DNA strands intercalated in the water layer between lipid bilayers with a repeat distance d~8.0-7.4 nm and another one with a repeat distance d~6.6-5.6 nm, which was interpreted as a phase formed by lipid bilayers only. Francescangeli et al. (2003) observed also the coexistence of two lamellar phases in aggregates of DOPC+DNA+Mn^{2+}. The coexistence of two lamellar phases in one aggregate formed by DOPC+DNA in the concentration range 0-76.5 mM of metal cations (Ca^{2+} or Mg^{2+}), effect of

temperature, DOPC:DNA molar ratio, and length of DNA fragments were studied in our recently published work (Uhríková et al., 2005a). The aggregates formed due to the interaction between DNA and dioleoylphosphatidylethanolamine (DOPE) in presence of 30 mM Mg^{2+} or Ca^{2+} show condensed columnar inverted hexagonal phase H_{II}^{c} (honeycomb-like structure) (Uhríková et al., 2005b). Francescangeli et al. (2004) reported the coexistence of condensed hexagonal phase H_{II}^{c} and H_{II} phase in DOPE+DNA aggregates in presence Fe^{2+}, Mn^{2+} and Co^{2+}. These experimental results indicate the ability of divalent cations to compact DNA into structures similar to those observed for DNA+lipid+cationic surfactant aggregates (Koltover et al., 1998; Radler et al., 1997). The ability of these aggregates to serve as vehicles for gene delivery was demonstrated in (Kovalenko et al., 1996; Sato et al., 2005; Zhdanov et al., 1997). However, the structure of the aggregates is of interest also as a model for contact sites between DNA and biomembranes (Kuvichkin, 1990).

The observed structural diversity of aggregates formed due to the interaction of DNA and neutral phosphatidylcholines (DPPC and DOPC) motivated us to study these aggregates in more detail. In this paper we investigate the effect of temperature, concentration of divalent cations, and the length of DNA fragments on the structure of aggregates formed due to DNA interaction with saturated zwitterionic phosphatidylcholines. Synchrotron small- and wide-angle X-ray diffraction (SAXD and WAXD) were used for the study of their structure.

Materials and methods

Sample preparation

Highly polymerized calf thymus DNA (Sigma Chemicals Co., USA) was dissolved in 0.5 mM NaCl, pH~7, at concentration ~2 mg/ml. The precise value of concentration was determined by measuring the absorbance A_λ at λ=260 nm. The purity of DNA was checked by measuring the absorbance A_λ at λ=260 nm and 280 nm, we have obtained A_{260}/A_{280}=1.8. Most of the samples were prepared with highly polymerized DNA (~12 kbp). The procedure of preparation of DNA with the length of fragments ~600 bp was decribed in detail in our previous work (Uhríková et al., 2005a).

Dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylcholine (DMPC) were purchased from Avanti Polar Lipids, USA. Solutions of $CaCl_2$ and $MgCl_2$ (Merck, Germany) were prepared in 0.5 mM NaCl, pH~7. Redistilled water was used for preparation of all solutions. Samples were prepared by hydration of a thin lipid film (~5 mg of lipid per sample) with a solution of DNA and divalent metal cations to obtain the required molar ratio

lipid : DNA base and concentration of ions. The samples were vortexed for a short time; a few minutes after the preparation a sediment formed in the sample. The supernatant was gently removed by a Pasteur pipette and the sediment was placed between two Kapton foils (Dupont, France), which constitute the windows of the sample holder for X-ray diffraction. Each sample was let at rest at least 30 min before to be transferred to the sample holder.

Multilamellar liposomes prepared from fully hydrated pure phosphatidylcholines were used as reference samples. Unilamellar liposomes were prepared by extrusion of multilamellar liposomes using polycarbonate filter with pores of 100 nm diameter according to (MacDonald et al., 2001).

X-ray diffraction

Small- (SAXD) and wide-angle (WAXD) synchrotron radiation diffraction experiments were performed at the soft-condensed matter beam line A2 at HASYLAB at the Deutsches Elektronen Synchrotron (DESY) in Hamburg (Germany), using a monochromatic radiation of wavelength λ=0.15 nm. The evacuated double-focusing camera was equipped with two linear delay line readout detectors. The sample was equilibrated at each selected temperature for 5 min before exposure to radiation. Temperature scans were performed at a scan rate 1 ^{o}C/min and the diffractograms were recorded for 10 s every minute. The raw data were normalized against the incident beam intensity using the signal of the ionization chamber. The SAXD detector was calibrated using rattail collagen (Roveri et al., 1980) and the WAXD detector by tripalmitin (Chapman, 1962; Kellens et al., 1990). Each diffraction peak of SAXD region was fitted with a Lorentzian above a linear background.

Results

The fully hydrated DPPC forms a lamellar phase in the temperature range 20-60 ^{o}C covered by our study. The diffractograms of DPPC+DNA+Ca^{2+} (DPPC:DNA=3:1 mol/base, 20 mM Ca^{2+}) aggregates are shown in Fig. 1. Two Bragg reflections at equidistant positions in the SAX region correspond to the lamellar phase with periodicity $d \approx 8.0$ nm in the gel state, resulting from the lipid bilayer stacking. The broad reflection with lower intensity, at $s \approx 0.196$ nm^{-1}, is identified as an evidence of DNA-DNA organization. We have found the interhelical DNA-DNA distance $d_{DNA} = 5.11 \pm 0.03$ nm at 20 ^{o}C. The diffractogram at 20 ^{o}C is

a typical diffractogram of a condensed lamellar phase L_β^c (Fig. 1, 20 ^oC). When heating the aggregate to higher temperatures, we have observed an increase in the DNA reflection width and decrease of its intensity (Fig. 1, 39 ^oC). The peak related to the DNA-DNA organization vanishes into the background when the sample is heated up to 70^oC and above (Fig. 1, 70 ^oC). The WAX region (in the range ~20-35^oC; Fig. 1, 20 ^oC) shows one asymmetric peak typical of $L_{\beta'}$ gel phase of nonoriented bilayers with lipid acyl chains tilted with respect to bilayer normal (Ranck et al., 1974). At higher temperatures ($t \approx 39$ ^oC) we observe a change in the shape of the peak: it becomes symmetric (Fig. 1, 39 ^oC). The symmetric shape of the peak in the WAX region is characteristic of the organization of the acyl chains perpendicular to the surface of the bilayer (gel L_β or P_β phase) (Ranck et al., 1974). Above 45 ^oC, the WAX region of the diffractograms shows a wide diffuse scattering typical of liquid-like acyl chains of DPPC molecules (Fig. 1, 70 ^oC). The SAX region was fitted by a superposition of three Lorentzians. From the positions of the peaks, we determine the repeat distance of lipid bilayer stacking ($d=1/s_1$, s_1 is the position of the first maximum), and the DNA-DNA interhelical distance ($d_{DNA}= 1/s_{DNA}$). The temperature dependences of the repeat distance d and d_{DNA} of DPPC+DNA+Ca^{2+} aggregates are shown in Fig. 2.

The temperature behaviour of fully hydrated pure DPPC is well known. In the temperature range 20-60 ^oC it shows two phase transitions: tilted gel $L_{\beta'} \rightarrow$ rippled gel $P_\beta \rightarrow$ liquid-crystalline L_α. Two abrupt changes of the repeat distance (Fig. 2) reflect the pretransition $T_p \approx 35$ ^oC ($L_{\beta'} \rightarrow P_\beta$) and the main phase transition $T_t \approx 42$ ^oC ($P_\beta \rightarrow L_\alpha$) and correspond to the literature (Albon and Sturtevant, 1978; Stumpel et al., 1983). We find the repeat distance $d = 6.33 \pm 0.01$ nm at 20 ^oC and this value increases only slightly in the temperature range of $L_{\beta'}$ phase. A marked change of the repeat distance in P_β phase ($d = 7.02$ nm at 38 ^oC) results partly from the changes in the lipid acyl chain organization, and mainly, as a consequence of a lipid - water interface rippling. This phase has SAXD peaks requiring two indices (h, k), where the period corresponding to h is the usual lamellar spacing (d) and the k index corresponds to the repeat distance (d_r) of ripples in the plane of the bilayer (Janiak et al., 1979). We observe a significant broadening (Δs_1=0.003 nm^{-1} and 0.007 nm^{-1} at 20 ^oC and 40 ^oC, respectively) and an asymmetry of peaks in the SAX region (not shown in figure) due to the superposition of (1,0) and (2,0) reflections of lipid bilayer stacking with (1,1) and (2,1) reflections of monoclinic lattice of P_β phase (Yao et al., 1991). Some of the diffractograms in this temperature range showed a poorly observable (0,1) reflection at $s \approx 0.072$ nm^{-1}, corresponding to the rippling repeat distance $d_r \approx 13.9 \pm 0.2$ nm. The repeat

distance d slightly decreases with increasing temperature in the liquid - crystalline L_α phase; we found d = 6.59±0.01 nm at 50 ^oC. Nagle and Tristram - Nagle (2000) reported for fully hydrated DPPC repeat distances 6.35 nm at 20 ^oC and 6.70 nm at 50 ^oC.

The repeat distance of lipid bilayer stacking of DPPC+DNA+Ca^{2+} aggregate shows only one phase transition; from gel to liquid - crystalline state at $T_t \approx 44\ ^oC$ (Fig. 2). Khusainova et al. (1999) reported $T_t = 44.1\ ^oC$ for the DPPC+DNA aggregate in 20 mM $CaCl_2$. In the temperature range of the gel state, the repeat distance d = 8.02 nm (at 20 ^oC) is constant within standard deviation ±0.01 nm. The repeat distance d in liquid-crystalline L_α phase gradually decreased with increasing temperature; we found d =7.55±0.01 nm at 50 ^oC. The distance d_{DNA} between DNA strands shows small fluctuations, its average value is d_{DNA}≈5.1 nm in the temperature range below 35 ^oC. In the range of temperatures corresponding to the rippled phase in pure DPPC (~35-42 ^oC), we observed an increase of DNA-DNA distance as well as its fluctuations. We determine the average value d_{DNA}= 5.31±0.07 nm in this range. Diffraction pattern of the aggregates in the gel state do not show rippling, nor broadening and asymmetry of diffraction peaks is observed. We found the half-widths of the first order peak at half maximum $\Delta s_1 \sim 0.003\ nm^{-1}$ in the whole temperature range below T_t. However, the WAX region of diffractograms in Fig. 1 indicates changes in the orientation of lipid acyl chains in a temperature range below T_t. The DNA strands located in the water layer between the lipid bilayers probably dump the rippling of the DPPC bilayer, what results in the observed d_{DNA} fluctuations. Above the main phase transition, the DNA-DNA distance slightly decreases, though the experimental error increases due to the broadening of the diffraction peak. We determined the DNA-DNA distance d_{DNA} = 5.02±0.11 nm at 50 ^oC. Structural parameters of the aggregate are summarized in Tab. 1. The structural parameters of DPPC+DNA+Ca^{2+} aggregates obtained are similar to those of DPPC+DNA+Mg^{2+} aggregates (Uhríková et al., 2001), i.e. d=8.12±0.01 nm and d_{DNA}=6.35±0.16 nm in the gel state (at 28 ^oC) and d=7.67±0.05 nm and d_{DNA}=6.12±0.07 nm in the liquid-crystalline state (at 53 ^oC). McManus et al. (2003a) observed regular packing of DNA strands with d_{DNA}= 5.14 nm in the temperature range below T_t in the aggregates DPPC:DNA=8:1 mol/base formed in presence of Ca^{2+} ions. The aggregates showed the coexistence of two lamellar phases interpreted by the authors as a phase with DNA strands packed between lipid bilayers with periodicity ~ 7.8 nm and a phase of uncomplexed lipid with the repeat distance ~ 6.3 nm.

To understand the structural changes of DPPC+DNA+Ca^{2+} aggregates induced by temperature, it is necessary to look at the structural parameters in more detail. The repeat distance d is the sum

$$d=d_s + d_w \quad \text{(Eq. 1)}$$

where d_s is the steric thickness of the lipid bilayer and d_w is the thickness of the aqueous layer between neighbouring bilayers which is filled with DNA strands in the aggregates. d_s includes molecules of water localized in the headgroup region of the lipid bilayer. In the lipid - water mixture, the volume of water between two phospholipid molecules of adjacent bilayers is equal to the interbilayer separation (d_w) times the area per lipid molecule A. Aggregates were prepared in excess of water. Not all the water that is added to the lipid is located between stacks of bilayers and so it is not trivial to determine the thickness d_s, and the amount of water between opposite lipids bilayers as expressed in Eq. 1 (Gawrisch et al., 1985; Nagle and Tristram-Nagle, 2000). The problem of determination of the thickness of the lipid bilayer and of the area per lipid molecule of fully hydrated bilayers was analysed in (Nagle and Tristram-Nagle, 2000).

The observed repeat distance d is a result of the electrostatic screening of repulsion between phosphate fragments of DPPC and DNA by metal ions. Earlier experiments (Izumitani, 1994; Shepherd and Buldt, 1978) have shown that the binding site for cations is near the negative phosphate group of the $P^- - N^+$ dipole of phospholipid headgroup. The binding or adsorption of metal cations influences the orientation and conformation of the choline headgroup. The positive end of the phosphocholine dipole is pushed out into the aqueous phase when positively charged metal cations are adsorbed to the lipid bilayer (Scherer and Seelig, 1989, and references therein). Ca^{2+} ions may form bridges between two neighboring phospholipid molecules, through their phosphate groups, which establishes an attractive electrostatic interaction. This attractive force may reduce the area per lipid molecule (Huster et al., 1999, and references therein). Neutralizing negative charge of phosphate group, the lipid bilayer becomes positively charged, and due to electrostatic interaction of the positive end of the $P^- - N^+$ dipole with the negatively charged phosphate groups of DNA, the aggregate is formed.

Huster et al. (1999) reported the reduction in the area per DMPC molecule ($\Delta A=0.027$ nm^2) in the liquid-crystalline phase when Ca^{2+} ions (15 mM, 37 ^oC) mediate the binding of anionic polyelectrolyte (sulfate dextrane) to the DMPC bilayers. These authors reported the increase of DMPC order parameter that characterizes the organization of lipid acyl chains. Higher order translates into more extended hydrocarbon chains and decreases the

average lipid area per molecule. We tried to evaluate the change in the steric lipid bilayer thickness due to the reduction of area per molecule A in DMPC. According to Nagle and Tristram-Nagle (2000) the steric lipid bilayer thickness is $d_s=2(d_c+d_H)$, where d_H is the steric headgroup thickness ($d_H=0.9$ nm for PC), and d_c is the thickness of hydrocarbon core, $d_c=V_c/A$. The V_c is the sum of volumes of chain methylene and methyl groups, for DMPC it is $V_c=0.782$ nm^3 at 30 ^{o}C. The area per DMPC molecule is $A=0.596$ nm^2 at 30 ^{o}C (Nagle and Tristram-Nagle, 2000). In the fluid lamellar phase, an increase of temperature should induce an increased population of gauche rotamers in lipid acyl chains accompanied by a lateral expansion of the bilayer, resulting in the increase of A and the decrease of the steric lipid bilayer thickness d_s. NMR experiments of (Huster et al., 1999) were performed at 37 ^{o}C. Evans and Needham (1987) have estimated the parameter of DMPC thermal area expansivity $\beta=0.00417\pm0.00020$ K^{-1}. The thermal volume expansivity $\gamma=0.00071\pm0.00002$ K^{-1} for the fluid lamellar phase of DMPC was reported in Nagle and Wilkinson (1978). Using the data of Nagle and Tristram-Nagle (2000) and the thermal expansivity parameters β, and γ, we obtained the area per DMPC molecule $A=0.614$ nm^2 and the steric lipid bilayer thickness $d_s=4.36$ nm at 37 ^{o}C (for the procedure in detail see e.g. Uhríková et al. 2002). Applying the reduction in the DMPC molecule area $\Delta A=0.027$ nm^2 (Huster et al., 1999), one can calculate the increase in the steric lipid bilayer thickness $\Delta d_s=0.12$ nm in the L_α phase, due to the adsorption of Ca^{2+} ions and dextrane on the DMPC lipid bilayer. Tatulian et al. (1991; and references therein) did not observe any changes of bilayer thickness of DPPC when the lipid was hydrated at 100 mM $CaCl_2$ within accuracy ±0.2 nm. To summarize the above mentioned data: due to adsorption of Ca^{2+}, the steric lipid bilayer thickness is not changed within accuracy ±0.2 nm. Nagle and Tristram-Nagle (2000) reported the steric lipid bilayer thickness of DPPC $d_s=5.24$ nm and 4.65 nm at 20 and 50 ^{o}C, respectively. By subtracting the thickness of lipid bilayer d_s (Eq. 1) we obtain the interbilayer distances $d_w\approx2.78$ nm and 2.90 nm at 20 and 50 ^{o}C, respectively. The transverse dimension of DNA is 2 nm. We see that DNA can easily fit between the lipid bilayers, and that there is a thin aqueous layer between its strands and bilayer surfaces.

Let us inspect shortly the structure of aggregates formed due to DNA interaction with cationic liposomes consisting of a mixture of neutral phospholipids and cationic lipid or cationic surfactants. After the intercalation of cationic amphiphiles into the bilayer between zwitterionic phospholipid molecules, the bilayer surface becomes positively charged (Haydon and Myers, 1973; Requena and Haydon, 1985) and the bilayer structure is disturbed

depending on the amphiphile concentration, alkyl chain length and counter-ion (Balgavý et al., 1984; Balgavý and Devínsky, 1996; Sarapuk et al., 1998). The smallest bilayer structural perturbation occurs when the cationic amphiphile and the lipid have approximately equal lengths of hydrocarbon chains (Balgavý and Devínsky, 1996; Gallová et al., 1990). To preserve the integrity of the lipid bilayer, neutral lipids and cationic surfactants with equal lengths of hydrocarbon chains are usually used for DNA carrier vectors. Polar headgroups of surfactant molecules provide a positive charge fixed to the bilayer surface by hydrophobic interactions between lipid acyl chains and alkyl chains of amphiphiles. The lipid bilayer allows for lateral diffusion of the molecules. In an isolated layer, the spatial distribution of the lipid and surfactant molecules will be, on average, uniform. The close proximity of oppositely charged DNA strands can induce substantial lateral redistribution of cationic surfactants that tend to concentrate near the DNA strands. The condensed lamellar phase L^c is a lamellar stack of cationic-nonionic lipid bilayers with intercalating monolayers of parallel DNA rods (May and Ben Shaul, 2004). Modelling of L^c phase shows a tendency of the cationic bilayers to partially wrap around the DNA rods. This DNA lattice may actually support the formation of periodic membrane undulations. It was found that the membranes undulate with a periodicity that is determined by the DNA interaxial distance (Schiessel and Aranda-Espinoza, 2001). The amplitude of this sinusoidal deformation depends on the composition-dependent material properties of the cationic lipid bilayer. As a consequence, the lamellar repeat distance changes resulting in a compression (or swelling) of the lamellar stack (Dan, 1996; Dan, 1997; Harries et al., 2003; May and Ben Shaul, 2004). Indeed, X - ray diffraction data (Radler et al., 1997; Koltover et al., 1999; Zantl et al., 1999; Koltover et al., 2000; Uhríková et al., 2002; Uhríková et al., 2004) indicate a "tight packing" of aggregates DNA - neutral phospholipid bilayer - cationic amphiphiles. Depending on the used lipid and its molar fraction in the mixture, the periodicity of lipid lamellar stacking $d \sim 6 - 7$ nm was observed in the liquid - crystalline phase. While the DNA-DNA distance varies in the range $2.5 \leq d_{DNA} < 6.2$ nm, d_{DNA} decreases as the mole fraction of the cationic amphiphile increases; the interbilayer thickness $d_w \sim 2.5$ nm is nearly constant in all lamellar complexes. Noting that the radius of DNA is 1 nm, it follows that the minimal spacing of DNA strands, $d_{DNA} = 2.5$ nm, corresponds to nearly close DNA-DNA contact, with only a thin hydration shell separating the strands. The thickness of d_w also corresponds to close contact between the hydrated DNA and the surfaces of lipid layers. The analysis of the line shape of the DNA peak suggests a 2D smectic order of strands with cross-correlations between the DNA strands of adjacent layers (Salditt et al., 1997; Salditt et al., 1998). Double stranded DNA is a rigid rod-like molecule showing negligible flexibility.

Salditt et al. (1998) suggested that the bending rigidity of the lipid bilayer must be locally orientation dependent, with the rigidity enhanced in the direction along the strands. Given the high persistence length of DNA of about 50 nm, thermal fluctuations of the bilayer along the normal must therefore be totally suppressed at least on smaller length scales, while the system allows bending in-plane of bilayer more easily. The compressional modes must also be affected by DNA, reducing the fluctuations of the lamellar periodicity d due to electrostatic and hard core interaction.

In the fully hydrated pure phospholipid bilayers, the strength of undulatory fluctuations is proportional to the square of the temperature (Helfrich, 1978). These fluctuations cause disorder in the relative position of unit cells (long-range disorder) resulting in broadening of diffraction peaks, and the magnitude of this effect increases with increasing diffraction order (Zhang et al., 1996; Petrache et al., 1998; Gordeliy et al., 2005). In our previous paper (Uhríková et al., 2002) we used the ratio of the widths of second and first diffraction peaks $\Delta s_2/\Delta s_1$, corresponding to the lipid bilayer stacking, to quantify temperature induced disorder in lipid bilayer organization. The ratio $\Delta s_2/\Delta s_1$ decreased slightly linearly with increasing temperature in the range 5 - 85 ^{o}C, what is an evidence that the DNA strands trapped between bilayers charged by cationic amphiphiles, improve the organization of bilayer stacking.

To compare structural parameters of our DPPC+DNA+Ca^{2+} aggregate and those formed with cationic amphiphiles, the difference in the aggregate packing is evident. In our aggregates, the repeat distance d and the interbilayer thickness d_w indicate a higher level of "freedom" of DNA strands. The DNA peaks exhibit a considerable line broadening, indicative of disorder in the DNA lattice (see Fig. 1). We found the half-widths of the DNA peak at half maximum Δs_{DNA}=0.051±0.003 nm^{-1} and 0.060±0.015 nm^{-1} at 20 and 60 ^{o}C, respectively, what is 2.5 - 3 times higher than Δs_{DNA} observed in aggregates with cationic amphiphiles (Salditt et al., 1998; Uhríková et al., 2002). The temperature dependence of the DNA peak intensity is shown in Fig. 3, inset. In the gel phase, the intensity slightly fluctuates within the range of experimental error. In the temperature range of gel→fluid phase transition the intensity decreases markedly. In the L_α phase, the intensity of DNA peak gradually decreases, and at ~70^{o}C the peak vanishes into the background (Fig. 1, 70 ^{o}C). Such marked changes of the intensity of the DNA peak were not observed in aggregates formed with cationic amphiphiles, even when samples were heated up to 90 ^{o}C (Zantl et al., 1998; Uhríková et al., 2002). With the aim to clarify if the temperature induced lipid bilayer fluctuations are responsible for observed

changes of the DNA peak intensity, we investigated the widths of first and second order peaks in diffractograms of DPPC+DNA+Ca^{2+} aggregate. While the width of the first order peak was nearly constant, $\Delta s_1 \sim 0.002\ nm^{-1}$, we observed a slight decrease of Δs_2 with increasing temperature in the L_α phase (not shown). The ratio of the widths of second and first diffraction peaks $\Delta s_2/\Delta s_1$ slightly decreases linearly with increasing temperature in the liquid-crystalline phase (Fig. 3). Qualitatively similar temperature induced changes of the $\Delta s_2/\Delta s_1$ width of first and second diffraction peak were observed when cationic amphiphiles were used for the formation of the aggregate (Uhríková et al., 2002). It should be noticed that the ratio $\Delta s_2/\Delta s_1$ increases nonlinearly with temperature in fully hydrated bilayers of pure phospholipids (Fig. 3) (see also Uhríková et al., 2002; 2005a). Our short analysis indicates no marked differences in temperature induced fluctuations of lipid bilayers trapped in aggregates formed with divalent metal cations and cationic amphiphiles. Consequently, the lipid bilayer fluctuations are not responsible for the DNA peak broadening observed in our diffractograms. So, the disorder in the DNA lattice is caused dominantly by in-plane fluctuations of the DNA strands. The metal cations, because of their high mobility, probably do not induce enough constraints to allow the DNA strands to assume such regular packing as it was observed in aggregates with cationic amphiphiles. The cations are not "anchored" in the hydrophobic region of the bilayer and probably do not induce "sinusoidal deformation" of the bilayer what helps to keep the regular packing of DNA strands in aggregates with cationic amphiphiles.

We studied also the thermal stability of DPPC+DNA+Ca^{2+} aggregate. After the temperature scan, the sample was heated up to 80 ^oC. We found the repeat distance $d = 7.23 \pm 0.01$ nm at 80 ^oC, and no marked changes in the shape of diffraction peaks were observed. After this heating procedure, the sample was intensely cooled to 20 ^oC, and measured 5 min later. As follows from Fig. 1, the aggregate keeps its structure, with regular packing of the DNA strands. We found the repeat distance of the lipid stacking $d = 7.96 \pm 0.01$ nm and the DNA-DNA distance $d_{DNA} = 5.10 \pm 0.04$ nm. Sample was measured again 2 days later. We observed no change in the repeat distance d, the DNA-DNA distance was changed within an experimental error ($d_{DNA} = 5.18 \pm 0.04$ nm). The width of DNA peak decreased from $\Delta s_{DNA} = 0.067 \pm 0.005\ nm^{-1}$ to $\Delta s_{DNA} = 0.057 \pm 0.004\ nm^{-1}$, what indicates a slight increase in the regularity of the DNA packing. Artzner et al. (1998) reported that the temperature cycling increased the DNA peak intensity and decreased its width in aggregates of DNA with DMPC and cationic surfactant DMTAP.

The structural diversity of aggregates

With increasing concentration of Ca^{2+}, we observed changes in diffraction patterns of DPPC+DNA+Ca^{2+} aggregates; see diffractograms of aggregates prepared in solutions of 1, 2 and 50 mM Ca^{2+}, respectively, on Fig. 4. The WAX region of all diffractograms exhibits a typical gel phase patterns (not shown).

The diffractogram of the aggregate DPPC:DNA=3:1 mol/base prepared at 1 mM Ca^{2+} (Fig. 4a) is interpreted as a superposition of two one-dimensional periodic structures. The reflections $L(1)^x$ and $L(2)^x$ correspond to the lamellar phase (L^x). The repeat distance d_{Lx} = 8.04±0.01 nm at 20 ^oC, provides enough space for DNA strands to be intercalated in the water layers between the DPPC bilayers. We do not observe any DNA reflection at 20 ^oC, even when the aggregates were heated between 20 - 60 ^oC. Because the packing of DNA strands in this phase is not obvious, we use the abbreviation L^x. The reflections $L(1)_{DPPC}$ and $L(2)_{DPPC}$ with the position of the first order maximum s=0.154 nm^{-1}, close to the position of the first order maximum of multilamellar DPPC structure (s=0.158 nm^{-1}), were identified as reflections due to the DPPC bilayer stacking (L_{DPPC}). We determined the repeat distance of L_{DPPC} phase, d_{DPPC} = 6.49±0.01 nm at 20 ^oC. Subtracting the DPPC lipid bilayer thickness d_s = 5.24 nm (Nagle and Tristram-Nagle, 2000), the interlamellar thickness d_w~1.25 nm do not offer enough space for DNA strands. The SAX diffraction pattern of neutral phospholipid bilayers already at low concentrations of Ca^{2+} or Mg^{2+} shows significant changes (Inoko et al. 1975, Tatulian et al. 1991): the addition of 1 mM $CaCl_2$ destroys the lamellar structure of neutral phosphatidylcholines and makes it to swell into excess water. The lamellar phase reappears when the concentration of $CaCl_2$ further increases. A partially disordered lamellar phase with the repeat distance 15-20 nm appears at about 10 mM, and d ~ 9-10 nm at 100 mM. Akashi et al. (1998) have shown that 1-30 mM solution of Ca^{2+} or Mg^{2+} promotes the formation of giant unilamellar vesicles. The repeat distance of L_{DPPC} phase, d_{DPPC} = 6.49±0.01 nm, detected in the DPPC+DNA+Ca^{2+} aggregate thus indicate no presence of Ca^{2+} cations. The existence of a “pure” phosphatidylcholine phase in samples prepared in solution of divalent cations is rather surprising. Such a coexistence of two lamellar phases is reported in aggregates of DOPC+DNA+Mn^{2+} (Francescangeli et al., 2003) and also in the liquid - crystalline phase of DPPC : DNA=8 : 1 (mol/base) aggregates at 1 - 100 mM Ca^{2+} (McManus et al., 2003b), where mechanism of interaction and models of coexistence of both phases were proposed. Our previous work (Uhríková et al., 2005a) documents that such

structure forms also DOPC + DNA in a wide range of cations concentrations (0 - 76.5 mM of Ca^{2+} or Mg^{2+}). The coexistence of two phases was investigated as a function of DOPC:DNA molar ratio, length of DNA fragments and temperature. If the amount of lipid increased, the fraction of L_{DOPC} phase was limited, depended on the portion of DNA in the sample and also on the length of DNA fragments. The coexistence of two phases can be explained by a lateral segregation of DNA and metal cations. It was suggested that the presence of negatively charged DNA between bilayers can induce a partial lateral segregation of cationic amphiphiles in bilayers to minimize the electrostatic energy of the whole system, i.e. lateral "demixing" in the plane of the bilayers can occur (Bruinsma and Mashl, 1998; Harries et al., 1998). Such a demixing was observed experimentally for aggregates with cationic amphiphiles (Macdonald et al., 1998; Mitrakos and Macdonald, 1996).

In the gel phase, diffractograms of DPPC+DNA in 1 mM Ca^{2+} do not show marked changes in the positions, intensities, and width of reflections of both phases with increasing temperature. In the liquid - crystalline phase, we observed the decrease in the intensity and the increase in the width of the second order reflection of L_{DPPC} phase at higher temperatures ($t \geq 50\ ^{o}C$). With regards to our experimental protocol, 1 mM solution of Ca^{2+} represents a molar balance DNA :Ca^{2+} ≈2.2 (base/mol) which indicates the excess of the DNA negative charge in the sample. The lack of positively charged cations could be thus responsible for the observed lateral segregation of DNA and temperature induced changes of the aggregate in the liquid-crystalline phase. However, it should be noticed that McManus et al. (2003a, b) have reported the coexistence of two lamellar phases in DPPC+DNA aggregates in the concentration range of 1 - 100 mM Ca^{2+}, which indicates that the preparation method, handling and history of the sample can play an important role in the formation of its structure.

Fig. 4b and c show diffractograms of DPPC:DNA=3:1 mol/base aggregate prepared in 2 mM Ca^{2+} solution at 20 and 37 ^{o}C, respectively. The diffractogram at 20 ^{o}C (Fig. 4b) shows the coexistence of two lamellar phases: L_{DPPC}, with the repeat distance d_{DPPC} = 6.54±0.01 nm, formed by lipid bilayers without DNA and Ca^{2+} ions, and the second phase, with d = 8.07±0.02 nm, identified as a condensed lamellar phase L^{c} with regularly organized DNA strands of periodicity d_{DNA} = 5.18±0.07 nm. A small increase in Ca^{2+} concentration improved the organization of DNA strands inside of L^{x} phase, although the DNA peak is broad (Δs_{DNA} = 0.058±0.08 nm^{-1}), indicating a poor organization of the DNA lattice. Heating the aggregate, we observed the decrease in the intensity of both reflections of L_{DPPC} phase. At ~37 ^{o}C, the L_{DPPC} phase "dissolved" completely, the diffractogram shows only the L^{c} phase

(Fig. 4c) in the aggregates. Experimental data indicate a slight increase of the DNA-DNA distance, d_{DNA} = 5.23±0.07 nm. The subsequent increase of temperature induced a disorder in the DNA packing, so the DNA peak vanished into the background. Structural parameters of the aggregates are summarized in Tab. 1.

Due to the interaction between DNA and DPPC (DPPC:DNA=3:1 mol/base) in 50 mM Ca^{2+} at 20 ^{o}C, a condensed lamellar phase L_{β}^{c} shows an additional well defined DNA peak at s ~ 0.164 nm^{-1}, as depicted in Fig. 4d. The lipid bilayer stacking gives two peaks corresponding to the periodicity d = 8.22±0.04 nm. As follows from Tab. 1, this value of d indicates a small increase in the interbilayer thickness. The concentration of Ca^{2+} cations in the interlamellar space of the L^{c} phase is probably higher than that necessary for the electrostatic screening of the repulsion between the DNA polyanion and DPPC phosphate fragments. The excess of Ca^{2+} creates a positive charge at the bilayer surface resulting in the observed increase of d. Also the increase of the DNA-DNA distance, d_{DNA} = 6.08±0.02 nm, could arise from the electrostatic repulsion between strands due to excess of positive charge. The increase of the distance between DNA strands in positively overcharged aggregates with cationic amphiphiles was reported (Koltover et al., 1999). The half-width at half maximum of the DNA peak, Δs_{DNA} = 0.012±0.01 nm^{-1}, indicates a better organization of the DNA lattice in comparison with that of the aggregates prepared at lower Ca^{2+} concentrations. Experimental data lead to the thought that electrostatic repulsion between strands helps "to keep the DNA-DNA distance", and so improves the organization of the DNA lattice. The temperature behaviour of the aggregates was similar to that of DPPC+DNA aggregates prepared at 20 mM Ca^{2+} described above. When heating the aggregates to higher temperatures, we have observed an increase in the DNA peak width and a decrease of its intensity, which gradually disappears in the background. After repeated heating and cooling process in the range 15 - 80 ^{o}C (2 times) we found (at 20 ^{o}C) a slight decrease in the periodicity of both the lipid bilayer stacking d as well as d_{DNA}. The DNA peak became broader, observable only when the intensity is displayed in a logarithmic scale. Structural parameters of the aggregates are given in Tab. 1.

We tested the temperature stability of the aggregates prepared using the DNA with shorter length of fragments (~600 bp). Fig. 5 shows SAX diffractograms of the DMPC : DNA=2:1 mol/base aggregate prepared at 50 mM Mg^{2+}. The aggregates form the condensed lamellar phase L^{c} (Fig. 5). Three marked peaks in the SAX region correspond to the lipid bilayer stacking of periodicity d = 7.98±0.02 nm at 20 ^{o}C. We found the DNA-DNA

distance d_{DNA} = 4.64±0.03 nm at 20 °C. In the covered temperature range 10 – 60 °C, the aggregates undergo the transition from the gel to the liquid crystalline phase, as documents the WAX region of the diffractograms. Fully hydrated DMPC shows the main phase transition at T_t = 23.9 °C (Lewis et al., 1987). The diffractogram of the DMPC+DNA+Mg^{2+} aggregate at 30 °C shows the DMPC repeat distance d = 7.60±0.01 nm. Nagle and Tristram-Nagle (2000) reported the steric lipid bilayer thickness d_s = 4.42 nm at 30 °C for fully hydrated DMPC. The interbilayer thickness $d_w = d-d_s \approx 3.1$ nm indicates again "high freedom" in the DNA strands packing probably due to excess of positive charge in the interlamellar space. Applying repeatedly heating-cooling cycles in the range 10 - 80 °C, the aggregates changed their structural organization, as shown in Fig. 5 (10 °C, upper diffractogram). The diffractogram shows the coexistence of two lamellar phases and no reflection corresponding to the DNA-DNA correlation. The process of heating-cooling induced a lateral segregation of the DNA strands and metal cations. At 20 °C, we determined the repeat distances d_{DMPC} = 5.76±0.01 nm of the L_{DMPC} phase and $d_L{}^x$ = 7.44±0.01 nm of the L^x phase with unknown organization of DNA strands. To compare the repeat distance L^x of the aggregates with the one before heating (L^C), one can see the decrease in the periodicity $\Delta d \approx 0.5$ nm of the phase with intercalated DNA strands. Temperature cycles induced structural changes in the aggregates.

When DNA aggregates with unilamellar liposomes from DPPC (DPPC:DNA=1:1 mol/base) in 20 mM Ca^{2+}, the diffractogram shows the presence of only one lamellar phase (L^x) with the repeat distance $d_L{}^x$ = 7.94±0.03 nm at 20 °C (not shown). The periodicity of lipid bilayer stacking indicates the presence of DNA strands between DPPC bilayers charged by Ca^{2+} cations. Unilamellar liposomes do not show long-range organization, so the diffraction pattern itself indicates the formation of an aggregate with an organized structure. We did not observe any peak due to DNA-DNA correlation, also when the sample was repeatedly cycled in the temperature range 20 - 60 °C. We observed a similar diffraction pattern, the lamellar phase L^x of periodicity $d_L{}^x$ = 7.49±0.03 nm (at 20 °C), when DNA aggregated with unilamellar DOPC liposomes (DOPC:DNA=3:1 mol/base, 20 mM Ca^{2+}). In contrast, McManus at al. (2004) documented an organization of DNA strands in a rectangular columnar phase with two-dimensional lattice constants a = 3.53 nm and $b = 2d_{lam}$ = 15.66 nm, where d_{lam} is the periodicity of lipid bilayer stacking. The aggregates were prepared from bath sonicated DPPC liposomes (at 50 °C) at DPPC:DNA=4:1 mol/base, 10 mM Ca^{2+}, and incubated 4 months at 4 °C. As these authors report, aggregates prepared at other

compositions and calcium concentrations did not appear to have this higher level of DNA organization, even after long period of time after preparation.

Conclusions

The aggregates due to DNA interaction with saturated zwittterionic diacylphosphatidylcholines (DPPC and DMPC) in presence of 1 - 50 mM divalent cations (Ca^{2+} or Mg^{2+}) show a structural diversity. In the gel state, we identified organization into: a condensed lamellar phase L_β^c; the coexistence of L_β^c phase with the phase of pure phospholipids (L_β^c and L_{PC}); the coexistence of L^x phase (unknown organization of DNA strands) with L_{PC} phase; or one lamellar phase L^x. The aggregates do not exhibit rippled P_β phase, typical for bilayers of saturated phosphatidylcholines. In the liquid-crystalline state we observed: the coexistence of the L^x and L_{PC} phase; condensed lamellar phase L_α^c; or L^x phase. The increase of temperature induces the decrease in the intensity and the increase in the width of the DNA peak, so L_α^c gradually converts into L^x phase. Our analysis of experimental data indicates that the temperature induced in-plane fluctuations of the DNA strands are responsible for the disorder in the DNA lattice. The aggregates showed different levels of temperature stability. After application of heating-cooling cycles, the aggregates converted into energetically more favorable structures: a condensed lamellar phase L^c (or L^x) was preserved or we observed lateral segregation of the DNA strands and metal cations (L^x phase) in coexistence with L_{PC} phase of pure phospholipids.

The DNA - cationic liposomes aggregates achieved maximal stability at the isoelectric point when the total lipid charge exactly balances the total DNA charge (May and Ben Shaul, 2004). One or two long hydrophobic alkyl chains of cationic amphiphile provide a high value of its partition coefficient, and so the amount of cationic amphiphile in bulk water is nearly zero. Applying simple mathematics one can determine the isoelectric point of DNA-cationic amphiphiles aggregate. To determine the isoelectric point of the aggregates DNA+neutral phospholipid+divalent metal cations is not a trivial task. Because of the high mobility of the metal cations, DNA binding to the lipid bilayers is a complicated balance of metal cations bridging and charge screening. In our previous experiments (Uhríková et al., 2005a), we detected the excess of lipid and positive charge in the sample at DOPC:DNA≥6:1 mol/base in 20 mM $CaCl_2$. McManus at al. (2003a) have found a compact arrangement of the DPPC:DNA=8:1 mol/base aggregate at 5 mM Ca^{2+}. These authors supposed stoichiometry ~1

Ca^{2+} cation for every two DPPC molecules. This suggests that calcium bridges two neighboring lipid molecules through their phosphate groups, thereby neutralizing the negative charge on each lipid. Gromelski and Brezesinski (2004) reported the stoichiometry of 2 molecules of dimirystoylphosphatidylethanolamine (DMPE) connected with 1 DNA phosphate in DMPE monolayers charged by magnesium ions. Kharakoz et al. (1999) found the stoichiometry of binding about 4.5-5 lipid molecules per nucleotide in DNA-DPPC aggregates prepared in 22 mM $CaCl_2$ in a temperature - scanning ultrasonic study, what corresponds to our previous findings (Uhríková et al., 2005a). The high diversity of stoichiometric data calls for futher investigation into structures of DNA+neutral phospholipid bilayer+divalent metal cations aggregates.

Our experiments uncovered higher heterogeneity in the structural organization of aggregates DNA + neutral phospholipid bilayer + divalent metal cations in comparison with the one observed in the aggregates DNA + cationic amphiphiles. The question is, if a high mobility of metal cations is responsible for this heterogeneity, or if it follows from a wide range of molar ratios of all constituents used in the experiments because of lack of information concerning the composition of the aggregates at isoelectric point.

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Tab. 1 The structural parameters of PC+DNA+metal cation^{2+} aggregates.

PC	PC:DNA (mol:base)	Cation^{2+}	c (mM)	t (^{o}C)	d (nm)	d_{DNA} (nm)	d_{PC} (nm)
DPPC	1:0	-	-	20	-	-	6.33±0.01
DPPC	1:0	-	-	50	-	-	6.59±0.01
DPPC	3:1	Ca	20	20	8.02±0.01	5.11±0.03	-
DPPC	3:1	Ca	20	50	7.55±0.01	5.02±0.11	-
DPPC*	3:1	Ca	20	20	7.96±0.01	5.10±0.04	-
DPPC	3:1	Ca	1	20	8.04±0.01	-	6.49±0.01
DPPC	3:1	Ca	1	50	7.77±0.01	-	6.84±0.01
DPPC	3:1	Ca	2	20	8.07±0.02	5.18±0.07	6.54±0.01
DPPC	3:1	Ca	2	37	8.06±0.03	5.23±0.07	-
DPPC	3:1	Ca	2	50	7.65±0.02	-	-
DPPC	3:1	Ca	50	20	8.22±0.04	6.08±0.02	-
DPPC	3:1	Ca	50	50	7.63±0.01	-	-
DPPC*	3:1	Ca	50	20	8.10±0.02	5.53±0.15	-
DPPC**	1:1	Ca	20	20	7.94±0.03	-	-
DMPC	2:1	Mg	50	20	7.98±0.02	4.64±0.03	-
DMPC	2:1	Mg	50	50	7.33±0.01	4.29±0.06	-
DMPC	2:1	Mg	50	20	7.44±0.01	-	5.76±0.01

*after heating-cooling cycle
**extruded unilamellar liposomes were used in preparation of the aggregate

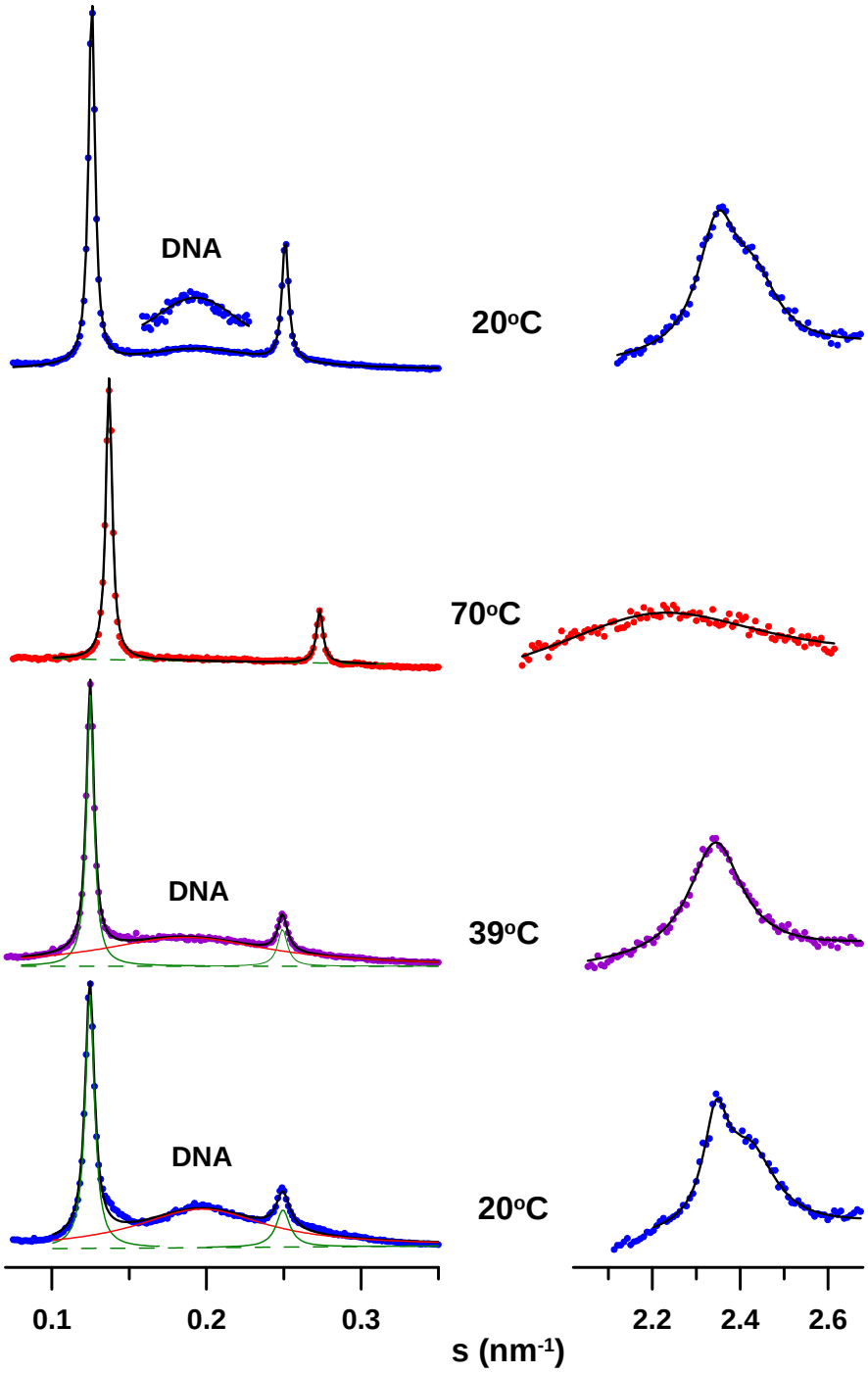

Fig. 1 SAX and WAX diffractograms of DPPC:DNA=3:1 mol/base aggregate at 20 mM Ca^{2+}. The upper diffractogram at 20 °C was measured after heating scan.

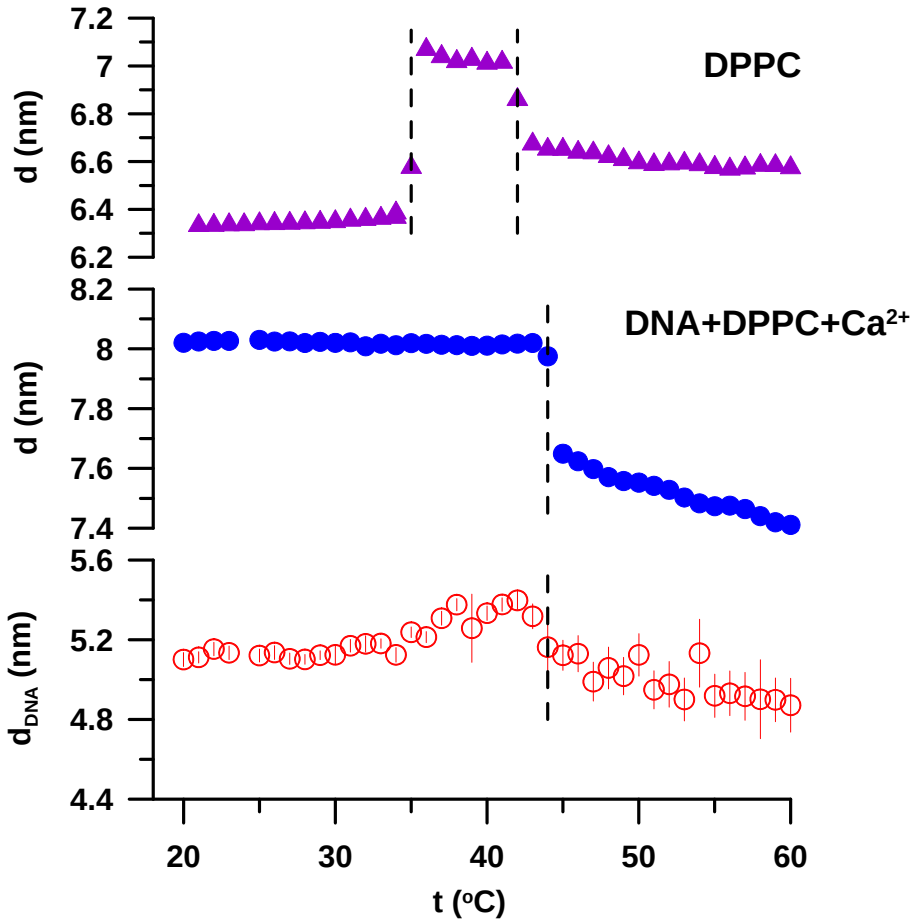

Fig. 2 Temperature dependences of the repeat distances (d) of fully hydrated DPPC bilayers and the lipid bilayer stacking of the DPPC:DNA=3:1 mol/base aggregate at 20 mM Ca^{2+}; d_{DNA} is the DNA-DNA interhelical distance.

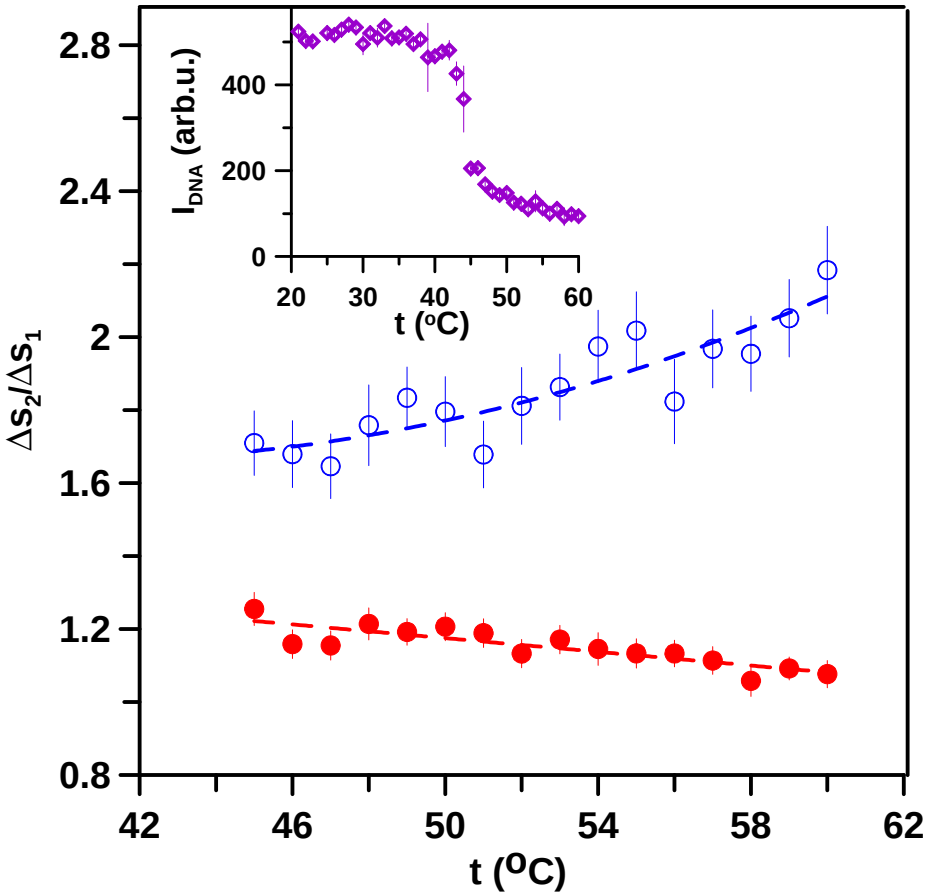

Fig. 3 Temperature dependence of the ratio of second and first order lamellar Bragg diffraction peak widths $\Delta s_2/\Delta s_1$ of fully hydrated DPPC bilayers (empty symbols) and DPPC+DNA+Ca^{2+} aggregate (full symbols); inset: temperature dependence of the intensity of DNA peak.

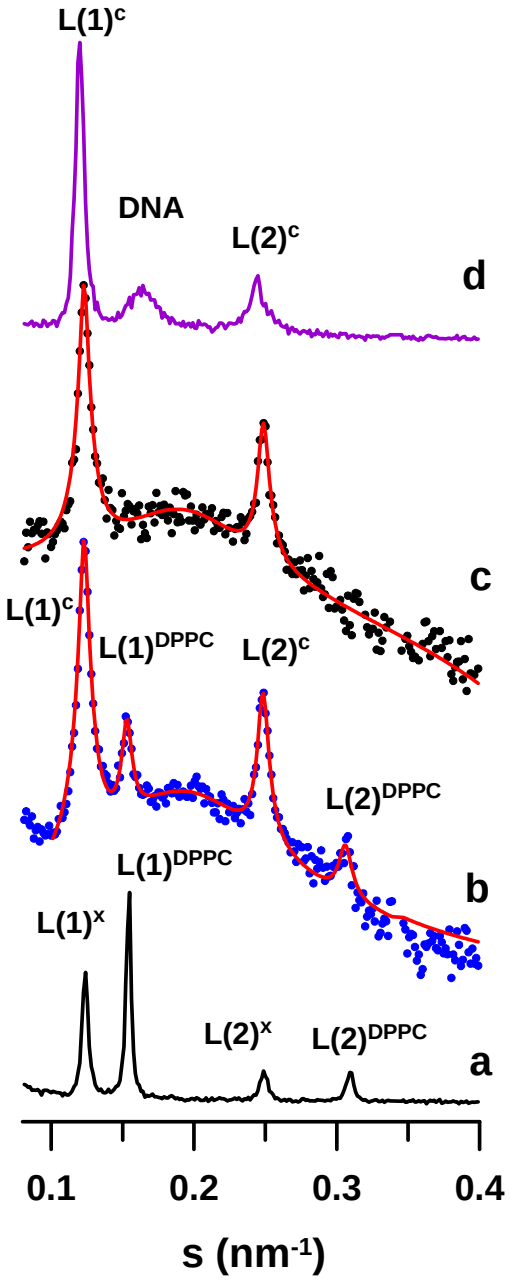

Fig. 4 SAX diffractograms of DPPC:DNA=3:1 mol/base aggregates prepared at a) 1 mM Ca^{2+}, 20 ^{o}C; b) 2 mM Ca^{2+}, 20 ^{o}C; c) 2 mM Ca^{2+}, 37 ^{o}C; d) 50 mM Ca^{2+}, 20 ^{o}C. The intensity of b) and c) is plotted in the logarithmic scale.

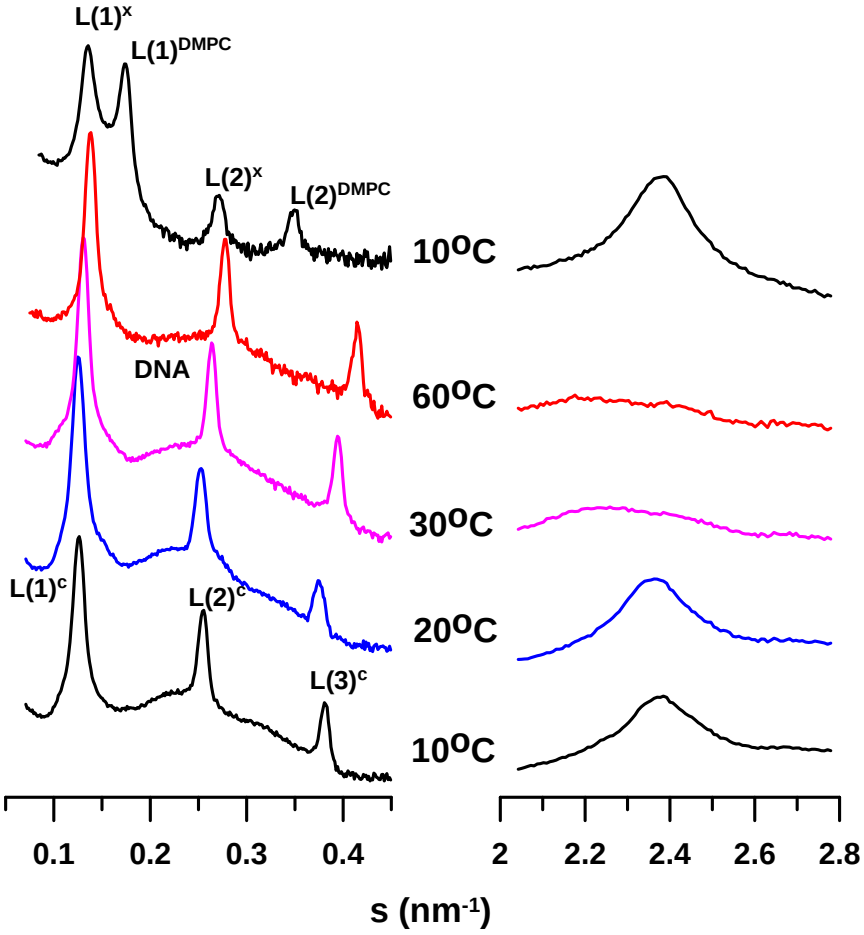

Fig. 5 SAX and WAX diffractograms of DMPC:DNA=2:1 mol/base at 50 mM Mg^{2+}. The upper diffractogram at 10 ^{o}C was measured after repeated heating-cooling cycles. The intensities of SAX diffractograms are plotted in the logarithmic scale.