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Small-angle neutron scattering study of the *n*-decane effect on the bilayer thickness in extruded unilamellar dioleoylphosphatidylcholine liposomes ☆

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Abstract

Dioleoylphosphatidylcholine (DOPC) and *n*-decane were mixed and hydrated afterwards in an excess of heavy water at 1 wt.% of DOPC. From this dispersion, unilamellar liposomes were prepared by extrusion through polycarbonate filter with 500-Å pores. Small-angle neutron scattering (SANS) was conducted on these liposomes. 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 $0.001\ \text{Å}^{-2} \le Q^2 \le 0.006\ \text{Å}^{-2}$, the liposome bilayer radius of gyration R_g and the bilayer thickness parameter $d_g = 12^{0.5}R_g$ were obtained. The values of d_g indicated that the bilayer thickness is within the experimental error constant up to *n*-decane/DOPC ~ 0.5 molar ratio, and then increases by 2.4 ± 1.3 Å up to *n*-decane/DOPC = 1.2 molar ratio.

Keywords: 1,2-Dioleoyl-3-phosphatidylcholine; Unilamellar liposomes; *n*-Decane; Bilayer thickness; Small-angle neutron scattering

1. Introduction

Alkanes have several pharmacological effects, which are believed to result from their interactions with constituents of biological membranes. In particular, *n*-alkanes are able to act as general anesthetics. It has been known for many years that a sudden drop in the anesthetic potency of *n*-alkanes (cut-off effect) occurs at a well-defined alkane chain length (e.g. 10 or 12 carbon atoms) depending on the species. Differences in capacitance between the phospholipid black lipid films formed using different chain length *n*-alkanes have been interpreted as indicating that short chain *n*-alkanes (6–10 carbon atoms) give rise to thicker bilayers than long chain *n*-alkanes (12–16 carbon atoms). This effect has been correlated with their anesthetic potency and the cut-off ef-

☆ Dedicated to Professor Pavel Švec on the occasion of his 60th birthday.

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fect [1,2], because the conductance of black lipid films containing certain ionophores has been also observed to be similarly dependent on the chain length of the *n*-alkane used as solvent in the films preparation [3]. However, the anesthetic potency and the cut-off effect could result also from binding of *n*-alkanes at hydrophobic sites in target proteins in central nervous system [4–7]. Similarly, a modulation of activity of transmembrane sarcoplasmic reticulum Ca–Mg–ATPase protein by *n*-alkanes, when reconstituted in phospholipids of defined acyl chain length, has been ascribed to the *n*-alkanes effect on the bilayer thickness [8] or to their binding to protein hydrophobic binding sites [9].

Using X-ray diffraction, McIntosh et al. [10] observed that short chain *n*-alkanes (6–8 carbons) increased the repeat period and the phosphate–phosphate transbilayer distance in multilamellar phases of dipalmitoylphosphatidylcholine, dilauroylphosphatidyl-choline and egg yolk phosphatidylcholine, while the long chain homologues (14–16 carbons) had a very small or no effect upon them. On the other hand, Pope et al. [11] did not see practically any effect of *n*-hexane, *n*-octane or *n*-dodecane upon the repeat period of the dimyristoylphosphatidylcholine lamellar phase. In the oriented dioleoylphosphatidylcholine lamellar phase, both the repeat period and the bilayer thickness remained unchanged up to 0.8 molar fractions of *n*-hexane in the lipid phase [12,13]. One possible cause of these conflicting results could be different bilayer hydration levels of phospholipid bilayers used by different groups of authors. At high concentrations, *n*-alkanes promote the formation of reversed hexagonal and isotropic phases in phospholipid dispersions in water [14–20].

The aim of the present contribution was to study the effects of *n*-decane on the large extruded unilamellar 1,2-dioleoyl-3-phosphatidylcholine (DOPC) liposomes by the small-angle neutron scattering method.

2. Material and methods

DOPC was purchased from Avanti Polar Lipids (Alabaster, USA), *n*-decane was from Sigma (St. Louis, USA) and heavy water (99.98% ^2H_2O) was obtained from Izotop (Moscow, Russia). Mixtures of DOPC and *n*-decane were prepared in a tube by simple addition of *n*-decane to dry lipid using a microsyringe. The amount of *n*-decane and lipid in samples was controlled by gravimetry. After that, heavy water was added to the sample to reach the DOPC concentration of 1 wt.%, the tube was purged with pure gaseous nitrogen and sealed with Parafilm M (American National Can, Greenwich, USA) or with a stopper. First, the multilamellar DOPC liposomes were prepared. The DOPC + *n*-decane in ^2H_2O was dispersed by hand shaking and sonication in a bath sonicator at room temperature. From this dispersion, extruded unilamellar liposomes were prepared according to MacDonald et al. [21]. The multilamellar liposomes were extruded through a polycarbonate filter (Nucleopore, Plesanton, USA) with pores of 500 Å diameter mounted in the Lipso-Fast Basic extruder (Avestin, Inc., Canada) fitted with two gas-tight Hamilton syringes (Hamilton, Reno, USA). The sample was subjected to 25 passes through the filter at room temperature. An odd number of passes were performed to avoid contamination of the sample by large and multilamellar vesicles, which might not have passed through the filter. The final DOPC concentration was not measured precisely, but it was at least ≤ 1 wt.% in all samples. The samples were flushed with the pure gaseous nitrogen, sealed and stored at room temperature. The maximum period between the sample preparation and its measurement was 7 h. This extrusion produces large unilamellar liposomes with a wide distribution of their diameters when using pure phospholipids without admixtures [21].

The SANS measurements were performed at the small-angle time-of-flight axially symmetric neutron scattering spectrometer MURN (now named YuMO in honor of the deceased Yu.M. Ostanevich) at the IBR-2 fast pulsed reactor of the Frank's Laboratory of Neutron Physics, Joint Institute for Nuclear Research in Dubna [22,23]. The samples were poured into quartz cells (Hellma, Müllheim, Germany) to provide the 2-mm sample thickness. The sample temperature was

set and controlled electronically at 20.0 ± 0.1°C. The sample in quartz cell was equilibrated minimally for 1 h at this temperature before measurement. The sample–detector distance was set to 10.553 m. The acquisition time for one sample was 1 h. The scattering patterns were corrected for background effects. The coherent scattering intensity was obtained by using a vanadium standard scatterer. The data matching and the resolution function of this spectrometer have been described in detail by Ostanevich [22].

3. Results and discussion

Fig. 1 shows the plots of the typical SANS scattering functions, $I(Q)$, obtained with unilamellar DOPC liposomes in our experiment, as a function of the scattering vector Q, which is defined as:

$$Q = 4\pi \sin\theta/\lambda \quad (1)$$

where 2θ is the scattering angle and λ is the wavelength of neutrons. In the scattering functions in Fig. 1, the absence of the pronounced first order Bragg diffraction peak is notable. The first order Bragg diffraction peak was observed in SANS experiments with multilamellar liposomes [24] and with a mixture of unilamellar and multilamellar liposomes prepared by sonication in a bath sonicator [25]. Since our experimental protocol involved a rather prolonged staying of samples before and during measurements, the liposomes could fuse and/or aggregate during the experiment with the possible formation of multilamellar structures. The absence of a Bragg peak is, therefore, evidence that the prepared unilamellar liposomes did not contain an appreciable amount of multilamellar liposomes.

The experimentally observed scattering intensity is for monodisperse system given by:

$$I(Q) \sim N_P P(Q) S(Q) \quad (2)$$

where N_P is the number of particles, $P(Q)$ is the particle structure factor and $S(Q)$ is the interparticle structure factor. In the first approximation,

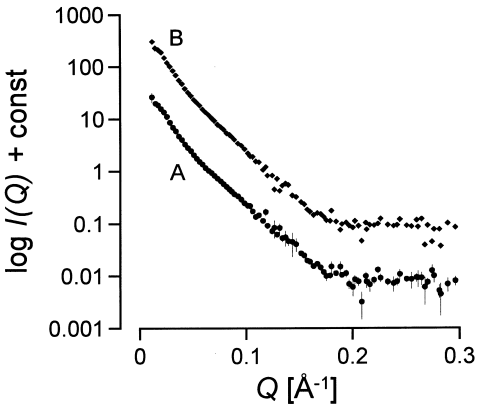

Fig. 1. The dependence of SANS intensity $I(Q)$ on the scattering vector Q for extruded unilamellar DOPC liposomes in the absence (a) and presence (b) of n-decane (molar ratio n-decane/DOPC = 1:1). The dots indicate the mean values obtained by averaging over seven circular detectors and the error bars the S.E. of the mean value.

the unilamellar liposomes are hollow spheres with the lipid bilayer shell separating the inside and outside aqueous compartments. For such particles, the particle structure factor P is equal to the mean squared form factor, which is a one-dimensional Fourier integral of the coherent neutron scattering length density. The interparticle structure factor $S(Q)$ is approximately equal to one for dilute and weakly interacting spherical system, which is an aqueous dispersion of uncharged unilamellar liposomes at the phospholipid concentration < 2 wt.% [25,26]. Indeed, we did not observe a so-called correlation peak in the scattering intensity in Fig. 1 in the studied Q range.

According to the Guinier approximation for very small scattering angles [27,28], one can then rewrite Eq. (2) as:

$$I(Q) \sim \exp\left(-Q^2 R_g^2/r\right) Q^{r-3} \quad (3)$$

where R_g is the object radius of gyration and $r \approx 1$, 2 and 3 holds for infinite sheet-like objects; for rod-like objects of infinite length and uniform cross section; and for a globular object, respectively [29,30]. The value $r \approx 1$ is a good approximation also for polydisperse hollow spheres with radii substantially larger than the constant shell

thickness, such as unilamellar liposomes [31]. The approximation [Eq. (3)] is valid for finite size objects when $L^{-1} \leq Q \leq R_g^{-1}$ 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 ($0.032\ \text{Å}^{-1} \leq Q \leq 0.077\ \text{Å}^{-1}$) such as in Fig. 1 by using Eq. (2) and a non-linear least squares program for the series of 15 samples with the molar ratio in the interval of $0 \leq n$-decane/DOPC ≤ 5. In the three parameter fit [$I(0)$ and R_g unconstrained, r constrained to $r \geq 1$] we have obtained the value of r in the range $1 \leq r \leq 1.018$ with the maximum S.D. of 0.135. These results indicate, that the n-decane + DOPC unilamellar liposomes are stable in a broad range of n-decane content in the bilayer, or/and that the unilamellar liposomes disaggregated into sheet-like objects (e.g. discoid micelles with very large lateral dimensions) due to interaction with n-decane. The SANS method as used in the present paper cannot discriminate between these two possibilities.

It is well known [27,28] that the thickness of the two-dimensional planar sheet d_g can be obtained from the radius of gyration R_g as:

$$d_g^2 \cong 12 R_g^2 \tag{4}$$

Eq. (4) can be also used for the estimation of bilayer thickness in unilamellar liposomes dispersed in heavy water [25,32]. The values of R_g can be obtained from the Kratky–Porod plots of experimental data such as presented in Fig. 2. It is seen that these plots are approximately linear in the $0.001\text{-Å}^{-2} \leq Q^2 \leq 0.006\ \text{Å}^{-2}$ region; however, in some plots the first 1–3 experimental points showed slight deviations from linearity. Supposing that there is no water penetration inside the polar region of the bilayer and that the liposomes are spherical and polydisperse, the computer simulations of scattering curves have shown that such deviations from linearity can occur in the indicated Q^2 region when the mean liposome radius decreases below 200 Å [31]. The mean radius of DOPC liposomes extruded through a 500-Å filter should be larger than 200 Å, however, the presence of n-decane liposomes could affect their polydispersity with a larger contribution of smaller and larger liposomes. This could bring about slight deviations from linearity. The referee of the present paper suggested in his/her comments some liposome aggregation as another source of these small deviations. We have thus tested if omitting of first 1–3 points would affect the values R_g and d_g substantially. We have observed slight improvements of liner fits, but the values of R_g (and d_g) obtained coincided within experimental error. For example, linear fit of all points in Fig. 1b gave $R_g^2 = 133.8 \pm 4.0\ \text{Å}^2$ with correlation coefficient $r^2 = 0.988$. After omitting the first two points one obtains $R_g^2 = 127.4 \pm 3.5\ \text{Å}^2$ with correlation coefficient $r^2 = 0.991$. Careful inspection of all data has shown no better improvement of the fits as above. We have thus decided to treat the data in a uniform way and include in the evaluation all the experimental points observed in the interval of $0.001\ \text{Å}^{-2} \leq Q^2 \leq 0.006\ \text{Å}^{-2}$. Our computer simulations of scattering curves have further shown that the value of d_g obtained from the data in the region of $0.001\ \text{Å}^{-2} \leq Q^2 \leq 0.006\ \text{Å}^{-2}$ is approximately equal to the thickness of the lipid bilayer in spherical unilamellar polydisperse liposomes [31,33]. Furthermore, we have found experimentally that the thickness parameter d_g is a linear function of the transbilayer phosphate–phosphate distance in unilamellar phosphatidylcholine liposomes [34].

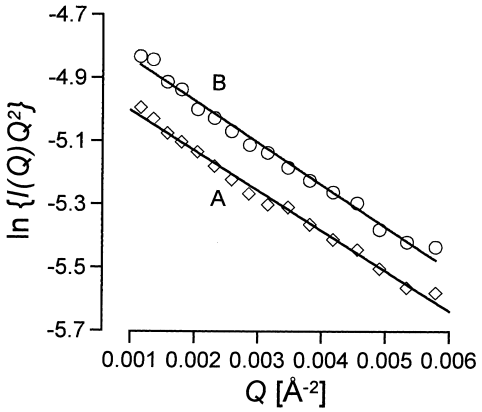

Fig. 2. Kratky–Porod plots of neutron scattering curves for extruded DOPC liposomes in the presence of n-decane at molar ratios n-decane/DOPC of 0.25:1 (curve A, diamonds) and 2:1 (curve B, circles).

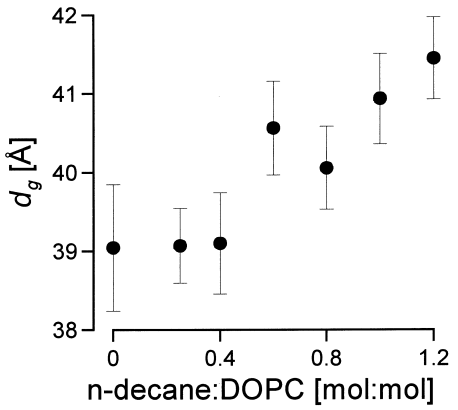

Fig. 3. Dependence of the DOPC bilayer thickness parameter d_g on the *n*-decane/DOPC molar ratio.

The bilayer thickness parameter d_g is thus a good measure of the bilayer thickness in unilamellar liposomes.

The dependence of d_g values obtained from the Kratky–Porod plots in the indicated Q region on the *n*-decane/DOPC molar ratio is shown in the Fig. 3. It is seen that *n*-decane has no effect within experimental error on the bilayer thickness up to *n*-decane/DOPC $\approx$ 0.5:1 molar ratio ('low molar ratio region'); and then it increases the bilayer thickness slightly. The change in d_g up to a 1.2:1 ratio is relatively small ($\sim 2.4 \pm 1.3$ Å) in comparison to that observed by McIntosh et al. [10] in lamellar phosphatidylcholine phases (~ 10 Å). We have measured the bilayer thickness also at higher molar ratios. Here the bilayer thickness increase was larger. For example, we have observed $d_g = 43.0 \pm 0.32$ Å, $d_g = 44.54 \pm 0.38$ Å and $d_g = 48.68 \pm 0.52$ Å at *n*-decane/DOPC molar ratios of 4, 4.5 and 5, respectively. The maximum change in the bilayer thickness with respect to the control sample is thus 9.64 ± 1.32 Å in the *n*-decane/DOPC molar ratio range studied.

It has been observed by using freeze-fracture and negative stain electron microscopy [35] that the morphology of bilayers containing *n*-tetradecane was different from bilayers containing *n*-hexane — while the smooth fracture faces of bilayers were unmodified by tetradecane, *n*-hexane affected the hydrophobic bilayer interior, producing large (20–50 nm) mounds and depressions in the fracture faces. The authors suggested that the long alkanes are primarily located between adjacent lipid hydrocarbon chains in each monolayer of the bilayer, while short alkanes can partition into the geometric center of the bilayer between opposing monolayers [35]. It is possible that *n*-decane, which has an intermediate length, changes its location in the bilayer of unilamellar DOPC liposomes in dependence on its concentration – in the 'low molar ratio region' it is located between DOPC acyl chains like the long alkanes, while in the 'high molar ratio region' it intercalates into the bilayer center between the opposing monolayers. This model could explain the dependence of the observed bilayer thickness on the *n*-decane concentration.

In conclusion, we have observed that the change of the DOPC bilayer thickness in extruded unilamellar liposomes is relatively small up to *n*-decane/DOPC = 1.2:1 molar ratio. This observation does not mean that the *n*-decane has no effect on the bilayer properties. Its location between the lipid acyl chains may affect, for example, the lipid surface area, the lateral diffusion of bilayer constituents and other bilayer dynamic properties. However, it is evident, that the change in the bilayer thickness is not the primary cause of the changes in the Ca-Mg–ATPase activity induced by *n*-decane as originally proposed by Johansson et al. [8]. The models of the anesthetic effect of normal alkanes should also take this into account.

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