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Formation of unilamellar dipalmitoylphosphatidylcholine vesicles promoted by Ca^{2+} ions: A small-angle neutron scattering study

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Abstract. Dipalmitoylphosphatidylcholine (DPPC) was hydrated in 0.2–60 mM solution of $CaCl_2$ in heavy water and thoroughly homogenized by freezing-thawing process. Small-angle neutron scattering (SANS) shows formation of unilamellar vesicles in the range 1–60 mM of $CaCl_2$. From the Kratky–Porod plot $\ln[I(Q)Q^2]$ vs. Q^2 of SANS intensity $I(Q)$ in the range of scattering vectors Q corresponding to the interval $0.001\ \text{Å}^{-2} \leqslant Q^2 \leqslant 0.006\ \text{Å}^{-2}$, the vesicle bilayer radius of gyration R_g and the bilayer thickness parameter d_g were obtained. The structure of the bilayer displays different behavior for the gel phase and the liquid-crystalline phase: In the gel phase (at 20°C), the values of d_g indicate nonlinear changes in the lipid bilayer thickness, with a maximum at ∼5 mM $CaCl_2$. In the liquid-crystalline phase (at 60°C), the parameter of the lipid bilayer thickness $d_g = 43.2 \pm 0.3$ Å is constant within the concentration range $1 \leqslant c_{Ca} \leqslant 40$ mM. Vesicles prepared at 60 mM $CaCl_2$ show within experimental error, the same values of d_g as pure DPPC unilamellar vesicles prepared by extrusion using polycarbonate filter with pores of diameter 500 Å.

Keywords: dipalmitoylphosphatidylcholine, Ca^{2+}, cationic vesicles, small-angle neutron scattering

1. Introduction

Calcium ions have an important role in many cellular processes [1–4]. The ions bind naturally to negatively charged phospholipids [5,6] but rather weakly to zwitterionic lipids as phosphatidylcholine (PC) and phosphatidylethanolamine. The binding mechanism and the effect of Ca^{2+} on PC bilayer has been studied using different physicochemical techniques: diffraction methods [7–12], calorimetry [13,14], NMR [15–18], force measuring method [19], infrared spectroscopy [20], and particle electrophoresis [21,22]. As a result of these studies, it is generally agreed that the preference for Ca^{2+} binding weakens with increasing degree of hydrocarbon chain unsaturation and it is also dependent on the phase

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state of phospholipid (gel $>$ liquid-crystal), with a variety of binding constants $\sim$1–400 M^{-1} depending on the lipid and on the experimental method. In spite of the "weak" affinity of calcium to neutral phospholipid bilayers, there is evidence of a large ability of changes of the structural organization of lipid bilayers due to calcium. A X-ray diffraction study of the effect of calcium on the dipalmitoylphosphatidylcholine (DPPC) – water system [7] revealed four different states depending on $CaCl_2$ concentration, c_{Ca2+}: a) State I, $c_{Ca2+} = 0$–1 mM: the system forms a lamellar phase with the repeat distance $d \sim 64.5$ Å (at 5°C) in excess of water; b) State II, $c_{Ca2+} = 1$–10 mM: destruction of the lamellar structure yielding it swelling into excess of water; c) State III, $c_{Ca2+} = 10$–200 mM: formation a partially disordered lamellar phase with a repeat distance $\sim$150–200 Å; d) State IV, c_{Ca2+} larger than 200 mM: a diffraction pattern quite similar to that of state I is observed. Lis et al. [10] confirmed these experimental results and suggested that the dominant force responsible for the destruction of the organization of DPPC bilayers in the lamellar phase is electrostatic repulsion. Izumitani [23,24] performed a theoretical analysis of the electrostatic cation – dipole interaction of DPPC-Ca^{2+} system observed in experiments of Inoko at al. [7] and Lis et al. [10]. Akashi et al. [25], based on microscopic observations, documented the spontaneous formation of giant unilamellar POPC vesicles with a diameter $\geqslant 25$ μm in the concentration range 1–100 mM of $CaCl_2$. The number of unilamellar liposomes decreases with increasing concentration of $CaCl_2$, from $\sim$1100 vesicles at 1 mM to $\sim$1 vesicle at 100 mM in 50 μl of solution prepared at 0.12 mg lipid/ml. Because unilamellar vesicles do not exhibit long-range order, the experiments of Akashi et al. [25] confirm the absence of any X-ray diffraction peak in State II experiments of Inoko et al. [7]. The study of the effect of calcium on the thickness of the lipid bilayer is not easy because of the swelling of multibilayer organization and absence of diffraction. Hydration experiments of Lis et al. [10] reported no effect of calcium on the DPPC bilayer thickness in a lamellar phase. The lipid was hydrated in 30 mM $CaCl_2$, and multibilayer swelling was limited by hydration in the range $\sim$90–30 wt% of lipid. X-ray data were analysed using Luzzati's approach ([26], or for a review see [27]), supposing that lipid and water form separate layers. Tatulian et al. [11], using the same approach for neutron diffraction data, reported the DPPC bilayer thickness in 100 mM $CaCl_2$: $d_L = 45.6$ and 35.5 Å at 20 and 55°C, respectively. For pure DPPC bilayers they found thicknesses of 43.1 and 33.9 Å at 20 and 55°C, respectively. These experiments were performed also at a limited hydration (^2H_2O:DPPC = 12:1 mol/mol), and the authors concluded that the thickness of the lipid bilayer does not depend on the presence of $CaCl_2$ within an accuracy of ± 2 Å.

In this paper we study the formation of unilamellar vesicles due to binding of Ca^{2+} ions to the DPPC bilayer, and the effect of ions on the lipid bilayer thickness. The samples were prepared in excess of water, in the concentration range 0.2–60 mM of $CaCl_2$. We focused our investigation on the concentration range 0.5–20 mM of $CaCl_2$, with the aim to follow an effect of calcium on the lipid bilayer underneath the concentration at which the formation of an electric double layer screens the effect of the charges. Small-angle neutron scattering (SANS) was used for the study, and scattering curves were analysed applying a treatment from our previous works [28–30].

2. Material and methods

Synthetic 1,2-dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids (Alabaster, USA). D_2O of isotopic purity 99.9% was purchased from Merck (Germany). DPPC 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 $CaCl_2$ solution prepared in D_2O (5 mM NaCl, pH $\sim$ 7). The concentration of $CaCl_2$ was changed in the range 0–60 mM. The dispersion was vortexed and homogenized in an ultrasound bath (at 60°C) and by at least tenfold freezing-thawing process to obtain a homogeneous distribution of positive charge between lipid multilayers. With increasing concentration of calcium, the samples showed a slight opalescence, typical for dispersions of unilamellar vesicles.

Unilamellar vesicles from DPPC (without Ca^{2+}) were prepared by extrusion of DPPC dispersion prepared as above through a polycarbonate filter (Nucleopore, Plesanton, USA) with pores of diameter 500 Å mounted in the LiposoFast Basic extruder (Avestin, Canada) fitted with two gas-tight Hamilton syringes (Hamilton, Reno, USA). The sample was subjected to 51 passes through the filter at about 50°C. An odd number of passes were performed to avoid contamination of the sample by large and oligolamellar vesicles, which might not have passed through the filter. The samples were filled into 1 mm quartz cells (Hellma, Germany), closed and stored at room temperature.

The neutron scattering experiments were performed on the *PAXE* spectrometer located at the G5 cold neutron guide of the Orphée reactor (Laboratoire Léon Brillouin, CEA Saclay, France). The sample to detector distance was 2750 mm and the neutron wavelength was $\lambda = 6$ Å, covering the scattering vector range 0.016–0.224 $Å^{-1}$. The temperature of the samples was set and controlled electronically within an accuracy of ± 0.1°C. The acquisition time for one sample was 40 minutes. The normalized SANS intensity $I(Q)$ as a function of the scattering vector Q was obtained as described previously [31].

Additional neutron scattering experiments (for samples in the Ca^{2+} concentration range 0.2–0.5 mM) were performed on the *Yellow Submarine* spectrometer operating on the cold neutron beam line at the Budapest Research Reactor [32]. The mean neutron wavelength $\lambda = 6$ Å and the sample – detector distance 2650 mm were used, covering the scattering vector range 0.012–0.138 $Å^{-1}$. The samples were measured using the same experimental protocol as above.

3. Results and discussion

Fig. 1a shows the plot of the SANS scattering function, $I(Q)$, obtained with multilamellar DPPC vesicles, as a function of the scattering vector Q, which is defined as

$$Q = 4\pi \sin\theta/\lambda, \tag{1}$$

where 2θ is the scattering angle and λ the wavelength of neutrons. The Bragg peak with a maximum at $Q \sim 0.094$ $Å^{-1}$ results from organization of lipid bilayers in one-dimensional periodic lattice of periodicity $d \sim 67$ Å, what is a typical repeat distance of fully hydrated DPPC multibilayers at $t \sim$ 50–60°C [27,33]. The addition of $CaCl_2$ destroys the periodic organization of bilayers what is evidenced by the reduction of intensity of the Bragg peak (Fig. 1b, c). Earlier experiments [23,34] have shown that the binding site for cations is near the negative phosphate group of the P^-–N^+ dipole of phospholipid headgroup. Neutralizing the negative charge of phosphate group, the lipid bilayer becomes positively charged, and the electrostatic repulsion between lamellae makes it to swell into excess water. The surface charge density higher than 1–2 $\mu C/cm^2$ promotes the formation of unilamellar vesicles [35].

In first approximation, calcium binding can be characterized by Langmuir adsorption isotherm of the form

$$\frac{X_b}{1 - nX_b} = K_{app} c_{\mathrm{Ca2+}}, \tag{2}$$

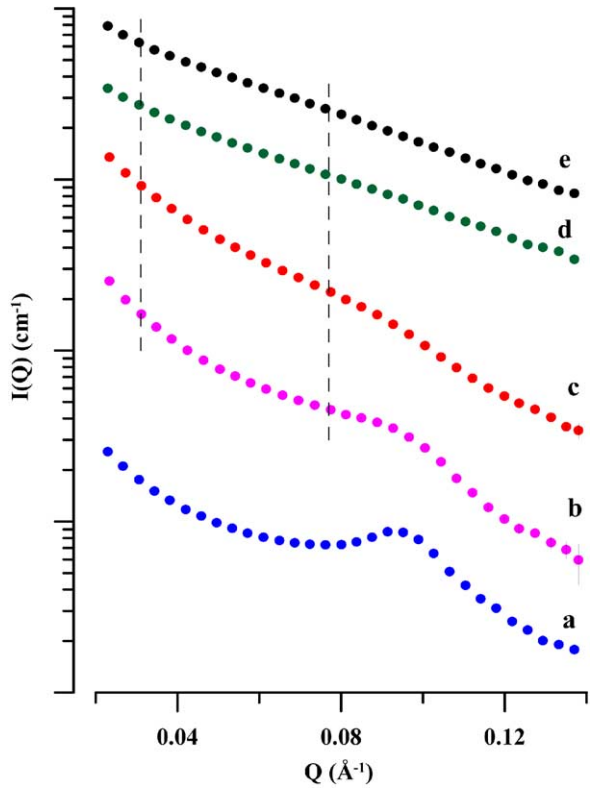

Fig. 1. Dependence of SANS intensity $I(Q)$ on the scattering vector Q for multilamellar DPPC vesicles (a) and DPPC vesicles prepared at 0.2 mM (b); 0.5 mM (c); 1 mM (d); and 60 mM (e) of $CaCl_2$ at 60°C. Dashed lines indicate Q range used for the Kratky–Porod plot.

where X_b is the number of associated calcium ions per lipid (mol/mol), n is the number of lipid molecules bound by one calcium ion [36,37], K_{app} denotes the apparent binding constant and $c_{\mathrm{Ca2+}}$ the total Ca^{2+} concentration. Calcium binds weakly to DPPC bilayers, the reported values of binding constants are scattered in the range 1–100 M^{-1}, and the binding is also sensitive to the phase state of bilayers, decreasing in the order gel state $>$ pretransition state $>$ liquid-crystalline state [16,19]. In the liquid-crystalline phase, the binding constant is in the range 10–20 M^{-1}, with $n = 1$ or 2 [15,16,19,22]. The scattering curve in Fig. 1c corresponds to DPPC vesicles prepared at 0.5 mM $CaCl_2$ ($t = 60$°C). By applying equation (2), one can calculate the binding stoichiometry of 1 Ca^{2+} ion per 100–200 molecules DPPC. Assuming that the area per DPPC molecule $A_L = 64$ $Å^2$ [27], one obtains the surface charge density ~0.25–0.50 $\mu C/cm^2$, what is underneath the surface charge density that would yield unilamellar vesicles. The slight non-linearity observed in Fig. 1c indicates that the sample contains a significant amount of multilamellar vesicles. At higher concentrations of Ca^{2+}, i.e. in the range of 1–60 mM $CaCl_2$, the scattering curves do not indicate any presence of multilamellar vesicles (Fig. 1d, e). Our experimental results agree with previous studies of modulation of intermembrane interaction by divalent cations [7,8,10] and confirm the spontaneous formation of unilamellar vesicles at concentrations of $Ca^{2+} \geqslant 1$ mM reported by [25] from microscopic observations.

The SANS curves of DPPC vesicles prepared at concentrations of $Ca^{2+} \geqslant 0.5$ mM were further analyzed with the aim to investigate the effect of calcium on the lipid bilayer thickness. For dispersions

of monodisperse centrosymmetric particles, the scattered intensity is given by

$$I(Q) = N_P \cdot P(Q) \cdot S(Q), \tag{3}$$

where N_P is the number density of particles, $P(Q)$ is their form factor and $S(Q)$ is the interparticle structure factor. In first approximation, unilamellar vesicles are hollow spheres with the lipid bilayer shell separating the inside and outside aqueous compartments. For such particles, the factor $P(Q)$ can be calculated by the one-dimensional Fourier integral of the coherent neutron scattering length density. The interparticle structure factor $S(Q)$ is approximately equal to 1 for dilute and weakly interacting particles, what is a good approximation in our case because the phospholipid concentration <2 wt.% [38, 39]. According to Guinier approximation, at small Q ($QR_g < 1$) [40,41], and assuming that $S(Q) = 1$, equation (3) can be written

$$I(Q) = I(0) \exp(-Q^2 R_g^2/r) Q^{r-3}, \tag{4}$$

where R_g is the radius of gyration of the particles and $r \approx 1$, 2, and 3 hold for infinite sheet-like object, for rod-like object of infinite length and uniform cross section, and for a globular object, respectively [42,43]; $r \approx 1$ is a good approximation also for polydisperse hollow spheres with radii substantially larger than the constant shell thickness, such as unilamellar vesicles [28]. The approximation (4) is valid for finite size objects when $L^{-1} \leqslant Q \leqslant R_g^{-1}$ where L is the longest size of the object. We have fitted the experimental values of $I(Q)$ in the region of small scattering vectors ($0.02\ \text{Å}^{-1} \leqslant Q \leqslant 0.13\ \text{Å}^{-1}$) by using eq. (4). In the three parameters fit ($I(0)$, R_g and r) we have obtained the value of r in the range $0.991 \leqslant r \leqslant 1.095$ with the maximum standard deviation of 0.063, what corresponds well to the r value characteristic for unilamellar vesicles.

It is well known [40] that the thickness of the two-dimensional planar sheet d_g can be obtained from the radius of gyration R_g as

$$d_g^2 \cong 12 R_g^2. \tag{5}$$

Equation (5) can be also used for the estimation of bilayer thickness in unilamellar vesicles dispersed in heavy water [28,38,44]. The values of R_g can be obtained from the Kratky–Porod plot of experimental data such as presented in Fig. 2, which confirm the validity of eq. (4). Supposing that there is no water penetration inside the polar region of the bilayer and that the vesicles are spherical, the computer simulations of scattering curves have shown that value of d_g obtained in the region of $0.001\ \text{Å}^{-2} \leqslant Q^2 \leqslant 0.006\ \text{Å}^{-2}$ (indicated in Fig. 1 by dashed lines) is approximately equal to the thickness of the lipid bilayer in these vesicles [28,45]. For unilamellar DPPC vesicles prepared by extrusion we have found $d_g = 45.0 \pm 1.1$ Å in the gel phase (at 20°C) and $d_g = 40.3 \pm 1.1$ Å in the liquid-crystalline phase (at 50°C), using the Kratky–Porod plot (Fig. 2, empty circles) and eq. (5). The thickness parameter d_g is a linear function of the transbilayer phosphate-phosphate distance d_{HH} in unilamellar phosphatidylcholine vesicles [30]. Applying correlation between d_g and d_{HH} (see Fig. 3 in [30]) one can obtain $d_{HH} = 42.5 \pm 1.3$ Å at 20°C and $d_{HH} = 36.8 \pm 1.3$ Å at 50°C. Kratky–Porod plots for DPPC vesicles prepared at concentrations $c_{\text{Ca2+}} < 60$ mM have shown excellent linearity as represented by the curves in Fig. 2 (full symbols), with correlation coefficients in the range $0.9998 \geqslant R^2 \geqslant 0.9963$. Plots for DPPC vesicles prepared at $c_{\text{Ca2+}} = 60$ mM have shown higher scatter of experimental points,

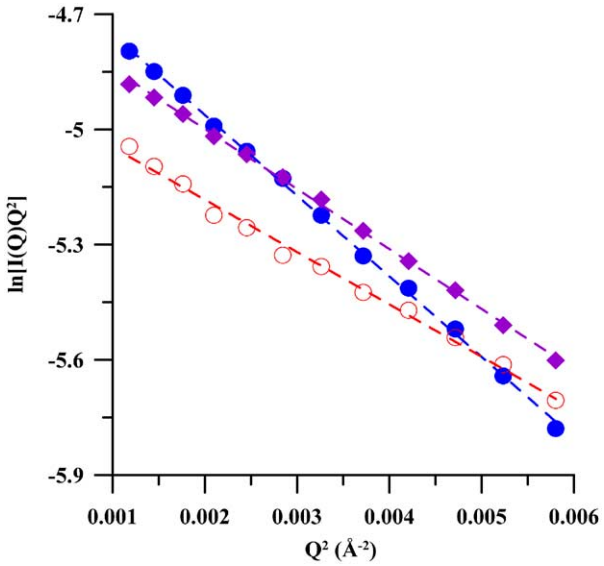

Fig. 2. Kratky–Porod plot of neutron scattering curves of DPPC unilamellar vesicles prepared by extrusion (empty circles, 20°C); DPPC vesicles at 2.5 mM of $CaCl_2$ (full circles, 20°C); at 15 mM $CaCl_2$ (full diamonds, 60°C).

$R^2 \sim 0.990$, what represents ~3% error in R_g. The dependence of d_g values obtained from the Kratky–Porod plots in the indicated Q region on the concentration of calcium c_{Ca2+} is shown in Fig. 3. Our experimental results indicate changes in the thickness of bilayer of DPPC vesicles due to binding Ca^{2+} cations. In the gel phase (at 20°C), the thickness parameter d_g sharply increases, reaching a maximum at ~5 mM Ca^{2+}. At the lowest concentration, $d_g = 47.3 \pm 0.1$ Å for DPPC vesicles prepared at 0.5 mM Ca^{2+}. In the indicated Q region, the plot shows excellent linearity ($R^2 = 0.9998$), in contrast with the situation at 60°C ($R^2 = 0.9880$). This discrepancy can explain higher affinity of Ca^{2+} to the DPPC in the gel state in comparison to liquid-crystalline state. The smaller area A_L per DPPC molecule in the gel state ($A_L = 47.9$ Å^2 [27]) and the higher value of binding constant (37 M^{-1} for DPPC – Ca^{2+} at 25°C [22]) provides sufficient surface charge density for the formation of unilamellar vesicles. The sharp increase of d_g in the concentration range 0.5–5 mM is rather surprising. In gel and also in liquid-crystalline phase, the P^-–N^+ dipole of phospholipid headgroup is nearly parallel to the bilayer surface and rotating freely around an axis perpendicular to the bilayer surface [15,34,46]. Physical arguments using electrostatic double layer theory predict that the binding plane for divalent cations is 3.5 Å away from the edge of hydrocarbon region of phospholipid bilayer [10] what corresponds well to ~4 Å determined by neutron diffraction experiments [12]. The electric field due to bound cations has sufficient magnitude to change the orientation of the P^-–N^+ dipole from the tangential to the normal direction with -$N^+(CH_3)_3$ group outward from the bilayer surface [23,47]. Binding of ions to the lipid vesicles surface thus causes a structural modification of the headgroup dipole. ^{2}H-NMR on deuterated lipids is a sensitive method for detection of changes in lipid chain packing [48]. The chain order parameter can be used to monitor structural changes. Higher order translates in more extended hydrocarbon chains and induces changes in the surface area per molecule [49] as well as in the lipid bilayer thickness. Shibata [18] indicated an increase in the order parameters of the segments both in the polar head group and in the hydrocarbon chain region of DPPC bilayer at $CaCl_2$ concentrations $\geqslant$1 mM. Huster et al. [50] reported an effect of Ca^{2+} on the organization of DMPC hydrocarbon chains at $c_{Ca2+} > 15$ mM (10 mM NaCl, 10 mM Hepes,

pH 7.4, 37°C), they observed a reduction in the surface area per DMPC molecule ($\Delta A_L = 2.7$ Å^2) in the liquid-crystalline phase when Ca^{2+} ions (15 mM, 37°C) mediated the binding of anionic polyelectrolyte (sulfate dextrane) to the DMPC bilayers. Zidovetzki et al. [17] observed measurable changes in the lipid bilayer packing at 25 mM of Ca^{2+}. This short review of published NMR results indicates an ability of calcium to affect the lipid bilayer organization. Our experimental results (Fig. 3) show that the effect of calcium on the lipid bilayer thickness is maximal at ∼5 mM $CaCl_2$. As follows from Fig. 3, the further increase in the Ca^{2+} concentration induces a decrease of the lipid bilayer thickness d_g. Two effects can explain this behaviour: First, an excess of positive charge on the lipid bilayer surface gives rise to electrostatic repulsion between polar headgroups, what induces a lateral expansion of bilayer and, consecutively, a decrease of the lipid bilayer thickness. Second, Ca^{2+} ions adsorbed on the surface and the mobile Ca^{2+} and Cl^- ions in water form an electrical double layer [51], so the effect of positive charge is screened. In fact, we found $d_g = 44.8 \pm 0.7$ Å at 60 mM Ca^{2+}, what corresponds, within the experimental error, to the d_g determined for pure DPPC liposomes prepared by extrusion. In the liquid-crystalline phase (Fig. 3, 60°C), the lipid bilayer thickness d_g does not manifest marked changes; we found the average value $d_g = 43.2 \pm 0.3$ Å within the concentration range $1 \leqslant c_{Ca2+} \leqslant 40$ mM. In the fluid lamellar phase, motions of phospholipid headgroups and conformational disorder of hydrocarbon chains result in the increase of the surface area per lipid and in the decrease of the lipid bilayer thickness as compared to the gel state. The increase of the surface area is related to the decrease of the number density of binding sites for Ca^{2+}, thus of surface charge density. The decrease of Ca^{2+} affinity together with thermal motions of head group and fluctuations of fluid bilayers (for a review see [27]) are most probably responsible for observed changes in d_g. At 60 mM Ca^{2+}, we find $d_g = 39.9 \pm 0.8$ Å, what again, corresponds to the d_g determined for pure DPPC liposomes prepared by extrusion. The electrical double layer formed at the vesicle surface screens the electrostatic repulsion between positively charged vesicles, what allows a mutual approach of bilayers. It is observed a completely rebuilding of multilamellar organization at high concentration of Ca^{2+} ([7], state IV). Akashi et al. [25] observed ∼90% reduction in the number of unilamellar vesicles at concentrations $c_{Ca2+} > 50$ mM, and these authors

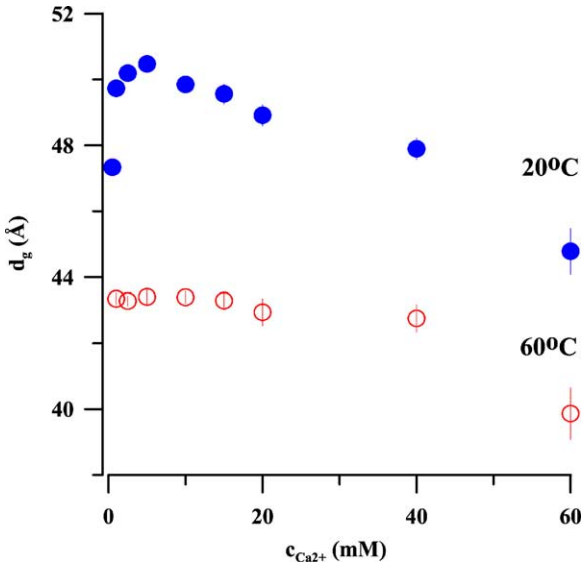

Fig. 3. Dependence of the lipid bilayer thickness parameter d_g on the concentration of $CaCl_2$.

reported the presence of "small particles of lipid". The X-ray diffraction pattern of DPPC at 50 mM Ca^{2+} showed two broad low-angle diffraction peaks corresponding to a periodicity $d = 120$ Å [7]. The decrease of the correlation coefficient $R^2 \sim 0.990$ of Kratky–Porod plots for DPPC vesicles prepared at $c_{Ca2+} = 60$ mM (and an increase of experimental error of d_g) thus probably follows from formation of oligolamellar or multilamellar vesicles due to reduced electrostatic repulsion between bilayer surfaces.

In conclusion, our SANS experiments show that due to binding of calcium to DPPC bilayer, unilamellar vesicles comparable to those prepared by extrusion are formed. The analysis of SANS reveals changes in the lipid bilayer thickness at low concentrations of calcium. The functions of many transmembrane proteins depend on the lipid bilayer physical properties ([4] and references therein, [52]). Calcium ions could affect these functions by affecting the lipid bilayer thickness.

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