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LIPID BILAYER THICKNESS IN EXTRUDED LIPOSOMES PREPARED FROM 1,2-DIACYLPHOSPHATIDYLCHOLINES WITH MONOUNSATURATED ACYL CHAINS: A SMALL–ANGLE NEUTRON SCATTERING STUDY*

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The thickness of the lipid bilayer affects protein properties in biological membranes. In the present paper, this thickness was studied in model bilayers in extruded unilamellar liposomes made of synthetic 1,2-diacyl-*sn*-glycero-3-phosphorylcholines with monounsaturated acyl chains (diCn:1PC, n = 14 - 24 is the number of acyl carbon atoms) at 30 °C using the small-angle neutron scattering (SANS). The experimental data were evaluated using the small–angle form of Kratky-Porod approximation $\ln[I(q)q^2]$ *vs.* q^2 of the SANS intensity $I(q)$ in the range of scattering vector values q corresponding to interval $0.14\ \text{nm}^{-2} < q^2 < 0.97\ \text{nm}^{-2}$ to obtain the bilayer gyration radius R_g taken perpendicularly to the bilayer, and the bilayer thickness parameter $d_g = 12^{0.5}R_g$. The dependence of this thickness parameter on the number of diCn:1PC acyl chain carbons was found to be linear with the slope 0.191 ± 0.013 nm *per* one acyl chain carbon. Using the known volumetric data and the known distances of diCn:1PC phosphate groups across the bilayer and from the Gibbs dividing surfaces between the hydrophobic and polar bilayer regions, the diCn:1PC surface area A at the bilayer – aqueous phase interface was obtained. The dependence of A on n displays a maximum at n=18.

Key words: *monounsaturated 1,2-diacyl-sn-glycero-3-phosphorylcholines – bilayer thickness – small-angle neutron scattering*

* Dedicated to Professor Dipl. Ing. K. Sarka, PhD. on the occasion of his 60th birthday

INTRODUCTION

Unilamellar liposomes are widely used in various pharmaceutical and cosmetic formulations as vehicles for delivery of drugs, DNA or proteins through cell membranes [1-4]. The phospholipid bilayer of unilamellar liposomes is also a convenient model of the lipid bilayer part of biological membranes [5]. The thickness of the lipid bilayer is an important physical parameter affecting strongly the properties of transbilayer polypeptides and proteins. It has been observed that the insertion and orientation of small polypeptides in the bilayer of liposomes prepared from 1,2-diacylphosphatidylcholines with monounsaturated acyl chains (diCn:1PC, n = 14 – 24 is the number of acyl chain carbon atoms) and the activity of several integral membrane proteins reconstituted into such bilayers depend critically on the diCn:1PC acyl chain length n ([6-11] and references therein). The mismatch between lengths of transmembrane segments of membrane proteins and bilayer thickness can affect thus protein sorting [12], protein translocation [13], and protein conformation [14] or lateral aggregation [15,16] in biological membranes.

In the present paper we study the thickness of bilayer in extruded unilamellar liposomes prepared from the diCn:1PC (n=14-24) homologues using the small-angle neutron scattering (SANS) method, and compare the values obtained with the data of Lewis and Engelman [17] who studied the bilayer thickness in ultrasonicated diCn:1PC (n=18,22,24) liposomes using the small-angle X-ray scattering (SAXS) method.

MATERIAL AND METHODS

Chemicals

The phospholipids 1,2-dimyristoleoyl-*sn*-glycero-3-phosphorylcholine (diC14:1PC), 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphorylcholine (diC16:1PC), 1,2-dioleoyl-*sn*-glycero-3-phosphorylcholine (diC18:1PC), 1,2-diecosenoyl-*sn*-glycero-3-phosphorylcholine (diC20:1PC), 1,2-dierucoyl-*sn*-glycero-3-phosphorylcholine (diC22:1PC) and 1,2-dinervonoyl-*sn*-glycero-3-phosphorylcholine (diC24:1PC) were purchased from Avanti Polar Lipids (Alabaster, USA) and heavy water (99.9 % ^2H_2O) from Isotec (Matheson, USA).

Sample preparation

The dry lipid powder was dispersed in heavy water at the 1 wt. % concentration. The dispersions were sonicated in a bath sonicator and homogenized by vortex mixing. The multilamellar liposomes were extruded according to [18] through two stacked polycarbonate filters (Nucleopore, Plesanton, USA) with pores of diameter 50 nm mounted in the LiposoFast Basic extruder (Avestin, Inc., Canada) fitted with two gas-tight Hamilton syringes (Hamilton, Reno, USA). Each sample was subjected to 25 passes through the filters at about 30 °C. The final phospholipid concentration was

≤ 1 wt. % in all samples because some amount of lipid could remain in the extruder. The samples were filled into 1 mm quartz cells (Hellma, Müllheim, Germany), closed and stored at room temperature. As the reference sample, the same cell containing heavy water was used. The maximum period between the sample preparation and its measurement was 5 hours.

SANS measurements and evaluation

The neutron scattering experiments were performed on the PAXE spectrometer [19] located at the end of the G5 cold neutron guide of the Orphée reactor (Laboratoire Léon Brillouin, CEA Saclay, France). The spectrometer is equipped with a two-dimensional 64 × 64 channels detector. In this work, the experiments were performed with the sample to detector distances D of 1700 mm and 5000 mm and the neutron wavelength of λ = 6 Å. The sample temperature was set and controlled electronically at 30.0 ± 0.1 °C. The acquisition time for one sample was 40 minutes. The normalized SANS intensity $I(q)$ as a function of the value of scattering vector $q = 4\pi\sin\theta/\lambda$, where 2θ is the scattering angle, was calculated using the equation

$$I(q) = F(\lambda)\left[\frac{I_s(q)}{T_s(q)t} - \frac{I_r(q)}{T_r(q)t} - I_{inc}(q)\right], \tag{1}$$

where $I_i(q)$ is the intensity and $T_i(q)$ the transmission measured with the sample ($i = s$) and with the reference sample ($i = r$), t is the sample thickness, $I_{inc}(q)$ – the intensity of incoherent background and $F(\lambda)$ the normalization factor. The normalization factor is

$$F(\lambda) = \frac{\Omega^2}{I_0(\lambda)}, \tag{2}$$

where Ω is the solid angle that corresponds to a cell of the detector. Because the area of each cell is equal to 1 cm^2, $\Omega = 1 / D^2$, with D expressed in cm. The intensity of the incident beam, $I_0(\lambda)$, is given by

$$I_0(\lambda) = A\Phi(\lambda)E(\lambda) \tag{3}$$

for the incident beam flux Φ of cross section A and the efficiency of the detector E. It was evaluated by multiplying the intensity of the incident beam attenuated using a slab of plexiglas of 4 mm thickness by the ratio of the intensities scattered by a block of graphite measured with and without the attenuator and extrapolated to $q \to 0$. The transmission measurements were performed with the same attenuator.

The experimentally observed $I(q)$ for monodisperse system of spherical particles is given by

$$I(q) \sim N_P P(q) S(q), \quad (4)$$

where N_P is the number of particles, $P(q)$ is the particle structure factor and $S(q)$ the interparticle structure factor [20,21]. The interparticle factor is equal to one for electrostatically neutral spherical liposomes at the lipid concentrations ~ 1 wt. % [22], so the $I(q)$ value is dominated by the particle structure factor of liposomes. The liposomes extruded through 50 nm pores are spherical and have a broad distribution of radii [18,23,24]. When their steric bilayer thickness d_S is small as compared to their radii, their neutron scattering can be approximated by the scattering on randomly oriented flat sheets having the same thickness [25,26]. For values of the scattering vector $\pi/R < q < 2\pi/d_S$, it is then possible to evaluate the experimental data by the small–angle form of Kratky-Porod approximation [27-29]

$$I(q) \sim q^{-2} \exp(-q^2 R_g^2), \quad (5)$$

where R_g is the flat sheet radius of gyration taken perpendicularly to the sheet surface. It can be used to obtain the bilayer thickness when using an appropriate model of the coherent scattering length density distribution in the bilayer [26,30]. In case of two-dimensional planar thin sheet with a homogeneous scattering density inside ($\rho_i(z) = \text{const}$) as well as outside (ρ_o) the sheet, the sheet thickness parameter d_g is obtained from R_g as

$$d_g^2 \cong 12 R_g^2 \quad (6)$$

under condition of sufficient contrast ($\rho_i \neq \rho_o$) between the inside and outside of the sheet, what is fulfilled for liposomes dispersed in heavy water [27-29]. When the lower limit of liposome radii is 2π nm, and the maximum bilayer thickness is 2π nm, one gets the limits of scattering vector values $0.5\ \text{nm}^{-1} < q < 1\ \text{nm}^{-1}$. After a careful inspection of the measured $I(q)$ curves we have set the limits of q to $0.372\ \text{nm}^{-1} < q < 0.985\ \text{nm}^{-1}$ where the simple method of data evaluation based on the Kratky-Porod approximation can be safely used for liposomes extruded through 50 nm pores.

RESULTS AND DISCUSSION

Typical Kratky-Porod plots of experimental values observed are shown in Fig. 1. From such plots, the squares of gyration radii R_g^2 were obtained using equation 5 and a non-linear least-squares fitting method (Table 1). From the values of R_g^2 obtained, the thickness parameters d_g were calculated using equation 6 (Table 1). When plotted as a function of diCn:1PC acyl chain length n, it is seen that the d_g increase with n can be approximated by a linear function (Fig. 2). Using the least squares fitting, we have obtained $d_g = (0.50 \pm 0.26) + (0.191 \pm 0.013) \cdot$ n (in nanometers) with correlation coefficient $r^2 = 0.98$

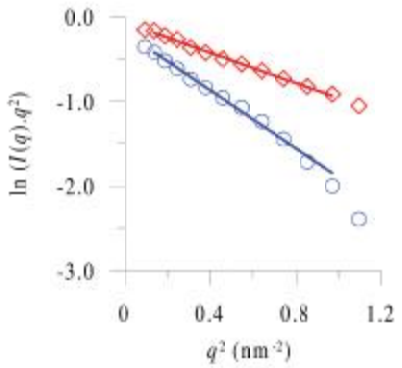

Fig. 1. Kratky-Porod plots of SANS intensity $I(q)$ for unilamellar diC14:1PC (○) and diC22:1PC (○) liposomes

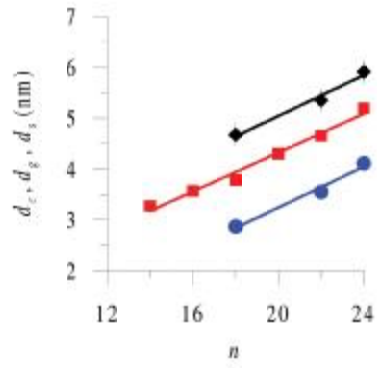

Fig. 2. Dependence of bilayer thickness parameters d_g (■), d_c (●) and d_s (♦) on diCn:1PC acyl length n at 30 °C

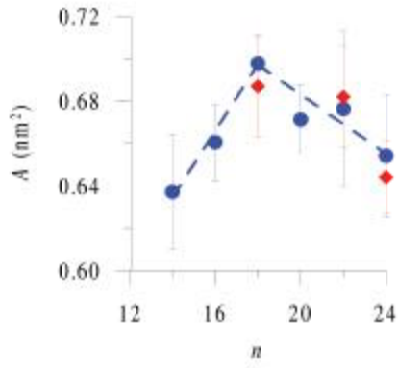

Fig. 3. Dependence of diCn:1PC surface area A on acyl chain length n at 30 °C: SANS (●), SAXS (♦)

Table 1. Structural parameters of diCn:1PC bilayers; the values of R_g^2, d_g, $V_{C,30}$, A_{30} and $d_{C,30}$ are at 30 °C and the values of d_{HH}, d_{HHc}, d_C, V_C and A at at 20 °C (#), 24 °C ($) and 36 °C (*); the t_c data were taken from [32-34]

n	T_c (°C)	R_g^2 (nm²)	d_g (nm)	d_{HH} (nm)	d_{HHc} (nm)	d_C (nm)	$V_{C,30}$ (nm³)	V_C (nm³)	A (nm²)	A_{30} (nm²)	$d_{C,30}$ (nm)	d_s (nm)
14	<-40	0.893±0.020	3.27±0.04				0.7579					
16	-38	1.063±0.016	3.57±0.03				0.8711					
18	-17.3	1.193±0.013	3.78±0.02	3.80±0.1[#]	3.90±0.1[#]	2.92±0.10[#]	0.9843	0.9739[#]	0.667±0.023[#]	0.687±0.024	2.87±0.10	4.67±0.22
20	-4.3	1.541±0.022	4.30±0.03				1.0975					
22	13.2	1.808±0.058	4.66±0.08	4.50±0.1[$]	4.60±0.1[$]	3.62±0.10[$]	1.2107	1.2034[$]	0.665±0.019[$]	0.682±0.024	3.55±0.10	5.35±0.22
24	26.7	2.250±0.059	5.20±0.07	4.85±0.1[*]	4.95±0.1[*]	3.97±0.10[*]	1.3239	1.3318[*]	0.671±0.017[*]	0.644±0.017	4.11±0.11	5.91±0.23

We will compare these data with the result of Lewis and Engelman [17]. They estimated the phosphate-phosphate separation d_{HH} across the bilayer from the position of the first positive non-origin peak in the Patterson function after inversion of the continuous small-angle X-ray scattering (SAXS) function of diC18:1PC, diC22:1PC and diC24:1PC liposomes. Their original d_{HH} values are collected in Table 1. Nagle and Tristram-Nagle [31] have shown that the values of d_{HH} obtained in [17] were underestimated by ~ 0.08 - 0.1 nm due to systematic truncation errors because the Patterson inversion of SAXS function was done in a limited range of scattering vector values. The phosphate-phosphate separations corrected for the truncation error, d_{HHc}, are shown in Table 1. The bilayer hydrophobic region thickness d_C, the lipid surface area A at the bilayer - aqueous phase interface and the steric bilayer thickness d_s can be calculated as

$$d_C = d_{HHc} - 2d_{HI} \tag{7}$$

$$A = 2V_C / d_C = 2(V_L - V_H) / d_C \tag{8}$$

$$d_s = d_C + 2d_H \tag{9}$$

under condition that the phosphate – hydrophobic region distance d_{HI}, the diCn:1PC molecular volume V_L, the volume of its polar headgroup (including acyl carbonyl groups) V_H, and the thickness of bilayer polar region d_H are known. For phosphatidylcholines with both saturated and unsaturated acyl chains, $d_{HI} = 0.49$ nm and $V_H = 0.319$ nm^3 can be used independent of temperature (see chapter 13 and Table 6 in [31]), the other parameters in equations 7 – 9 are temperature dependent. Lewis and Engelman [17] estimated the phosphate-phosphate separation d_{HH} in diC18:1PC, diC22:1PC and diC24:1PC liposomes at 20 °C, 24 °C and 36 °C, respectively, while our experiments were performed at 30 °C; we have corrected thus their data in Table 1 for temperature effects. First, we calculated the diCn:1PC hydrophobic volumes at 30 °C, $V_{C,30}$, from the known volumes $V_L = 0.13033$ nm^3 of diC18:1PC [35] and $V_H = 0.319$ nm^3 [31] by simply adding an appropriate number of methylene group volumes $V(CH_2) = 0.0283$ nm^3 (J. F. Nagle, personal communication). Second, the hydrophobic volumes V_C of diC18:1PC, diC22:1PC and diC24:1PC at 20 °C, 24 °C and 36 °C, respectively, were calculated using the thermal volume expansion coefficient

$$\alpha = \partial(\ln V_L) / \partial T \tag{10}$$

where T is the absolute temperature. For this correction, the value $\alpha = 0.008$ K^{-1} found experimentally for diC18:1PC [35] was used for the all diCn:1PC homologues. Third, the surface areas A of diC18:1PC, diC22:1PC and diC24:1PC at 20 °C, 24 °C and 36 °C, respectively, were calculated using equation 8 and the V_C volumes and d_C thicknesses obtained at these temperatures (Table 1). Fourth, these surface areas were corrected for temperature effects using the thermal area expansion coefficient

$$\beta = \partial(\ln A) / \partial T \qquad (11)$$

For diC1:18PC liposomes studied well above the gel – fluid phase transition temperature t_c (see Table 1), the value $\beta = 0.003$ K^{-1} was used as in [31]. Larger values of $\beta = 0.00417$ K^{-1} and $\beta = 0.00681$ K^{-1} were obtained in giant unilamellar 1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine (diC14:0PC) liposomes 5 K and 11 K above t_c, respectively [36,37]. Since no other β values for phosphatidylcholines near the t_c temperature are available, we used $\beta = 0.00417$ K^{-1} for diC22:1PC and $\beta = 0.00681$ K^{-1} for diC24:1PC to obtain the corrected surface areas A_{30} at 30 °C. Fifth, the bilayer hydrophobic region thickness $d_{C,30}$ at 30 °C was obtained from the $V_{C,30}$ and A_{30} values using equation 8. Finally, the steric bilayer thickness d_s was calculated (equation 9) supposing that the thickness of the bilayer polar region is $d_H = 0.90 \pm 0.12$ nm. This value was obtained from results of neutron diffraction studies of specifically deuterated 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (diC16:0PC) in oriented multibilayers [31,38-40].

The diCn:1PC bilayer thickness parameters d_C, d_g and d_s obtained at 30 °C as described above are compared in Fig. 2. It is seen that $d_C < d_g < d_s$, i.e. the evaluation of SANS data by using the simple Kratky-Porod approximation (equations 5 and 6) yields intermediate bilayer thickness values between the steric and hydrophobic thicknesses. When fitted by linear functions, the slopes of both d_C and d_s *vs.* acyl chain length n dependencies are $\partial d_C / \partial n = \partial d_s / \partial n = 0.201 \pm 0.027$ nm, within experimental error the same as $\partial d_g / \partial n = 0.191 \pm 0.013$ nm. The linear correlation ($r^2 = 0.99$) between d_g obtained from SANS data and d_C evaluated from SAXS results strongly supports the use of d_g in studies of relative bilayer thickness changes, e.g. under influence of various amphiphilic and hydrophobic drugs [41-44]. This linear correlation can also be used to obtain the d_C values from experimental d_g data and to calculate the surface areas A for all diCn:1PC homologues studied by using equation 8. The results of this calculation are shown in Fig. 3 and compared with the SAXS results corrected for temperature effects. For diC18:1PC, the results of three different methods $A = 0.687 \pm 0.024$ nm^2 (SAXS), $A = 0.698 \pm 0.013$ nm^2 (SANS) and $A = 0.722 \pm 0.011$ nm^2 small-angle X-ray diffraction (SAXD) study of fully hydrated diC18:1PC in multilamellar liposomes [35]) coincide within experimental errors at $A = 0.711$ nm^2, close to $A = 0.694 \pm 0.012$ nm^2 found by SAXD for fully hydrated multilamellar liposomes prepared from natural hen egg phosphatidylcholine [45] which average acyl chain length is 17.8 carbon atoms with 1.2 double bonds – i.e. diC17.8:1.2PC [46]. Even though the experimental errors of A are relatively large, it is seen in Fig. 3 that A increases with the diCn:1PC acyl chain length up to $n = 18$ carbon atoms and than decreases till $n = 24$. The value of lipid surface area A is the result of attractive and repulsive forces acting at the aqueous phase - bilayer interface. The main attractive components are the van der Waals forces between acyl chains and headgroup dipolar interactions, and the main repulsive components include steric interactions, hydration forces, and entropic contributions due to acyl chain ordering; the equilibrium area A is thus given by the balance of these forces that minimizes the interfacial free energy. At constant temperature, the increase of acyl chain length will increase the van der Waals attraction and this will reduce the A value.

However, the reduction of A will result at the same time in the reduction of *gauche* conformers formation in chains, decreasing thus the chain disorder, i.e. the entropy. These two effects which act in opposite directions could cause the peculiar dependence of A in diCn:1PC bilayers observed. The decrease of A with the length of acyl chain has been observed earlier in bilayers prepared from 1,2-diacyl-*sn*-glycero-3-phosphorylcholines with saturated acyl chains (diCn:0PC) in multilamellar liposomes in the solid-like gel state for $n = 16 - 18$ by SAXD [47] and in the fluid state above t_c for $n = 12 - 18$ by ^{2}H NMR [48,49] and in unilamellar liposomes in the fluid state above t_c for $n = 10 - 18$ by SANS [30]. The specific dependence of A in diCn:1PC in comparison to diCn:0PC could be caused by the presence of the double bond in the diCn:1PC acyl chains; it is well known that this reduces the chain melting phase transition temperature t_c significantly [5].

In conclusion, we have found using SANS that the bilayer thickness parameter d_g increases linearly with the acyl chain length in unilamellar diCn:1PC ($n = 14 - 24$) liposomes at 30 °C, while the lipid surface area A shows a maximum at $n = 18$. Since the polypeptides and membrane proteins are incorporated frequently into bilayers made of lipids with unsaturated chains and because the cell membranes contain phospholipids with unsaturated chains, besides the bilayer thickness also the lateral bilayer parameters such as phospholipid surface area A on polypeptide and protein properties should be taken into account.

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HRÚBKA LIPIDOVEJ DVOJVRSTVY V EXTRÚDOVANÝCH LIPOZÓMOCH PRIPRAVENÝCH Z 1,2-DIACYLFOSPHATIDYLCHOLÍNOV S MONONENASÝTENÝMI ACYLOVÝMI REŤAZCAMI ŠTUDOVANÁ POMOCOU MALOUHLOVÉHO ROZPTYLU NEUTRÓNOV

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Hrúbka lipidovej dvojvrstvy v extrúdovaných unilamelárnych lipozómoch pripravených zo syntetických 1,2-diacyl-*sn*-glycero-3-fosforylcholínov s mononenasýtenými acylovými reťazcami

(diCn:1PC, n = 14 - 24 je počet acylových uhlíkov) bola sledovaná pri 30 °C pomocou malouhlového rozptylu neutrónov (SANS). Experimentálne údaje sa vyhodnotili malouhlovou aproximáciou SANS intenzity $I(q)$ podľa Kratkeho-Poroda $\ln[I(q)q^2]$ *vs.* q^2 v oblasti hodnôt rozptylového vektora q zodpovedajúcej intervalu $0.14\ nm^{-2} < q^2 < 0.97\ nm^{-2}$. Zo získaných hodnôt gyračného polomeru R_g v kolmom smere k dvojvrstve sa určil parameter hrúbky dvojvrstvy ako $d_g = 12^{0.5}R_g$. Zistili sme, že tento parameter je lineárnou funkciou počtu atómov uhlíka v acylovom reťazci diCnPC so smernicou 0.191 ± 0.013 nm na jednu metylénovú skupinu. Zo známych vzdialeností fosfátových skupín diCn:1PC naprieč dvojvrstvou a od Gibbsovej roviny oddeľujúcej polárnu a hydrofóbnu oblasť dvojvrstvy a zo známych hodnôt parciálnych mólových objemov diCn:1PC v dvojvrstve sa vypočítal plošný obsah A pripadajúci na jednu molekulu diCn:1PC na medzifázovom rozhraní dvojvrstva – vodná fáza. Závislosť A od n vykazuje maximum pre n=18.

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