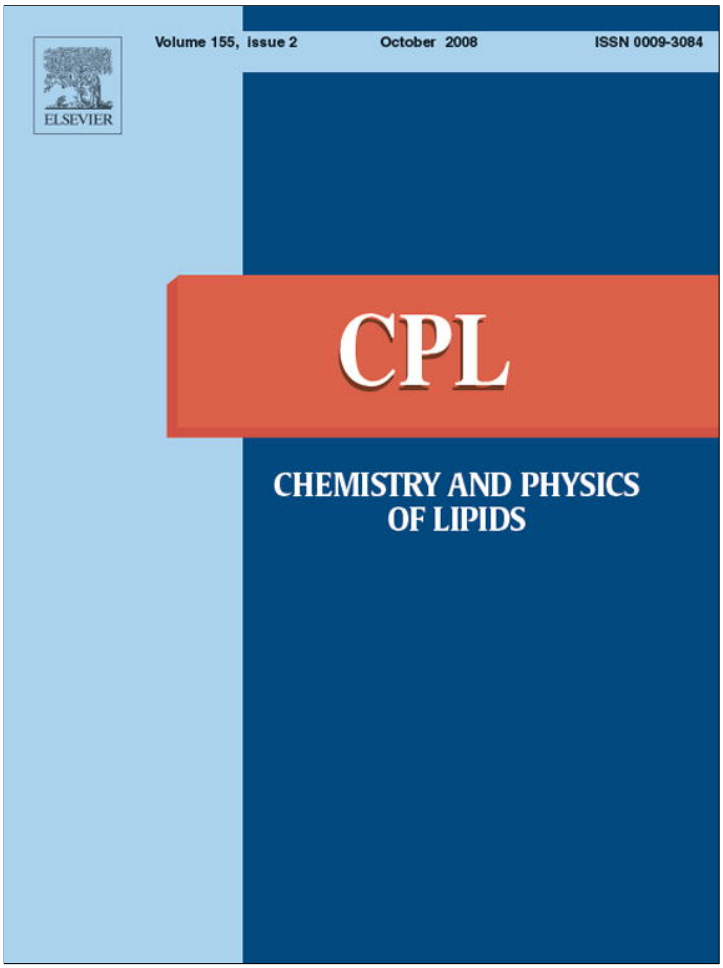

Chemistry and Physics of Lipids 155 (2008) 80–89

Contents lists available at ScienceDirect

Chemistry and Physics of Lipids

journal homepage: www.elsevier.com/locate/chemphyslip

Structural changes in dipalmitoylphosphatidylcholine bilayer promoted by Ca^{2+} ions: a small-angle neutron scattering study

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ARTICLE INFO

Article history:
Received 28 December 2007
Received in revised form 16 July 2008
Accepted 18 July 2008
Available online 31 July 2008

Dedicated to Prof. F. Kopecký on the occasion of his 70th birthday.

Keywords:
Dipalmitoylphosphatidylcholine
Ca^{2+}
DPPC lipid bilayer thickness
Area per DPPC molecule
Small-angle neutron scattering

ABSTRACT

Small-angle neutron scattering (SANS) curves of unilamellar dipalmitoylphosphatidylcholine (DPPC) vesicles in 1–60 mM $CaCl_2$ were analyzed using a strip-function model of the phospholipid bilayer. The fraction of Ca^{2+} ions bound in the DPPC polar head group region was determined using Langmuir adsorption isotherm. In the gel phase, at 20 °C, the lipid bilayer thickness, d_L, goes through a maximum as a function of $CaCl_2$ concentration (d_L = 54.4 Å at ~2.5 mM of $CaCl_2$). Simultaneously, both the area per DPPC molecule A_L, and the number of water molecules n_W located in the polar head group region decrease ($\Delta A_L = A_{L(DPPC)} - A_{L(DPPC+Ca)} = 2.3$ Å^2 and $\Delta n = n_{W(DPPC)} - n_{W(DPPC+Ca)} = 0.8$ mol/mol at ~2.5 mM of $CaCl_2$). In the fluid phase, at 60 °C, the structural parameters d_L, A_L, and n_W show evident changes with increasing Ca^{2+} up to a concentration $c_{Ca^{2+}} \leq 10$ mM. DPPC bilayers affected by the calcium binding are compared to unilamellar vesicles prepared by extrusion. The structural parameters of DPPC vesicles prepared in 60 mM $CaCl_2$ (at 20 and 60 °C) are nearly the same as those for unilamellar vesicles without Ca^{2+}.

1. Introduction

Calcium ions have an important role in many cellular processes (Lee et al., 1993; Petersen et al., 2005; Lee, 2004). Various experimental methods have been applied in the past to shed light on the interaction of cations with biological and model membranes. The cations bind naturally to negatively charged phospholipids (Macdonald and Seelig, 1987; Sinn et al., 2006) but rather weakly to zwitterionic lipids as phosphatidylcholine (PC) and phosphatidylethanolamine (Altenbach and Seelig, 1984; Marra and Israelachvili, 1985; Satoh, 1995). The binding mechanism and effect of Ca^{2+} on PC bilayer has been studied using different physicochemical techniques: diffraction methods (Inoko et al., 1975; Lis et al., 1981a,b; Tatulian et al., 1991; Herbette et al., 1984; Yamada et al., 2005), calorimetry (Ganesan et al., 1982; Lehrmann and Seelig, 1994), NMR (Akutsu and Seelig, 1981; Altenbach and Seelig, 1984; Zidovetzki et al., 1989; Shibata, 1990; Huster et al., 2000), interbilayer force measuring (Marra and Israelachvili, 1985), infrared spectroscopy (Binder and Zschornig, 2002), and particle electrophoresis (Tatulian, 1987; Satoh, 1995). As a result of these studies, it is generally agreed that the preference for Ca^{2+} binding weakens with increasing degree of unsaturation of hydrocarbon chain, and that it depends on the phospholipid phase (gel > fluid), with a variety of binding constants ~1–400 M^{-1} depending on the lipid and on the experimental method. X-ray and neutron scattering experiments (Inoko et al., 1975; Yamada et al., 2005; Uhríková et al., 2007b) as well as microscopic observations (Akashi et al., 1998) document the changes of the structural organization of neutral PC bilayers due to calcium: at concentrations $c_{Ca^{2+}} < 1$ mM the neutral PC + $CaCl_2$ system forms a lamellar phase with the repeat distance $d \sim 65$ Å when dispersed in an excess of water. The destruction of lamellar structure and the spontaneous formation of unilamellar vesicles is observed for $1 \leq c_{Ca^{2+}} <\sim 60$ mM (depending on the temperature and PC concentration). Further increase of $CaCl_2$ concentration induces formation of a partially disordered lamellar phase with the repeat distance ~120–200 Å, and finally, the lamel-

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doi:10.1016/j.chemphyslip.2008.07.010

lar phase with $d \sim 65$ Å is rebuilt at $c_{Ca^{2+}} > 200$ mM (Inoko et al., 1975; Yamada et al., 2005). The dominant force responsible for the destruction of this lamellar phase is the electrostatic repulsion (Lis et al., 1981b). The binding site for cations is close to the negative phosphate group of the P^-–N^+ dipole of phospholipid headgroup (Hauser et al., 1975; Izumitani, 1994, 1996).

In spite of many studies of Ca^{2+}adsorption on PC membranes, the information concerning the influence of cations on lipid bilayer itself is rather scattered. NMR experiments (Akutsu and Seelig, 1981; Altenbach and Seelig, 1984) suggest a conformational change in the polar region of dipalmitoylphosphatidylcholine (DPPC) bilayers. Shibata (1990) indicates an increase in order parameters in the polar head group segments as well as in the hydrocarbon chains in case of DPPC bilayer at $CaCl_2$ concentrations $\geq$1 mM. The chain order parameter can be used to monitor structural changes (Koenig et al., 1997). Huster et al. (1999) reports an effect of Ca^{2+} on the organization of dimyristoylphosphatidylcholine (DMPC) hydrocarbon chains at $c_{Ca^{2+}} > 15$ mM (10 mM NaCl, 10 mM Hepes, pH 7.4, 37 °C). They observe 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) mediate the binding of anionic polyelectrolyte (sulfate dextrane) to the DMPC bilayers. Zidovetzki et al. (1989) observe measurable changes in lipid bilayer packing at 25 mM of Ca^{2+}. On the other hand, hydration experiments of Lis et al. (1981b) report 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 ~90–30 wt.% of lipid. Their X-ray data were analyzed using the Luzzati's approach (Luzzati, 1968 or see Nagle and Tristram-Nagle, 2000 for a review), supposing that lipid and water form separate layers. The study of the calcium effect on the thickness of lipid bilayer in excess water is not easy due to the swelling of multibilayer organization and absence of diffraction. It is worth to mention that the interaction of calcium with PC membranes was already reported to be associated with dehydration of the lipids. Infrared spectroscopy data suggest a deep penetration of divalent ions into the bilayer polar region causing the partial dehydration of head groups (Binder and Zschornig, 2002). The presence of 5–10 mM calcium induces significant changes in compressibility of DPPC monolayers (Banerjee and Bellare, 2001) as well as vesicles (Aruga et al., 1985). The current state of the art leaves a considerable room for the question: "Does calcium affect the lipid bilayer thickness?"

In our previous work (Uhríková et al., 2007b) we have followed the formation of unilamellar DPPC vesicles promoted by $CaCl_2$ (0.2–60 mM) using small-angle neutron scattering (SANS). SANS shows the formation of unilamellar vesicles in the range 1–60 mM of $CaCl_2$. SANS intensity $I(q)$ was analyzed in the range of scattering vector q corresponding to the interval 0.001 Å$^{-2} \leq q^2 \leq 0.006$ Å^{-2} using the Kratky–Porod plot ($\ln[I(q)q^2]$ vs. q^2). The bilayer radius of gyration R_g and the bilayer thickness parameter d_g were obtained. In the gel phase (at 20 °C), the values of d_g indicated 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 $d_g = 43.2$ Å was constant within the concentration range $1 \leq c_{Ca^{2+}} \leq 40$ mM. Vesicles prepared at 60 mM $CaCl_2$ showed, within experimental error, the same values of d_g as DPPC unilamellar vesicles without any calcium, prepared by extrusion.

In this paper, we extend our SANS studies of DPPC vesicles. We analyze the experimental SANS curves of vesicles prepared in 1–60 mM solution of $CaCl_2$ using a strip-function model to obtain the lipid bilayer thickness (d_L), the interfacial area per single DPPC molecule (A_L), and the number of water molecules located in the lipid polar head group (n_W). In this model, the bilayer is divided into three strips corresponding to polar head group regions (one on each side of the bilayer) with the hydrocarbon region spanning the bilayer center. The model includes the volumes of bilayer regions defined above and their coherent scattering length densities, which were taken from the literature. A contrast variation technique (D_2O/H_2O) was employed for DPPC vesicles prepared at 20 mM $CaCl_2$ with the aim to recognize the effect of Ca^{2+} cations on the polar head group region. The values of d_L, A_L and n_W in the gel (at 20 °C) and in the liquid–crystalline phase (at 60 °C) are compared with those obtained for DPPC unilamellar vesicles prepared by extrusion.

2. Materials and methods

2.1. Sample preparation

Synthetic 1,2-DPPC was purchased from Avanti Polar Lipids (Alabaster, USA). Heavy water of isotopic purity 99.9% D_2O 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 ~ 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 calcium ions between lipid multilayers. After this procedure, the samples showed a slight opalescence, typical for dispersions of lipid vesicles.

DPPC vesicles in 20 mM $CaCl_2$ were prepared also at 80%, 60%, and 50% D_2O. Different contrasts were reached by varying the D_2O/H_2O ratio in aqueous phase. Redistilled H_2O was used in experiment. Samples were measured at 20 and 50 °C.

Unilamellar vesicles from DPPC (without Ca^{2+}) were prepared from DPPC dispersion prepared as above and extruded through a polycarbonate filter (Nucleopore, Plesanton, USA) with pores of diameter 500 Å. Filters were mounted in the LiposoFast Basic extruder (Avestin, Canada) and 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. DPPC unilamellar vesicles were prepared immediately before measurement to avoid spontaneous formation of oligolamellar vesicles. DPPC + Ca^{2+} vesicles were prepared ~3 days before measurement and stored at ~5 °C. The samples were filled into 2 mm thick quartz cells (Hellma, Germany).

2.2. SANS experiments

The neutron scattering 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 $\lambda = 6$ Å ($\Delta\lambda/\lambda = 10\%$) covering the scattering vector range 0.016–0.224 Å^{-1}. The temperature of the samples was set to 20 and 60 °C (50 °C for the contrast variation), controlled electronically within an accuracy of ±0.1 °C. The acquisition time for one sample in pure D_2O was 40 min. The acquisition time for samples in 80%, 60% and 50% D_2O was 50, 60 and 70 min, respectively. The normalized SANS intensity $I(q)$ as a function of the scattering vector q was obtained as described previously (Kučerka et al., 2003).

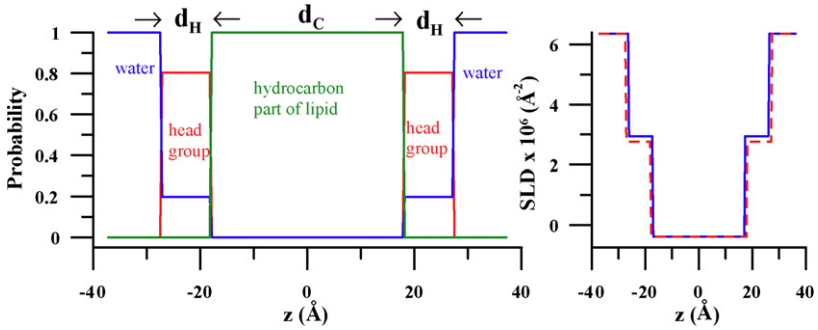

Fig. 1. Strip-function 3S model, and corresponding neutron scattering length density (SLD) profile. SLD profiles: full line corresponds to DPPC bilayer (V_H = 325 Å^3, d_H = 9 Å, 20 °C), dashed line corresponds to DPPC bilayer in 5 mM $CaCl_2$ (V_H = 325 Å^3, d_H = 9 Å, 20 °C). (Probability expressed according to Kučerka et al., 2007.)

2.3. Data analysis

For dispersions of monodisperse centrosymmetric particles, the scattered intensity is given by

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

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 phospholipid concentrations <2 wt.% (Knoll et al., 1981; Nawroth et al., 1989). However, the multilamellar structure gives rise to the characteristic interaction peak. This can be approximated by Gaussian distribution function as

$$S(q) = 1 + k \exp\left(-\frac{(q - q_0)^2}{2\sigma^2}\right) \tag{2}$$

where σ is the width of the interference peak, and q_0 is the position of its center, which is inversely related to the distance between bilayers. The coefficient k corresponds to the amount of multilamellar structures.

The vesicles are hollow spheres with the single or multiple lipid bilayer shells separating the inside and outside aqueous compartments. In the strip-function model (Fig. 1), the bilayer is divided into three strips corresponding to two polar head group regions (one on each side of the bilayer) and the bilayer center spanning hydrocarbon region. 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 represented by strip model it is given by (Kučerka et al., 2004):

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

where $\Delta\rho_i(r)$ is the SLD contrast and $\Delta d_i = R_i - R_{i-1}$ is the thickness of the ith strip. The total lipid bilayer thickness d_L is then given by $d_L = 2d_H + d_C$, where d_H and d_C are thicknesses of the polar head group region and hydrocarbon part, respectively. The SLD in the head group region is

$$\Delta\rho_H = \frac{B_H + n_W B_W}{V_H + n_W V_W} - \rho_W \tag{4}$$

where n_W is the number of water molecules located in this region, V the volume, B the coherent scattering length, and subscripts W and H abbreviate water and head group, ρ_W is the neutron SLD of the aqueous solvent. The SLD of the hydrocarbon region is determined by

$$\Delta\rho_C = \frac{B_C}{V_C} - \rho_W \tag{5}$$

where the volume of hydrocarbon part is $V_C = V_L - V_H$, and V_L is the volume of lipid molecule at given temperature. Evaluation of n_W in Eq. (4) is equivalent to the evaluation of the ratio $\Delta\rho_C/\Delta\rho_H$, which puts the structural model on an absolute scale.

The following relation, involving the area per lipid A_L, the thickness d_H of the polar region, and the thickness d_C of the hydrocarbon region

$$A_L = \frac{V_H + n_W V_W}{d_H} = \frac{V_C}{d_C} \tag{6}$$

provides a constraint between the three model parameters d_H, d_C, and n_W reducing the number of independent parameters to two. Volumetric values used in the data analysis are given in Table 1 and coherent scattering lengths of required model groups were calculated using the known coherent scattering lengths of nuclei (Sears, 1986). Fig. 1 displays an example of one-dimensional SLD profile representing the fully protonated DPPC bilayer dispersed in D_2O. There is a large contrast between the hydrocarbon region and solvent, which decreases in stair-like way to characterize molecules of solvent (D_2O) distributed in the polar region of bilayer.

The binding site for calcium is near the negative phosphate group of the P^-–N^+ dipole of phospholipid headgroup (Hauser et al., 1975; Herbette et al., 1984; Binder and Zschornig, 2002). In our model the calcium ions are represented by spheres of radius 1 Å (Shannon, 1976) distributed in polar head group region. The partitioning of probability distributions for lipid and calcium is then done based on a simple complementarity. A portion of Ca^{2+} ions bound to polar head group region was determined using the Lang-

Table 1
Volumetric data

	20 °C	60 °C
V_{DPPC} (Å^3)	1144[a]	1244[b]
V_H (Å^3)	325 ± 6[c]	325 ± 6
V_{D_2O} (Å^3)[d]	30.1	30.5
V_{H_2O} (Å^3)[d]	30	30.4
$V_{Ca^{2+}}$ (Å^3)	4.2[e]	4.2
d_H (Å)	9 ± 1.2[f]	9 ± 1.2

[a] Nagle and Tristram-Nagle (2000).
[b] Tristram-Nagle and Nagle (2004).
[c] Small (1967); Sun et al. (1994); Tristram-Nagle et al. (2002).
[d] Handbook of Chemistry and Physics (1969).
[e] Shannon (1976).
[f] Pabst et al. (2000).

muir adsorption isotherm of the form

$$\frac{X_b}{1-nX_b} = K(c_{Ca^{2+}} - X_b c_L) \quad (7)$$

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 (Seelig and Macdonald, 1989; Huster and Arnold, 1998), K denotes the binding constant, c_L is the concentration of DPPC, and $c_{Ca^{2+}}$ the total Ca^{2+} concentration. The binding constants 37 and 10 M^{-1} for gel and liquid–crystalline phase with binding stoichiometry 1:1 (Satoh, 1995) were used for the X_b evaluation. Our samples are prepared in 5 mM NaCl. The binding constants of 0.28 M^{-1} for Cl^-, and 0.25 M^{-1} for Na^+ (Satoh, 1995) at the NaCl concentration used indicate a negligible portion of bound ions (Na^+, Cl^-), and they are not included in the fitting. We do not consider more detailed specifications of electrostatic interactions in our structural analysis.

Structural parameters are refined in terms of an iterative model-fitting approach that results in the bilayer SLD profile. Experimentally obtained scattering curves have been fitted with those calculated theoretically using the function minimization and error analysis program Minuit (CERN Program Library entry D506). The vesicle structure factor derived from the model described above was convoluted with the Schulz distribution function of vesicle radii $G(R)$ (Gradzielski and Hoffmann, 1992, for more details see Kučerka et al., 2007), and with the PAXE spectrometer resolution function $T(q)$

$$I(q) = N_P \int_{q'} T(q') \int_R G(R) I(R, q') \mathrm{d}R \, \mathrm{d}q' \quad (8)$$

The convolution due to the polydispersity can be solved analytically following Pencer et al. (2006). The scattered intensity is then calculated as the square of the planar bilayer form factor and multiplied by the function which includes the particle's "sphericity" and the system's polydispersity, $P_{TS}(q)$, and is written as follows:

$$I(q) = N_P \int_{q'} T(q') P_{TS}(q') S(q') F^2(q') \mathrm{d}q' \quad (9)$$

$$P_{TS}(q) = \frac{8\pi^2(z+1)(z+2)}{s^2 q^2} \times \left\{ 1 - \left(1 + \frac{4q^2}{s^2} \right)^{-(z+3)/2} \cos\left[(z+3) \arctan\left(\frac{2q}{s} \right) \right] \right\} \quad (10)$$

where $s = R/\sigma_R^2$ and $z = R^2/(\sigma_R^2 - 1)$ are the products of the vesicle mean radius R and σ_R represents the system's polydispersity. The final formula (Eq. (9)) for the scattering intensity used in our calculation thus comprises Eqs. (10) and (2), and the bilayer form factor $F(q)$ for symmetric flat bilayer written as

$$F(q) = \frac{1}{q}\{\Delta\rho_H[\sin(q(d_C + d_H)) - \sin(qd_C)] + \Delta\rho_C \sin(qd_C)\} \quad (11)$$

where the SLD contrasts $\Delta\rho_i$ and thicknesses d_i are defined in Eqs. (4)–(6).

3. Results

3.1. Unilamellar DPPC vesicles

Fig. 2a shows the SANS scattering curve $I(q)$ vs. q of multilamellar DPPC vesicles. 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

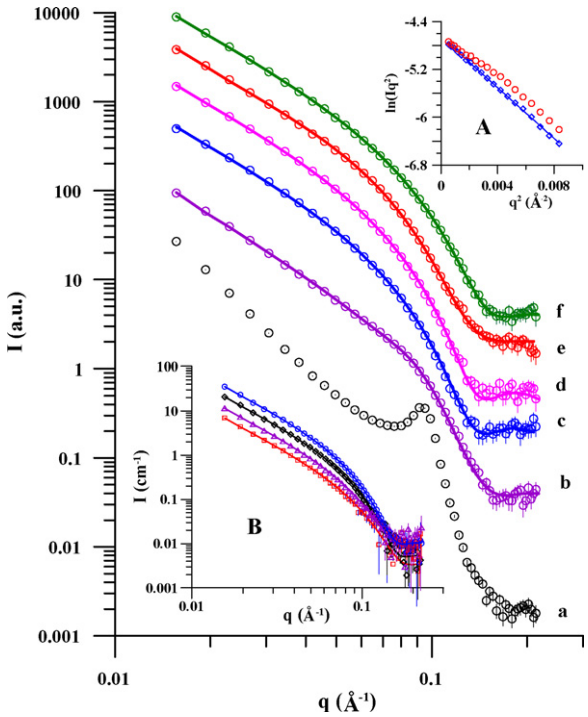

Fig. 2. Dependence of SANS intensity $I(q)$ on the scattering vector q for DPPC multilamellar vesicles at 60 °C (a), DPPC unilamellar vesicles prepared by extrusion at 60 °C (b), and DPPC vesicles prepared at $CaCl_2$ concentrations: 1 mM (c), 10 mM (d), 60 mM (e) at 20 °C; 2.5 mM (f) at 60 °C. Inset A: Kratky–Porod plot for curves c (◊) and e (○). Inset B: DPPC vesicles in 20 mM $CaCl_2$ at 50 °C in 100% (○), 80% (◊), 60% (△) and 50% (□) D_2O. Full lines represent the best fits.

of fully hydrated DPPC multibilayers at $t \sim 50$–60 °C (Nagle and Tristram-Nagle, 2000; Uhríková et al., 2007a). From the thermodynamical point of view, lipids dispersed in an excess of water form a two phases system, where the second phase is the bulk water. Not all the water that is added to the lipid is located between stacks of bilayers with uniform spacing and so it is difficult to determine the thickness d_L and the amount of water between opposite lipid bilayers. Moreover, except water located between lipid's lamellae there is the water located in the polar head group region (n_W). The problem of determination of the thickness of the lipid bilayer and of the area per lipid molecule of fully hydrated PC bilayers was analyzed in (Nagle and Tristram-Nagle, 2000), where different methods are reviewed. Structural parameters of neutral PC bilayers organized in multilamellar vesicles correspond well to data obtained from SANS on unilamellar vesicles (see, e.g. Balgavý et al., 2001a).

The extrusion procedure of (MacDonald et al., 2001) destroys periodic bilayers organization, and lipid bilayers form the system of unilamellar vesicles. SANS curves of unilamellar vesicles then show no diffraction peak (Fig. 2b) and can be analyzed using Eq. (1) and $S(q) = 1$. We have analyzed our data obtained for DPPC unilamellar vesicles in gel (at 20 °C) and liquid–crystalline (at 60 °C) phases by using the 3-strip model (3S) with the aim to examine the Ca^{2+} effect on the lipid bilayer thickness d_L, the area per one DPPC molecule A_L and the number of water molecules located in the polar head group region n_W.

There are two separate groups of parameters that determine the whole SANS curve: in the low-q region, the scattering is sensitive to large length-scales, i.e. the overall size of the vesicles, while the information about bilayer structure is content in the mid- and high-q region. Two parameters describe the size distribution function of unilamellar vesicles (radius R and polydispersity σ_R) and two parameters define our model of bilayer (A_L and n_W). Together

with two linear parameters corresponding to a multiplicative scaling coefficient and an additive background constant, overall, there are six adjustable parameters in our analysis. Unfortunately, a lack of experimental data in the low-q region ($q < 0.016$ Å^{-1}) restrained us from determining the vesicle radii and their polydispersities in much detail. Nevertheless, our current data were consistent with the values of radii $R \sim 300$–350 Å and polydispersities $\sigma_R \sim 80$–90 Å, evaluated previously for unilamellar vesicles extruded through 500 Å filter (Balgavý et al., 2001a,b).

As can be seen from Eqs. (4)–(6) and from Fig. 1, our model is based on volumetric distributions. We have used the well-known volumes of DPPC molecule, reported by Nagle and Tristram-Nagle (2000), $V_L = 1144$ Å^3 at 20 °C, and $V_L = 1244$ Å^3 at 60 °C (Tristram-Nagle and Nagle, 2004) (Table 1). In addition, the volume of the head group V_H is essential for the determination of the area per molecule A_L from Eq. (6). The X-ray diffraction on gel lamellar phases provided $V_H = 319$ Å^3 for DPPC at 24 °C (Sun et al., 1994), and $V_H = 331$ Å^3 for DMPC at 10 °C (Tristram-Nagle et al., 2002). The error of these estimates was ±6 Å^3, with the overlap at $V_H = 325$ Å^3, which is nearly the same as the molecular volume of 324.5 Å^3 reported for glycerylphosphorylcholine (Small, 1967). The thickness of the polar region d_H can be deduced from the data of other authors as well. Pabst et al. (2000) obtained $d_H = 9.0 \pm 1.2$ Å from neutron diffraction data of oriented, partially dehydrated DPPC bilayers (Büldt et al., 1979; Zaccai et al., 1979). McIntosh et al. (1986) estimated $d_H = 10$ Å from space-filling models of PC bilayers. The value $d_H = 9.0$ Å was used for different PCs (Nagle and Tristram-Nagle, 2000; Petrache et al., 1998). To cover all the different data above, we used values $V_H = 325 \pm 6$ Å^3 and $d_H = 9 \pm 1.2$ Å in our analysis. Table 2 summarizes the structural parameters (d_L, A_L, and n_W) of DPPC lipid bilayers at 20 and 60 °C obtained from the fitting procedure for different combinations of V_H and d_H. In the gel phase, the best fit provided $d_L = 52.6 \pm 0.3$ Å, $A_L = 47.4 \pm 0.4$ Å, and $n_W = 3.5 \pm 0.1$ mol/mol with $V_L = 325$ Å^3, $d_H = 9$ Å which agree well with the data of Nagle and Tristram-Nagle (2000). In addition, the change of ±6 Å^3 in the volume of polar head group V_H (while $d_H = 9.0$ Å) induces only minor changes in the structural parameters. On the other hand, the results were more scattered when we constrained the thickness of the polar head group region d_H to one of the extreme values (7.8 and 10.2 Å). As expected, combinations of values $V_L = 331$ Å^3, $d_H = 7.8$ Å and $V_L = 319$ Å^3, $d_H = 10.2$ Å represent the outermost results, e.g. we have obtained $d_L = 47.4$ and 58.5 Å, respectively. In the liquid–crystalline phase, the best fit (Fig. 2b) using $V_L = 325$ Å^3, $d_H = 9$ Å gives $d_L = 48.0 \pm 0.5$ Å, $A_L = 61.3 \pm 1$ Å, and $n_W = 7.5 \pm 0.3$ mol/mol. Table 2 compares the structural parameters obtained in our analysis with the structural parameters of DPPC bilayer obtained by Nagle and Tristram-Nagle (2000) at 50 °C which we have recalculated to 60 °C using the volume $V_L = 1244$ Å^3 and thermal area expansivity 0.003 K^{-1} (Evans and Needham, 1987). As one can see, there are larger differences in structural parameters than we have observed at 20 °C. Similar differences are reported also for unilamellar dioleoylphosphatidylcholine vesicles (at 30 °C) and may depend on the experimental method (X-ray and/or neutron scattering) as discussed in Kučerka et al. (2007).

Despite the differences in the bilayer structure on the absolute scale that depends on input parameters, the relative changes can be readily obtained. In the following, we will use for comparison structural parameters obtained from the best fits of SANS curves (at 20 and 60 °C), as the aim of this work is to study relative effects of the calcium binding to the DPPC bilayer.

3.2. DPPC + Ca^{2+} vesicles

The destruction of the periodic organization of DPPC bilayers due to addition of $CaCl_2$ is born out by the reduction of intensity of Bragg peaks in X-ray diffraction patterns (Inoko et al., 1975; Yamada et al., 2005) as well as in SANS curves published recently (Uhríková et al., 2007b). The binding site for cations is near the negative phosphate group of the P^-–N^+ dipole of phospholipid headgroup. As the negative charge of phosphate group is neutralized, the lipid bilayer becomes positively charged, and the electrostatic repulsion between bilayers makes them to swell in excess water. The surface charge density higher than 1–2 μC/cm^2 promotes the formation of unilamellar vesicles (Hauser, 1993). Fig. 2c–f shows typical SANS curves of DPPC vesicles in solutions of 1–60 mM $CaCl_2$. Our previous work (Uhríková et al., 2007b) shows that the obtained curves correspond to the scattering from polydisperse hollow spheres (i.e. unilamellar vesicles) (Balgavý et al., 1998).

Full lines in Fig. 2 represent the best fits to SANS curves obtained by our model. Structural parameters of DPPC in the gel phase (at 20 °C), i.e. the lipid bilayer thickness d_L, the area per DPPC molecule A_L, and the number of water molecules located in the polar head group region n_W are shown in Fig. 3 as a function of the calcium concentration. Dashed lines represent the structural parameters of DPPC unilamellar vesicles without calcium issued from the same model.

PCs gently hydrated in solution of calcium at low concentration spontaneously form giant unilamellar liposomes (Akashi et al., 1998). Ultrasound waves as well as freezing-thawing cycles, applied at homogenization of our samples, are processes resulting in a reduction of vesicles size and their polydispersity (Kaasgaard et al., 2003; Siow et al., 2007; Barenholz et al., 1977). The curves fitting

Table 2
Representative structural parameters of DPPC unilamellar vesicles prepared by extrusion as obtained from fitting of SANS curves using 3S model

Model	V_H (Å^3)	d_H (Å)	t (°C)	d_L (Å)	A_L (Å^2)	n_W (mol/mol)
3S	325	9	20	52.6 ± 0.3	47.4 ± 0.4	3.5 ± 0.1
3S	319	9	20	53.1 ± 0.2	47.1 ± 0.2	3.5 ± 0.1
3S	331	9	20	52.2 ± 0.7	47.6 ± 0.9	3.2 ± 0.3
3S	325	7.8	20	51.5 ± 0.9	45.6 ± 1.2	1.0 ± 0.3
3S	325	10.2	20	58.4 ± 0.5	43.1 ± 0.6	3.8 ± 0.2
Ref.[a]	319	9	20	52.4	47.9	3.7
3S	325	9	60	48.0 ± 0.5	61.3 ± 1	7.5 ± 0.3
3S	319	9	60	48.3 ± 0.5	61.1 ± 0.9	7.6 ± 0.3
3S	331	9	60	47.7 ± 0.5	61.5 ± 0.9	7.3 ± 0.3
3S	331	7.8	60	44.9 ± 0.5	62.3 ± 1	5.1 ± 0.3
3S	319	10.2	60	52.0 ± 0.5	58.5 ± 1	9.1 ± 0.3
3S	325	7.8	60	45.1 ± 0.5	62.3 ± 0.3	5.3 ± 0.3
3S	325	10.2	60	51.6 ± 0.6	59.0 ± 1	9.1 ± 0.3
Ref.[a]	319	9	60[b]	46.1	65.9	9

[a] Ref. data from Nagle and Tristram-Nagle (2000).
[b] Data from Nagle and Tristram-Nagle (2000) recalculated to 60 °C using the volume $V_L = 1244$ Å^3 and the thermal area expansivity 0.003 K^{-1} (Evans and Needham, 1987).

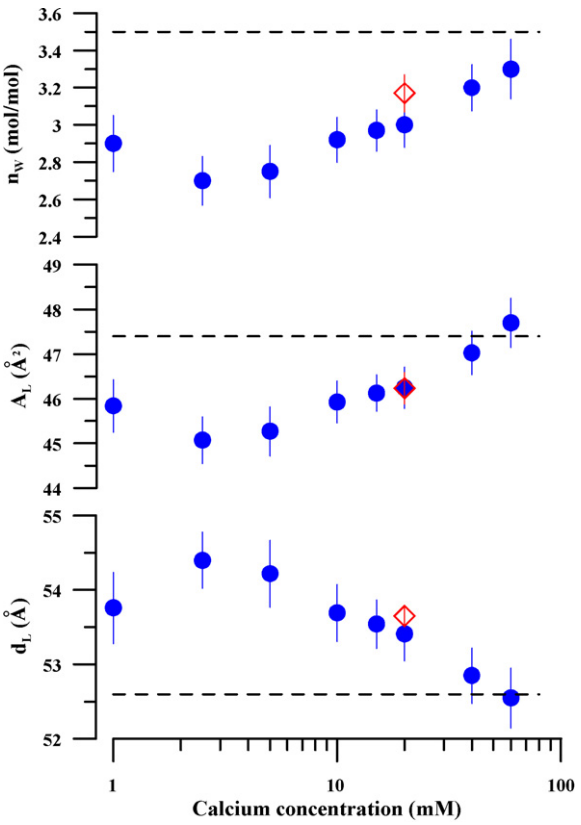

Fig. 3. Structural parameters (d_L, A_L, and n_W) of DPPC bilayer in 1–60 mM $CaCl_2$ (20 °C). Dashed lines represent structural parameters of DPPC unilamellar vesicles without calcium obtained with the same model. Diamonds correspond to data from the contrast variation technique.

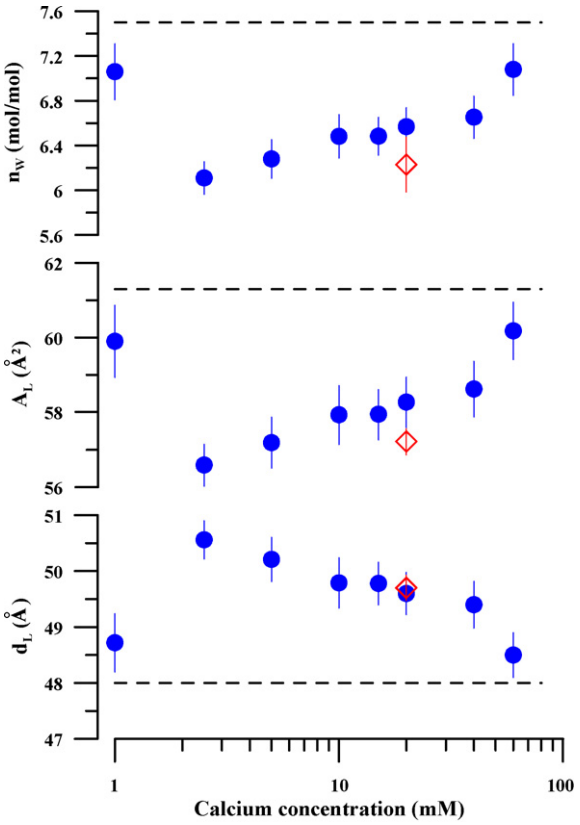

Fig. 4. Structural parameters (d_L, A_L, and n_W) of DPPC bilayer in 1–60 mM $CaCl_2$ (60 °C). Dashed lines represent structural parameters of DPPC unilamellar vesicles without calcium obtained with the same model. Diamonds correspond to data from the contrast variation technique (at 50 °C).

have indicated the vesicle radii $R \sim 350$–380 Å and polydispersities $\sigma_R \sim 80$–95 Å. We do not report the dependence of the vesicle radius on calcium concentration in much detail. Our experimental setup has covered the scattering vector range 0.016–0.224 $Å^{-1}$, in this region the intensity is insensitive to the precise values of R and σ_R or to deviations from sphericity due to undulation fluctuations and it is much more sensitive to the local structure of the bilayer, which is the subject of interest in this paper (for more details see Kučerka et al., 2004).

The consequences of addition of calcium are the following: in the gel phase (20 °C), the lipid bilayer thickness goes through a maximum $d_{L(DPPC+Ca)} = 54.4 \pm 0.4$ Å at 2.5 mM $CaCl_2$. The total change is $\Delta d_L = d_{L(DPPC+Ca)} - d_{L(DPPC)} = 1.8 \pm 0.7$ Å, where $d_{L(DPPC)}$ is the thickness of DPPC unilamellar vesicles prepared by extrusion. At higher concentration of calcium ($c_{Ca^{2+}} > 2.5$ mM), d_L decreases. The lipid bilayer thickness at $c_{Ca^{2+}} \geq 40$ mM is the same as that observed for DPPC vesicles without Ca^{2+} ions.

A watchful inspection of scattering curves in Fig. 2 one can see a difference in the shape of scattering profiles Fig. 2c and d in comparison to Fig. 2e. A non-perfect linearity of the Kratky–Porod plot of the DPPC vesicles in 60 mM $CaCl_2$ curve (in comparison to that prepared at the lower concentration, Fig. 2, inset A) warns about a possible structural changes in the dispersion (discussed also in Uhríková et al., 2007a,b). Actually, the fitting of SANS curve obtained for DPPC vesicles prepared at 60 mM $CaCl_2$ reveals the presence of a small broad peak at $q = 0.060 \pm 0.006$ $Å^{-1}$, suggesting the existence of pauci-lamellar vesicles, i.e. formed by more than one bilayer (Kučerka et al., 2007). The intensity of the peak is much lower in comparison to that observed for multilamellar vesicles in Fig. 2a, the peak is hidden under scattering curve, slightly deforming its shape. To account for pauci-lamellarity, we have included an additional Gaussian function in $S(q)$ as given in Eq. (2), which improved the quality of fit substantially. The peak associated periodicity, $d = 105 \pm 11$ Å, corresponds with the formation of a partially disordered lamellar phase, which is observed at higher concentration of Ca^{2+} (Inoko et al., 1975; Yamada et al., 2005). Yamada et al. (2005) reported a repeat distance $d \sim 123$ Å for DPPC vesicles in 60 mM $CaCl_2$ at 30 °C.

A_L corresponds to the lateral area of the lipid molecule (including a partial fraction of calcium); it is calculated from the fitted thickness and volumetric data. A_L goes through a minimum at 2.5 mM $CaCl_2$, and the total change is $\Delta A_L = A_{L(DPPC)} - A_{L(DPPC+Ca)} = 2.3 \pm 0.9$ $Å^2$. Then A_L increases monotonically up to the value observed for unilamellar DPPC vesicles ($A_L = 47.4 \pm 0.4$ $Å^2$). Finally, n_W varies as A_L. The minimum hydration is observed at 2.5 mM $CaCl_2$, with reduction $\Delta n_W = n_{W(DPPC)} - n_{W(DPPC+Ca)} = 0.8 \pm 0.2$ mol water/mol DPPC, while for pure DPPC bilayer, $n_W = 3.5 \pm 0.1$ mol/mol.

The same parameters (d_L, A_L, and n_W) at 60 °C (liquid–crystalline phase) are shown in Fig. 4. At 2.5 mM $CaCl_2$ we observe a maximum in the lipid bilayer thickness, $d_{L(DPPC+Ca)} = 50.6 \pm 0.3$ Å. The area per DPPC molecule and hydration show minima with $A_{L(DPPC+Ca)} = 56.6 \pm 0.6$ $Å^2$ and $n_{W(DPPC+Ca^{2+})} = 6.1 \pm 0.1$ mol/mol, respectively. These values indicate a considerable change in the lipid bilayer when compared to those for DPPC without any calcium, given in Table 2: $d_{L(DPPC)} = 48.0 \pm 0.5$ Å, $A_{L(DPPC)} = 61.3 \pm 1$ $Å^2$, and $n_{W(DPPC)} = 7.5 \pm 0.3$ mol/mol. Apart from the observed extremity, the change of structural parameters with the further Ca^{2+} increase ($c_{Ca^{2+}} > 10$ mM) is not that obvious as we found in the gel phase (Fig. 3). Similar to the gel phase, the scattering curve

of DPPC vesicles in 60 mM $CaCl_2$ revealed the presence of partially disordered lamellar phase. From the position of the peak at $q = 0.056 \pm 0.005$ Å^{-1}, we found the periodicity $d = 112 \pm 10$ Å. Yamada et al. (2005) reported $d \sim 136$ Å obtained for DPPC in 60 mM $CaCl_2$ at 50 °C in. In 60 mM $CaCl_2$, d_L, A_L, and n_W within an fitting error agree with the structural parameters of DPPC without any calcium. To determine the effect of calcium on the fluid lipid bilayer, it is worth noting the concentration range $c_{Ca^{2+}} \leq 10$ mM, and of course, structural parameters of the lipid bilayer itself should be evaluated carefully.

To test the variability of our results, we fitted SANS curves of DPPC + $CaCl_2$ using the 3S model and two different polar head group volumes $V_H = 319$ and 331 Å^3. The obtained structural parameters (d_L, A_L, and n_W) coincide with data shown in Figs. 3 and 4 within presented fitting errors. Similarly, when the thickness of polar head group region d_H was constrained either to 10.2 or 7.8 Å, dependences of the obtained structural parameters on calcium concentration have shown the same tendencies. Nevertheless, the data were shifted proportionally to values given for DPPC unilamellar vesicles (Table 2).

4. Discussion

The observed changes in the lipid bilayer thickness $\Delta d_L \sim 1.8$–2.6 Å, and area per DPPC molecule $\Delta A_L \sim 2.3$–4.7 Å^2 (gel–liquid-crystal) are in scale with conformational changes of lipid molecules located in the bilayer.

In both gel and liquid–crystalline phases, the P^-–N^+ dipole of phospholipid head group is nearly parallel to the bilayer surface and rotates freely around an axis perpendicular to the bilayer surface (Akutsu and Seelig, 1981; Shepherd and Buldt, 1978; Seelig et al., 1977). The binding plane for divalent cations is 3.5–4 Å away from the edge of hydrocarbon region of phospholipid bilayer (Lis et al., 1981b; Herbette et al., 1984). The electric field due to bound cations is large enough 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 (Izumitani, 1994; Seelig, 1990). Conformational changes in the lipid molecule due to calcium binding were confirmed experimentally by NMR spectroscopy (Zidovetzki et al., 1989; Shibata, 1990; Huster et al., 1999) and IR study (Binder and Zschornig, 2002). Kataoka et al. (1985) and Aruga et al. (1985) have proposed a molecular model for Ca^{2+}–DPPC interaction: The positive net charge of the complex Ca^{2+}–DPPC produces an electric field not only in the direction perpendicular to the bilayer but also in the plane of bilayer. Due to electric field in the plane of bilayer, the zwitterions of the surrounding DPPC molecules are rearranged, so that the negatively charged groups are oriented to the bound Ca^{2+}. Consequently, long-range attraction between bound Ca^{2+} and the surrounding DPPC molecules produces a larger lateral pressure in the hydrophobic part of the bilayer, than in the absence of Ca^{2+}, and closer packing of hydrocarbon chains. Thus, with increasing concentration of calcium, the distance between Ca^{2+} cations decreases, the average values of lateral pressure must increase, and also the Debye screening length is changed. The Debye screening length b (Moore, 1972)

$$b = \left(\frac{e^2}{\varepsilon_0 \varepsilon_r k_B T} \sum_i \chi_i z_i^2 \right)^{-1/2} \quad (12)$$

where ε_0, ε_r are permitivities, k_B is the Boltzmann constant, T is temperature, χ_i is the number density of ith ion with its charge number z_i, decreases with increasing concentration, e.g. $b \sim 56$–17 Å for 1–10 mM $CaCl_2$ solution, which is long enough with respect to the molecular dimension. The analysis of our SANS curves quantifies structural changes in the DPPC bilayer due to Ca^{2+} binding in the range 1–60 mM of $CaCl_2$.

Calcium binds weakly to DPPC bilayers. The reported values of binding constants are scattered in the range 1–120 M^{-1} (Akutsu and Seelig, 1981; Altenbach and Seelig, 1984; Marra and Israelachvili, 1985; Satoh, 1995). We tested, whether the steric effect due to calcium binding plays any role in our fitting. The results presented in Figs. 3 and 4 are obtained approximating the calcium ion by a sphere with Pauling radius of Ca^{2+} (1 Å), taking the binding constant for gel and liquid–crystalline phase ($K = 37$ and 10 M^{-1}, $n = 1$ from Satoh, 1995). The volume occupied by the ion in the polar head group region is very small in comparison to the volume of the polar head group itself, even in comparison to the volume of water molecules. For the test we selected the SANS curve obtained at 40 mM $CaCl_2$ and 20 °C. Structural parameters obtained using the 3S model were not changed (in the range of fitting error) when the number of associated calcium ions per lipid X_b was determined from Eq. (7) using either $K = 74$ M^{-1} and $n = 2$ (Satoh, 1995) or $K = 37.7$ M^{-1} and $n = 3.4$ (Huster and Arnold, 1998). It did not change significantly even when we used data from Fig. 5 in the work of Satoh (1995) where the ratio of ion bound DPPC molecules to the whole DPPC (~7% at 40 mM $CaCl_2$) is recalculated using the surface charge density obtained from zeta-potential measurements. The test revealed that the steric effect of calcium localized in the polar head group region of bilayer plays only minor role in structural changes detected by our analysis using the 3S model.

With the aim to recognize what is the effect of Ca^{2+} on the polar head group, we employed contrast variations technique for DPPC liposomes prepared at 20 mM $CaCl_2$. The SANS curves at four different contrasts (Fig. 2, inset B) were fitted simultaneously with the 3S model, taking the volume of the polar head group $V_H = 325 \pm 6$ Å^3 without constraining the polar head group thickness d_H. The obtained structural parameters of the bilayer (d_L, A_L, n_W) are plotted in Figs. 3 and 4, and correspond well with data of samples prepared in pure D_2O. Samples were measured at 50 °C in liquid–crystalline phase what results in more marked differences in A_L and n_W (Fig. 4). For $V_H = 325 \pm 6$ Å^3, we obtain the average value of the polar head group thickness $d_H = 9.15 \pm 0.10$ Å in the gel phase, where the deviation indicates the changes in d_H when either $V_H = 319$ Å^3 or $V_H = 331$ Å^3 were taken. In the liquid–crystalline phase, at 50 °C we find $d_H = 9.02 \pm 0.13$ Å using the same procedure. The contrast variation experiment has shown that the chosen range of $d_H = 9 \pm 1.2$ Å covers sufficiently the possible changes in the polar head group thickness induced by Ca^{2+}, and the thickness d_H constrained to 9 Å does not carry weighty error in our analysis.

The next point under discussion is why structural parameters of the bilayer (d_L, A_L, n_W) show extremities due to Ca^{2+} binding. The analysis of SANS curves using the 3S model reveals a maximum in the lipid bilayer thickness at ~2.5 mM $CaCl_2$. As it was mentioned in Section 1, we found a maximum in the bilayer thickness parameter d_g at ~5 mM $CaCl_2$ in our previous work (Uhríková et al., 2007b). The d_g parameter was determined from SANS data using the small-angle Kratky–Porod approximation and an assumption of the bilayer (together with calcium ions) having a uniform SLD. This "shift" results from differences in SLD of both models. In the 3S model, there is a change of SLD in the polar head group region, characterized quantitatively by the number of water molecules (n_W) and also by the Ca^{2+} ions (X_b) inside this region (Fig. 1). Ultrasonic experiments of Aruga et al. (1985) have shown a biphasic change in the ultrasonic velocity, the ultrasonic absorption, and the bulk modulus of DPPC liposomes with an extremity at 10 mM $CaCl_2$. They have examined the effect of Ca^{2+} on a gel–liquid-crystal phase transition of DPPC. The temperature of the main transition increased from 41.4 to 44.2 °C at 300 mM $CaCl_2$. The DPPC gel-to-liquid crystal transition is of weak first order phase transition (see

Mitaku et al. (1983) and citation therein) in absence of Ca^{2+} and approaches a critical point as the Ca^{2+} concentration is increased to 10 mM. The authors (Aruga et al., 1985 and references therein) refer to the critical fluctuations at critical point with changes in the lateral pressure. The phase transition is of first order at the lateral pressure below π_c, whereas it is of second order when $\pi = \pi_c$, π_c is the lateral pressure at critical point. This model explains the observed extremity in temperature range close to the phase transition temperature. However, there has been reported a biphasic change (with the minimum at 10 mM $CaCl_2$) in a bulk modulus of DPPC + Ca^{2+} bilayer at 30 °C (Aruga et al., 1985), what is far from the DPPC phase transition temperature. Suezaki et al. (1987) using a thermodynamic approach solve the problem through the fluctuation in the number of adsorbed Ca^{2+} at the phospholipid bilayer surface that can account for the volume fluctuation of the bilayer system. They show that the system goes to the extremity when the adsorption sites of phospholipid are half filled. Let go back to our experimental results: our experiments were performed at 20 and 60 °C, far from the main phase transition temperature, thus to relate the observed extremities to the critical fluctuations of the bilayers at phase transition does not sound physically reliable. We found the extremity in structural parameters at lower concentration of Ca^{2+} (~2.5–5 mM $CaCl_2$, taking into account also our previous results Uhríková et al., 2007b) in comparison to ~10 mM $CaCl_2$ (Aruga et al., 1985). This difference may result from several reasons: different experimental techniques were used, for the bulk modulus determination the pressure is applied on the lipid bilayer, the techniques used require different lipid concentrations, there is also a difference in the Debye screening length due to different ionic strength of solution ($CaCl_2$ + 5 mM NaCl was used in our experiments). Monovalent ions improve phospholipids acyl chain packing, and do no induce changes in the lipid bilayer thickness (Garcia-Manyes et al., 2005; Petrache et al., 2006; Pabst et al., 2007). The extremity at ~2.5 mM $CaCl_2$ was observed in both phases, in the gel as well as in the liquid–crystalline phase, in spite of massive structural changes in bilayer organization due to phase transition. The total molar balance of our sample prepared at 2.5 mM $CaCl_2$ corresponds to the ratio ~5:1 mol DPPC/mol Ca^{2+}, i.e. the excess of lipid. At low ion concentrations (bound ions ≪ lipid concentration), the binding of cations can be characterized by a simple nonspecific 1:1 partitioning, known as Henry–Dalton partition equilibrium:

$$X_b = K c^S_{Ca^{2+}} \quad (13)$$

where X_b is proportional to the concentration of calcium in the vicinity of the bilayer $c^S_{Ca^{2+}}$, and K (M^{-1}) is the binding constant. However, it is not easy to determine K, as it changes with concentration at least by a factor of 10 (Altenbach and Seelig, 1984). According to Seelig (1990), the concentration of Ca^{2+} ions in the plane of the phospholipid head groups can be enhanced by at least two orders of magnitude compared to bulk solution. Thus to determine the concentration of calcium when the adsorption sites are half filled is not a trivial task. To conclude, the observed extremities in the structural parameters of DPPC bilayer due to calcium binding result from the synergetic effect of the number of Ca^{2+} bounded, their distances in the polar head group, the Debye screening length and induced changes in the lateral pressure in the hydrophobic part of bilayer. The increase in the A_L, disorder in the hydrophobic part of bilayer due to higher mobility of the acyl chains and the bilayer fluctuations in the fluid phase modulate this effect and thus changes in the structural parameters become with decreasing Debye screening length less evident.

The reorientation of the P^-–N^+ dipole as well as more extended hydrocarbon chains induce changes in the surface area per molecule. Our experiments show the decrease in the area per DPPC molecule with the maximum $\Delta A_L \sim 2.3$–4.7 Å^2 (gel–liquid-crystal) due to calcium binding. As Figs. 3 and 4 document, changes in A_L are a function of calcium concentration and temperature. It is known that the A_L depends on the length of lipid's acyl chains as well as on their unsaturation degree (see, e.g. Nagle and Tristram-Nagle, 2000; Kučerka et al., 2003). Double bonds create defect in the hydrocarbon chains packing that can damp changes in the lateral pressure and thus minimize the effect of cation binding. Huster et al. (2000) report such "inconspicuous" effect of calcium on the lipid bilayers in the liquid-crystalline phase. They observed an increase in the average ^{2}H NMR order parameter of perdeuterated acyl chain in monounsaturated lipids (C_n:0–C_n:1, n is the number of carbons in the acyl chain) after the addition of 5 mM Ca^{2+} ions equivalent to a decrease in area per molecule of ~1 Å^2. The decrease in the area per lipid molecule was more pronounced in C_{18}:0–C_{18}:1 than in C_{18}:0–C_{22}:6, as obtained from the mixtures of phosphatidyl-choline, -ethanolamine, and -serine (PC:PE:PS = 4:4:1 mol/mol/mol).

Our analysis of SANS curves indicates changes in the hydration of DPPC polar head group due to calcium. For pure DPPC vesicles we found n_W equal to 3.5 and 7.5 mol water/mol DPPC in gel and liquid–crystalline phase, respectively. Due to calcium binding there is a decrease in the hydration (Figs. 3 and 4) with the maximum in $\Delta n_W \sim 0.8$–1.4 mol water/mol DPPC depending on the lipid phase. As one can see from Table 1, the volume of water molecule is significantly higher (~30 Å^3) in comparison to that of Ca^{2+} (4.2 Å^3), thus the observed changes in the hydration do not result from simple substitution of water molecules with calcium in the polar head group region. A conformational changes in the lipid's head group due to calcium binding confirmed by NMR and IR are the most probably responsible for that decrease the ability of lipid to bind the water molecule.

5. Conclusions

Our experiments quantify the structural changes of the DPPC bilayer induced by Ca^{2+} binding in the concentration range 1–60 mM where unilamellar vesicles form spontaneously.

The analysis of SANS curves using the strip-function model reveals changes in DPPC bilayer in the gel and the liquid–crystalline phase due to calcium binding. In the gel phase, the lipid bilayer thickness shows a maximum at ~2.5 mM $CaCl_2$. For 60 mM $CaCl_2$, the lipid bilayer thickness d_L is nearly the same as observed for unilamellar vesicles prepared by extrusion without Ca^{2+}, and a partially disordered lamellar phase due to Ca^{2+} screening is observed. Simultaneously, both the area per DPPC molecule, and the number of water molecules located in the polar head group region have opposite dependences on Ca^{2+} concentration with minima at ~2.5 mM $CaCl_2$. In the liquid–crystalline phase, the same structural parameters d_L, A_L, and n_W show unambiguous changes with increasing Ca^{2+} up to concentration $c_{Ca^{2+}} \leq 10$ mM. At higher concentration $c_{Ca^{2+}} > 10$ mM, changes in structural parameters are less clear, although they are still different in comparison to parameters of unilamellar DPPC vesicles without calcium.

Our experiments revealed that biphasic changes in the structural parameters of the bilayer with the extremities at 2.5 mM $CaCl_2$ are observable both in the gel as well as in the liquid–crystalline phase, at temperatures far from the main phase transition (20 and 60 °C). In addition, the concentration of the lipid and the Debye screening length of bounded cations are determining parameters for the extremity.

The data presented demonstrate the ability of calcium to modify the structure of DPPC lipid bilayers: to increase its thickness at the expense of the area per molecule at interface, and hydration. Functions of many transmembrane proteins depend on the

lipid bilayer physical properties (Lee, 2004 and references therein, Karlovská et al., 2006). Physiological Ca^{2+} concentrations generally do not exceed 10 mM, so calcium ions could affect these functions by affecting the bilayer thickness, lipid surface and hydration of the polar head group region.

Acknowledgements

The experiments were supported by the European Commission through the Access Activities of the Integrated Infrastructure Initiative for Neutron Scattering and Muon Spectroscopy (NMI3), supported by the European Commission under the 6th Framework Programme through the Key Action: Strengthening the European Research Area, Research Infrastructures, Contract no.: RII3-CT-2003-505925; by the JINR project 07-4-1031-99/2008, by the VEGA grant 1/3029/06 to DU, and by the APVV grant 51-027 404. DU thanks the LLB for their hospitality.

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