Lipid bilayer – DNA interaction mediated by divalent metal cations: SANS and SAXD study

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Abstract. The structure of aggregates formed due to DNA interaction with dipalmitoylphosphatidylcholine (DPPC) in presence of Ca^{2+} and Zn^{2+} is examined using small-angle synchrotron X-ray diffraction (SAXD) and small-angle neutron scattering (SANS). SAXD detected two structures: L^C - condensed lamellar phase and L^X - lamellar phase with DNA strands intercalated between the adjacent lipid bilayers, without regular packing at low cation concentration (~1 mM). The high concentration of Zn^{2+} induces a macroscopic phase separation in mixtures. The SANS curves of DPPC+ions^{2+} vesicles evaluated using the strip function model have shown different modes of cations binding to the DPPC bilayers.

1. Introduction

More than three decades ago Budker at al. [1] reported the ability of divalent metal cations to mediate the interaction between DNA and phosphatidylcholine vesicles. Electron freeze fracture micrographs of DNA – phosphatidylcholine - Ca^{2+} mixtures suggested structures with long-range organization [2,3]. The small angle X-ray diffraction confirmed the presence of condensed lamellar phase (L^C) with DNA strands regularly packed between phospholipid bilayers in DNA – dipalmitoylphosphatidylcholine (DPPC) - Mg^{2+} and Ca^{2+} system [4,5]. In addition to biologically relevant divalent cations, calcium and magnesium, also Fe^{2+}, Co^{2+} and Mn^{2+} have shown the ability to mediate DNA – neutral phospholipid binding [6]. The DNA interaction with neutral phospholipid bilayer in the presence of divalent metal cations (Ca^{2+}, Mg^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}) was thoroughly studied in our laboratory [7,8]. All studied cations facilitate regular packing of DNA strands between DPPC bilayers forming the condensed lamellar phase (L_β^C) in the temperature range corresponding to the gel state of DPPC. However, the DNA regular packing is disrupted when the lipid goes into a liquid-

crystalline state, as shown in [9]. Aggregates phospholipid – DNA – cations have shown high structural heterogeneity as a function of temperature and cations concentration.

In this contribution we try to shed more light onto observed structural variety in DNA+phospholipid+ion^{2+} system, particularly in aggregates with zinc. Zinc plays a fundamental role in several critical cellular functions such as protein metabolism, gene expression, structural and functional integrity of biomembranes, and in metabolic processes [10]. Compared with other micronutrients, zinc is present in biological systems in high concentrations, particularly in biomembranes. According to Williams [11], the concentration of zinc in animal systems range from $<10^{-9}$ M in cytoplasm to $>10^{-3}$ M in some membrane vesicles.

Generally, in the system consisiting of DNA, neutral phospholipid and divalent cations, in addition to a formation of triple complex (DNA + phospholipid + ion^{2+}), one must consider also the interaction DNA + ion^{2+} and phospholipid + ion^{2+}. Alkaline earth metal cations (Ca^{2+}, Mg^{2+}) preferentially interact with the phosphate groups of DNA, thereby reducing the charge repulsion between the opposing strands of double-helix [12] and stabilizing the polynucleotide molecule. Transition metal cations interact more extensively with DNA bases causing disruption of base pairing and destabilization of DNA molecule [12,13]. According to Duguid et al. [14], the affinity of divalent cations to DNA bases decreases in the order Hg^{2+}>Cu^{2+}>Pb^{2+}>Cd^{2+}>Zn^{2+}>Mn^{2+}>Ni^{2+}, Co^{2+}>Fe^{2+}> Ca^{2+}>Mg^{2+}, Ba^{2+}. Our fluorescence and UV-VIS experiments have confirmed the ability of zinc to condense DNA in presence of lipid bilayer, and to protect it against thermal denaturation up to the level comparable with calcium [8].

In this contribution we demonstrate the structural differences in aggregates prepared due to DNA interaction with DPPC bilayer in the presence of calcium and zinc observed in our SAXD experiments. We have studied recently polymorphic behaviour of the DNA-DPPC in a solution of divalent transition metals and Zn^{2+} in large concentration range [7,15]. In aggregates with phosphatidylcholines (DPPC, DMPC, DOPC), we have identified structures: L^C - condensed lamellar phase; L^X - lamellar phase with DNA strands intercalated between the adjacent lipid bilayers, without regular packing; coexistence of a lamellar phase L^{PC} of pure lipid with L^X or L^C phase. High concentrations of transition metal ions (Co^{2+}, Ni^{2+}) and Zn^{2+} induce a macroscopic phase separation. In addition to the condensed lamellar phase a partially ordered lamellar phase L^V was identified, showing the periodicity ~ 20 - 8 nm, formed due to screening of the phospholipid positive surface charge at high concentration of ions in the solution.

With the aim to understand the observed polymorphic behaviour we have studied the effect of two cations, calcium and zinc, on the DPPC bilayer itself employing small-angle neutron scattering (SANS). The study of DPPC+Ca^{2+} system has revealed structural changes in DPPC bilayer due to calcium binding [16]. The analysis of SANS curves of DPPC+Zn^{2+} system in the concentration range 1 – 60 mM of ZnCl$_2$ indicates different mode of ions binding to DPPC bilayer that offers an explanation for the macroscopic phase separation observed in DNA+DPPC+Zn^{2+} mixtures.

2. Material and Methods

2.1. Sample preparation

SAXD experiment: 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. Dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids, USA. Solutions of CaCl$_2$ and ZnCl$_2$ (Merck, Germany) were prepared in 0.5 mM NaCl, pH~7. Redistilled water was used for the preparation of all solutions. Samples were prepared by the 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 (3:1) 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 DPPC were used as a reference sample.

SANS experiment: Heavy water of isotopic purity 99.9% D_2O was purchased from Merck (Germany). Solutions of $CaCl_2$ and $ZnCl_2$ were prepared in D_2O (5 mM NaCl, pH~7). Lipid was dissolved in methanol and portioned (10 mg per sample) into plastic tubes. The solvent was gently evaporated under a stream of gaseous nitrogen to create a thin lipid film. Traces of solvent were removed by an oil vacuum pump. The dry lipid was hydrated by adding 1 ml of the XCl_2 solution (X = Ca^{2+}, Zn^{2+}) at wished concentration. The dispersion was vortexed and homogenized in an ultrasound bath (at 60 ^{o}C) and by at least tenfold freezing-thawing process to obtain a homogeneous distribution of calcium ions between lipid multilayers. After this procedure, the samples showed a slight opalescence, typical for dispersions of lipid vesicles. The reference sample of unilamellar vesicles from DPPC was prepared by extrusion of DPPC multilamellar dispersion through a polycarbonate filter (Nuclepore, Plesanton, USA) with pores of 50 nm diameter, using the LiposoFast Basic extruder (Avestin, Ottawa, Canada) fitted with two gas-tight Hamilton syringes (Hamilton, Reno, USA) as described by MacDonald et al. [17]. The samples were subjected to 51 passes through the filter at a temperature above the main phase transition temperature of pure phospholipid. The prepared samples were placed in 2-mm quartz cells (Hellma, Müllheim, Germany), closed and kept at room temperature before measurement.

2.2. Experimental methods

Small-angle X-ray diffraction: SAXD 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 selected temperature for 5 min before exposure to radiation. 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 [18]. Each diffraction peak of SAXD region was fitted with a Lorentzian above a linear background.

Small-angle neutron scattering: SANS experiments were performed on the *PAXE* spectrometer located at the G5 cold neutron guide of the Orphée reactor (Laboratoire Léon Brillouin, Saclay, France). The sample to detector distance was 2.75 m and the neutron wavelength was λ=6 Å ($\Delta\lambda/\lambda$=10%) covering the scattering vector range 0.016-0.224 Å^{-1}. The temperature of the sample was set to 20 ^{o}C, controlled electronically within an accuracy of ±0.1°C. The acquisition time for one sample was 30 minutes. The normalized SANS intensity *I(q)* as a function of the scattering vector modulus *q* was obtained as described previously [19].

3. Results and Discussion

3.1. SAXD of DNA – DPPC – ion^{2+}

Fully hydrated DPPC at 20 ^{o}C forms a lamellar phase (Figure 1a), we determined the repeat distance *d* = $2\pi/(q_2-q_1)$ = 6.36 ±0.01 nm, where q_i is the position of *i*-th peak's maximum in reciprocal space units. Typical diffractograms observed in the DNA-DPPC-ion^{2+} (Ca^{2+}, Zn^{2+}) mixture at 20 ^{o}C are shown in Figure 1b-h. The diffractogram of the aggregate DPPC:DNA=3:1 mol/base prepared at 1 mM Ca^{2+} (Figure 1b) 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). We determined its repeat distance $d_{Lx} = 8.04\pm0.01$ nm at 20 °C. Supposing the DPPC bilayer thickness $d_L \sim 5.3$ nm [16], the water layer thickness $d_W = d - d_L$ provides enough space to accommodate DNA strands with diameter ~2.5 nm [20]. Because the packing of DNA strands in this phase is not obvious, we denote this phase as L^x. The reflections $L(1)^{DPPC}$ and $L(2)^{DPPC}$ were identified as reflections due to the DPPC bilayer stacking (lamellar phase L^{DPPC}). Such coexistence of two phases in one aggregate was observed and discussed earlier in [5,6,21]. An increase in Ca^{2+} concentration improved the organization of DNA strands inside of L^x phase, although the DNA peak is broad, indicating a poor organization of the DNA lattice. Diffractograms in Figure 1c, d, and f are typical diffractograms of a condensed lamellar phase with regularly packed DNA strands. Structural parameters are given in Table 1. Aggregates prepared at low concentration of zinc ($c_{ZnCl2} \leq 20$ mM) show similar microstructure (Figure 1e and f). The diffraction pattern of DNA+DPPC in 40 mM of $ZnCl_2$ (Figure 1g) is different in comparison to the previous one. A deconvolution of peaks revealed the superposition of two phases: the condensed lamellar phase with repeat distances $d=8.54$ nm and $d_{DNA}=6.14$ nm, and a lamellar phase L^V with periodicity $d=13.5$ nm. The periodicity $\sim$ 13 nm (with the water gap d_W ~8 nm) is too big in comparison to structural parameters of L^C phase, also to accommodate DNA strands or to be formed from neutral lipid itself. This indicates a destruction of the lamellar structure yielding its swelling into excess of water. The periodicity of the L^V phase is modulated with increasing concentration of zinc

Figure 1.
SAXD diffractograms (at 20 °C) of:
a) DPPC
DNA+DPPC in:
b) 1 mM $CaCl_2$
c) 20 mM $CaCl_2$
d) 50 mM $CaCl_2$
e) 1 mM $ZnCl_2$
f) 20 mM $ZnCl_2$ (*Is*=65 mM)
g) 40 mM $ZnCl_2$
h) 20 mM $ZnCl_2$ (*Is*=122 mM)*

The peak related to the DNA-DNA packing is marked by arrow.
Is is ionic strength of solution
* adapted from [15].

(Table 1) that was studied in [15]. We believe that only a fraction of lipid is bound by DNA and Zn^{2+} forming L^C phase, the rest of DPPC forms L^V phase, which shows the periodicity decreasing with increasing concentration of ions in solution, and which is macroscopically separated from L^C.

The driving force for mutual condensation of DNA by cationic vesicles to form an ordered, composite phase is the gain in electrostatic free energy. The electrostatic free energy depends on the surface charge densities of the separated macro-ions, the structure and composition of the condensed phases, and the salt concentration in solution [22]. A detailed analysis of the phase behaviour of DNA, cationic amphiphiles and neutral phospholipids mixtures can be found in [22-24]. If salt is added to the system, the mobile salt ions screen electrostatic interactions between fixed charges along DNA and the $P^- - N^+$ dipole of phospholipid headgroups. High ionic strength can screen the DNA – lipid interaction up to level the aggregate dissolution [4]. Calcium has shown an ability to destroy and to rebuild lamellar organization of DPPC bilayers as a function of concentration [25-27]. Yamada et al. [26] detected so-called "unbound state" of DPPC bilayers (e.g. unilamellar vesicles) up to ~ 50 mM of $CaCl_2$ at 20 wt% of DPPC in the liquid-crystalline phase, and this phenomenon was dependent on the DPPC concentration. In the gel phase, the DPPC + Ca^{2+} mixture forms a lamellar phase with decreasing periodicity ~ 33 – 6.5 nm in the concentration range 4 – 400 mM of $CaCl_2$. The phenomenon was discussed through electrostatic cation – dipole interaction [28]. Figure 1h displays the diffractogram of DNA+DPPC in 20 mM of $ZnCl_2$, where the ionic strength of solution was modulated by NaCl (total ionic strength Is=122 mM). The structural parameters (Table 1) are close to the system DNA+DPPC in 40 mM of $ZnCl_2$. Neither the repeat distance nor the lipid bilayer thickness of neutral phospholipid changed in 1–500 mM NaCl [29]. Evidently, the electrostatic screening of Zn^{2+} charge due to ions accumulation and formation of a diffuse double layer are responsible for the observed structural changes. It should be stressed that 0. 144 M of NaCl was found to destroy the L^C phase in DNA+DPPC+Mg^{2+} mixture [4].

Table 1. Structural parameters of DNA+DPPC+ion^{2+} aggregates

Cation	Concentration (mM)	d (nm)	d_{DNA} (nm)	d_{DPPC} (nm)	d^V (nm)
-	0	6.36±0.01	-	-	-
Ca^{2+}	1	8.04±0.01	-	6.49±0.01	-
Ca^{2+}	20	8.19±0.01	4.87±0.05	-	-
Ca^{2+}	50	8.33±0.01	6.08±0.01	-	-
Zn^{2+}	1	8.27±0.01	-	6.53±0.01	-
Zn^{2+}	20	8.48±0.01	5.25±0.01	-	-
Zn^{2+}	40	8.54±0.01	6.14±0.02	-	13.47±0.03
Zn^{2+}	50	8.54±0.01	6.38±0.01	-	12.19±0.01
Zn^{2+}	20*	8.18±0.01	5.70±0.02	-	13.12±0.03

* ionic strength Is=122 mM, data from [15]

3.2. SANS on DPPC – ion^{2+}

We employed SANS to examine the effect of cations (Ca^{2+} and Zn^{2+}) on DPPC bilayer. Figure 2 shows typical scattering curves. For dispersions of monodisperse centrosymmetric particles, the SANS intensity is given by

$$I(q) = N_P \cdot \left| F(q) \right|^2 \cdot S(q) \tag{1}$$

where q is the scattering vector q=$4\pi\sin\theta / \lambda$, 2θ the scattering angle, N_P the number density of particles, $F(q)$ their form factor and $S(q)$ the interparticle structure factor. The interparticle structure

factor $S(q)$ is approximately equal to 1 for dilute and weakly interacting particles, what is a good approximation for unilamellar vesicles at PC concentrations <2 wt. % [30,31]. The model of weakly interacting particles describes well unilamellar vesicles prepared by extrusion [32]. A typical SANS curve of unilamellar vesicles prepared by extrusion is shown in Figure 2a. Unilamellar vesicles are also formed spontaneously when the surface charge density is higher than 1-2 μC/cm^2 [33], e.g. multilamellar DPPC dispersion in 1 mM CaCl$_2$ completely converts into unilamellar vesicles [25, 34]. Figure 2b-e shows SANS curves of unilamellar DPPC vesicles formed spontaneously in solutions of studied ions.

The SANS curves were analyzed using a strip-function model of the phospholipid bilayer [35]. In this model, the bilayer is divided into three strips corresponding to two polar head group regions, each of thickness d_H, (one on each side of the bilayer) and the bilayer centre spanning the hydrocarbon region (with thickness d_C). The form factor $F(q)$ is the Fourier transform of the contrast $\Delta\rho(r)$ between the coherent neutron-scattering length density (SLD) of the bilayer and the solvent. For unilamellar vesicles bilayer, the strip model gives:

$$F(q) = 4\pi \sum_{i=1}^{3} \int_{R_{i-1}}^{R_i} \Delta\rho_i \frac{\sin(qr)}{qr} r^2 dr \qquad (2)$$

where $\Delta\rho_i(r)$ is the SLD contrast and $\Delta d_i = R_i - R_{i-1}$ is the thickness of the i-th strip. The total lipid bilayer thickness d_L is then given by $d_L = 2d_H + d_C$. In addition to d_L, the model includes the number of water molecules n_W per lipid located in the head group region of lipid, and the surface area per 1 lipid molecule on water lipid interface A_L. The obtained structural parameters of the DPPC bilayer and effect of both cations as a function of their concentration (at 20 ^{o}C) is summarized in Figure 2. With increasing concentration of zinc (Figure 2, empty symbols) we observe the increase in the DPPC bilayer thickness d_L, accompanied by the decrease in A_L and in the hydration of the polar head group region. For comparison, full symbols in Figure 2 represent data with calcium thoroughly discussed in our previous study [16]. Evidently, the effect of both ions on the DPPC bilayer is different. The observed changes follow from the nature of the ion itself. The electronic structure of zinc is different
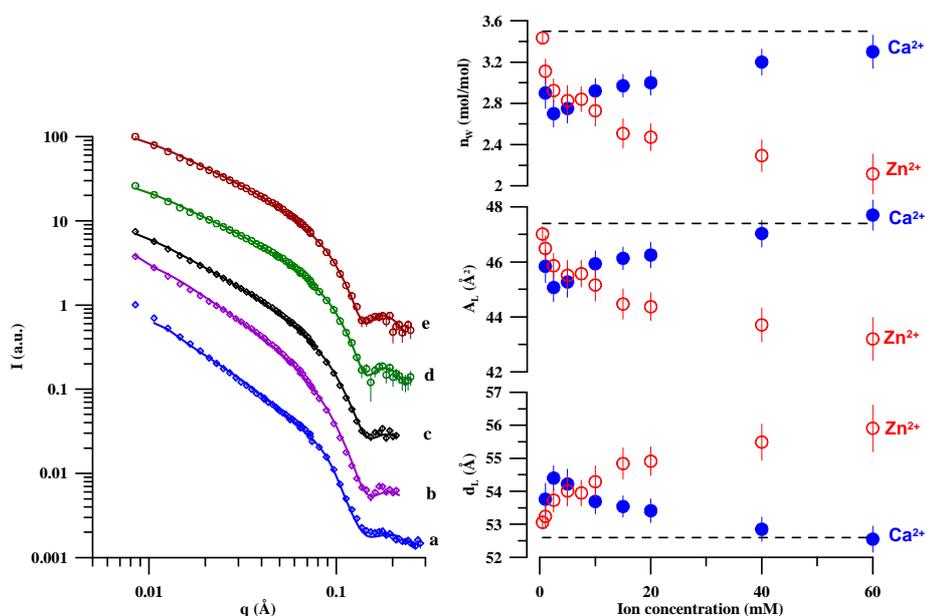
Figure 2. SANS curves of DPPC unilamellar vesicles (a) and DPPC vesicles prepared in 20 mM of CaCl$_2$ (b); and in ZnCl$_2$ at 0.5 mM (c), 5 mM (d) and 20 mM (e). Structural parameters of DPPC bilayer as a function of ion concentration: ZnCl$_2$ (empty symbols) and CaCl$_2$ [16] (full symbols).

from that of divalent alkali earth metal ions. Zinc cation possesses a higher affinity to electronegative groups [36], and therefore also other electronegative moieties such as ester oxygens and/or carbonyl groups of the lipid headgroup can be directly involved in complex formation. According to [37], Zn^{2+} bridges neighboring zwitterionic lipid molecules, forming a complex lipid : $Zn^{2+} = 2 : 1$, reaching the ratio $1 : 1$ at saturation. The interaction of phospholipid headgroups with Zn^{2+} is conveniently described by a hydrated zinc-phosphate complex, which key energy contribution has a more covalent than electrostatic nature. Our analysis has shown the decrease in both, the area per lipid A_L and hydration of polar headgroup (n_W) of DPPC what supports the finding mentioned above. The dependence of d_L *vs. ion concentration* shows a course of "a classic adsorption", the most probably indicating a formation of a diffuse layer of the zinc and counter-ions at the DPPC surface.

The minima in electrostatic free energy of DNA – cationic liposomes complexes occur when the fixed negative charges on DNA surface are balanced by the same number of positive charges on the bilayer surface, i.e., at the isoelectric point. Because of the high mobility of metal cations, the evaluation of the isoelectric point in our system is not a trivial task. Our experiments do not allow us to determine the composition of the mixture at the isoelectric point. However, a short summarization can provide a picture "how compactly are the aggregates packed". We found the repeat distance of L_β^C phase $d = 8.51 \pm 0.03$ nm in the concentration range $20 - 50$ mM of $ZnCl_2$. The steric lipid bilayer thickness of DPPC in $20 - 60$ mM of $ZnCl_2$ at 20^oC is $d_L = 5.54 \pm 0.05$ nm. Thus, for the interbilayer distance with the DNA accommodated, we get $d_W = d - d_L = 2.97 \pm 0.08$ nm. The diameter of DNA strands is 2 nm. The minimal interbilayer distance 2.6 nm is usually treated in theoretical works [22,24], representing the short-range repulsive forces arising from hydration, protrusion, and other excluded volume interactions [38]. The packing of DNA in aggregates is thus rather "soft". The obtained d_W offers enough room for a layer of ions between DNA and the DPPC bilayer surfaces. It has been proved that millimolar concentrations of zinc cause the sedimentation of DNA [39]. Thus at higher zinc concentrations, binding sites of both, DNA and DPPC are saturated, and zinc does not mediate the binding. This saturation is responsible for the macroscopic phase separation observed in the studied mixtures, and the temperature instability of DNA + DPPC + Zn^{2+} phase particularly in aggregates with short fragmented DNA as shown in [15,40].

4. Conclusion

As follows from our experiments, both ions mediate DNA binding to DPPC bilayer forming the condensed lamellar phase. With alkaline earth metals, in a large concentration range of ions, either the condensed lamellar phase with regularly packed DNA strands or coexistence of two phases have been detected at $20\ ^oC$ [5,6,9,21]. In aggregates with zinc, at low concentration (≤ 20 mM $ZnCl_2$) we detected the condensed lamellar phase. High concentrations of zinc induce a macroscopic phase separation in the mixture, and in the addition to L_β^C phase, some volume fraction of lipid forms a partially ordered lamellar phase with periodicities significantly dependent on the ionic strength of solution. The observed structural polymorphism results from the nature of cations themselves, their affinity and way of binding to electronegative groups. Our experiments revealed that zinc at very low concentrations (of the order of millimoles) mediates the DNA-DPPC binding. These concentrations can be physiologically relevant, meaning that our experimental results could contribute to understanding of the role of zinc in biological processes.

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