acta physica slovaca vol. 51 No. 1, 53 – 68 **February 2001**

EVALUATION OF SMALL-ANGLE NEUTRON SCATTERING CURVES OF UNILAMELLAR PHOSPHATIDYLCHOLINE LIPOSOMES USING A MULTISHELL MODEL OF BILAYER NEUTRON SCATTERING LENGTH DENSITY

P. Balgavý [1]
Department of Physical Chemistry, Faculty of Pharmacy, J. A. Comenius University, Odbojárov 10, 832 32 Bratislava, Slovakia

N. Kučerka
Department of Chemical Theory of Drugs, Faculty of Pharmacy, J. A. Comenius University, Kalinčiakova 8, 832 32 Bratislava, Slovakia

V. I. Gordeliy[ab], **V. G. Cherezov**[b]
(a) IBI-2: Structural Biology, Research Centre Juelich, 52425 Juelich, Germany
(b) Frank's Laboratory of Neutron Physics, JINR, 141980 Dubna, Russia

Received 10 October 2000, in final form 15 Decembar 2000, accepted 15 December 2000

Computer simulated small-angle neutron scattering (SANS) curves of spherical polydisperse extruded unilamellar liposomes from saturated 1,2-diacylphosphatidylcholines in the aqueous phase are evaluated by using a multishell model, which divides the lipid bilayer of liposomes into the polar head group region, and the nonpolar hydrocarbon region consisting of the chains of methylene groups and of the region of methyl groups. In the each of these regions, the coherent neutron scattering length density is supposed to be homogeneous. The evaluation is based on obtaining of radius of gyration from the Kratky-Porod plot of SANS data in the Guinier region of small scattering vector values. From radii of gyration obtained at several different molar fractions $N_{\mathrm{D_2O}}/(N_{\mathrm{D_2O}} + N_{\mathrm{H_2O}})$ in the aqueous phase (contrasts) and independent volumetric data, the lipid surface area A_{L} (or the bilayer thickness d_{L}) and the number of water molecules N_{L} penetrated into the bilayer polar region can be evaluated. Using this method and the SANS curves of unilamellar 1,2-dimyristoylphosphatidylcholine (DMPC) liposomes measured at 30 °C and contrasts $N_{\mathrm{D_2O}}/(N_{\mathrm{D_2O}} + N_{\mathrm{H_2O}})$=1.0, 0.8, 0.6 and 0.4 the values d_{L}=42.6 ± 0.5 Å^3 and A_{L}=62.0 ± 0.7 Å^2 at N_{L}=7.3 were obtained.

PACS: 87.16.Dg, 87.64.Bx, 82.70.Uv, 83.80.Qr

[1] E-mail address: pavol.balgavy@fpharm.uniba.sk

1 Introduction

The study of the biological membranes is one of the most popular parts of biophysics. Its questions consist of the determination of membrane components, their physical properties and interactions, determination of solvent effects and changes of their physical properties due to some admixtures, e.g. drug molecules. Because the biological membranes are very complex objects, it is convenient to study their physical properties by using model systems. The most popular models of the phospholipid part of biological membranes are various mesomorphic phases, which form from phospholipids in contact with the aqueous phase [1, 2]. The lamellar phases of phospholipids wherein stacked phospholipid bilayers are separated by layers of the aqueous phase closely resemble phospholipid bilayers of biological membranes. Phospholipids are amphiphilic molecules composed of hydrophilic part consisting of the lipid polar head group, and of hydrophobic part - long hydrocarbon chains. In the bilayer, the hydrophobic part is closed inside of bilayer and the hydrophilic part is localized on the interface with the aqueous phase. The lamellar phases convert spontaneously into multilamellar liposomes at increased water content [3, 4]. Unilamellar liposomes produced from multilamellar liposomes are hollow spheroidal particles consisting of single phospholipid bilayer shell with the aqueous phase inside and outside of this shell.

Basic physical parameters of these systems are the thickness of the phospholipid bilayer, d_{L}, the surface area per lipid on the bilayer-aqueous phase interface, A_{L}, and the number of water molecules per lipid penetrated into the polar region of the bilayer, N_{L}. The simplest method of obtaining d_{L} and/or A_{L} is the evaluation of small-angle X-ray diffraction (SAXD) or small-angle neutron diffraction (SAND) on lamellar phases of phospholipids. Usually, the experimentally measured scattering intensity function is separated into a structure factor and a form factor which depend on a scattering vector $\boldsymbol{Q}$. Knoll *et al* [5] were the first who realized that one can use the Guinier approximation of the small-angle neutron scattering (SANS) curve for obtaining the bilayer thickness in unilamellar liposomes in the small region of scattering vector values $\boldsymbol{Q}$. In the Guinier approximation, the scattering intensity for planar two-dimensional sheets can be written as function of the scattering vector value

$$I(Q) = I(0) \cdot Q^{-2} \cdot \exp(-R_{\mathrm{t}}^2 Q^2), \tag{1}$$

where R_{t} is the radius of gyration of the sheet characterizing the thickness of the sheet d_{t} by equation

$$d_{\mathrm{t}}^2 \cong 12 \cdot R_{\mathrm{t}}^2. \tag{2}$$

The radius of gyration is defined as

$$R_{\mathrm{t}}^2 = \frac{\int \rho(r) r^2 \mathrm{d}r}{\int \rho(r) \mathrm{d}r} \tag{3}$$

[6, 7]. However, this method is explicitly applicable only in the case of planar phospholipid bilayer which thickness is small in comparison with its lateral dimensions, i.e.

$$2\pi / S^{1/2} \leq Q \leq 1/R_{\mathrm{t}} \tag{4}$$

where S is the total area of the bilayer [8]. Its application to the liposome bilayer, which is curved, is thus questionable. Komura *et al.* [9] supposed, that unilamellar liposomes are spherical and

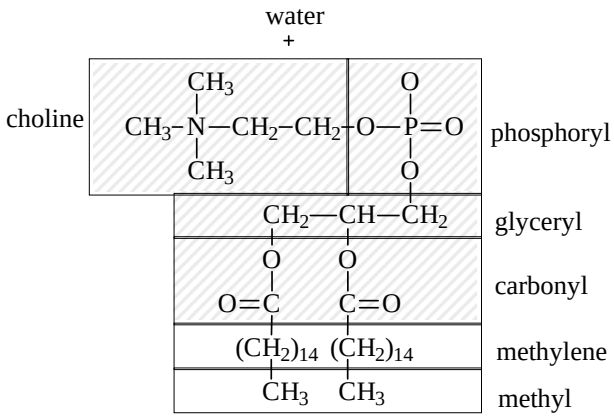

Fig. 1. Chemical structure of 1,2-dipalmitoylphosphatidylcholine. The shaded area is the polar head group region.

polydisperse with respect to their radii. They further supposed that the neutron scattering length density in the bilayer could be taken as homogenous when the liposomes were dispersed in heavy water. The single shell model has been recently used also by other groups of authors [4, 10-12]. However, the single shell model neglects the inner structure of the bilayer and divides the system into the bilayer region and excess water only. The more realistic model is a multishell model, which divides the bilayer into several small homogenous parts. Fig. 1 shows the chemical composition of one simple phospholipid molecule – 1,2-dipalmitoylphosphatidylcholine (DPPC). The basic parts of this phospholipid molecule are the polar head group region displayed by shaded area, and the nonpolar hydrocarbon region consisting of the chains of methylene groups (CH_2) and of the region of methyl groups (CH_3). In the bilayer, some limited number of water molecules can penetrate the head group region. Such a model was used in [13] for the interpretation SAND experiments on the oriented lamellar phospholipid phases.

In the present paper we develop this model for the interpretation of SANS data of unilamellar phosphatidylcholine liposomes. We extract the thickness of the phospholipid bilayer, the surface area per lipid on the bilayer-aqueous phase interface and the number of water molecules penetrated into the polar region of the bilayer from computer simulated SANS curves. We use this method for the evaluation of experimental SANS data of Cherezov [14] obtained with unilamellar 1,2-dimyristoylphosphatidylcholine (DMPC) liposomes.

2 Scattering of neutrons on unilamellar liposomes

The experimentally observed scattering intensity, the coherent part of the differential cross section, is for monodisperse system given by

$$I(\boldsymbol{Q}) = N_{\mathrm{P}} \cdot P(\boldsymbol{Q}) \cdot S(\boldsymbol{Q}), \tag{5}$$

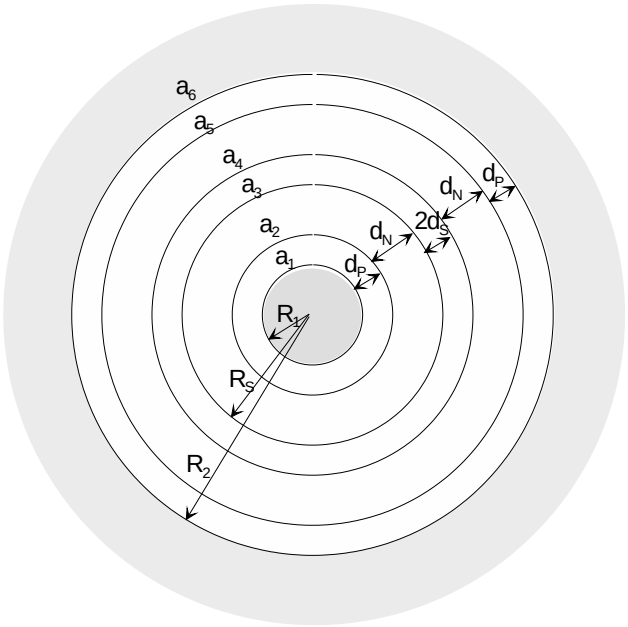

Fig. 2. Multishell model of unilamellar liposome. The shaded area is the aqueous phase.

where N_{P} is the number of particles, $P(\boldsymbol{Q})$ is the particle structure factor and $S(\boldsymbol{Q})$ is the interparticle structure factor. $S(\boldsymbol{Q})$ is approximately equal to 1 for dilute and weakly interacting spherical system, what is an aqueous dispersion of uncharged unilamellar liposomes at the phospholipid concentration $\leq$2 wt. % [15]. Particle structure factor $P(\boldsymbol{Q})$ is equal to the square of form factor, which is the one-dimensional Fourier integral of the scattering length density for the centrosymmetric particles ($F(\boldsymbol{Q}) = F(Q)$)

$$F(Q) = -4\pi \int_0^\infty r^2 \rho(r) \frac{\sin(Qr)}{Qr} \mathrm{d}r, \tag{6}$$

where the integration is over the whole space [16]. If one wants to take only the part of neutron scattering on the particle, needs to subtract the background and then to integrate only over the space of particle.

We suppose that each of the two monolayers in the bilayer in unilamellar liposomes consists of three shells. Polar head group shell is characterized by the thickness d_{P} and the nonpolar hydrocarbon shell consisting of methylene and methyl subshells is characterized by thicknesses d_{N} and d_{S}, respectively (Fig. 2). The bilayer thickness is then $d_{\mathrm{L}} = 2d_{\mathrm{P}} + 2d_{\mathrm{N}} + 2d_{\mathrm{S}}$. Another model parameter is the neutron scattering length density, ρ_i, different for each shell of the bilayer. The Fourier integral is thus divided into the integrals over the bilayer shells. Then the scattering

intensity without the background is given by equations

$$I(Q) = N_{\mathrm{P}} \cdot \left(\frac{4\pi}{Q^3}\right)^2 \left\{\sum_{i=1}^{6} \Delta\rho_i \cdot (A_{i+1} - A_i)\right\}^2$$
$$A_i = Q \cdot a_i \cdot \cos(Q \cdot a_i) - \sin(Q \cdot a_i), \tag{7}$$

where $\Delta\rho_i = \rho_w - \rho_i$ are the contrast values of neutron scattering length densities against aqueous phase for the individual shells, and $a_1, a_2, a_3...$ are borders of these shells (see Fig. 2). These equations are valid for monodisperse systems. However, experimentally studied unilamellar liposomes have some degree of polydispersity of the outer radius of liposome R_2 at constant values of thickness parameters d_{P}, d_{N}, d_{S}. Komura *et al.* [9] suggested, that this polydispersity may be described by a Gaussian distribution function in the form

$$f(R_2) = \frac{1}{\sqrt{2\pi} \cdot \sigma_{\mathrm{R}}} \exp\left[-\frac{(R_2 - R_{2,mean})^2}{2\sigma_{\mathrm{R}^2}}\right], \tag{8}$$

where $R_{2,mean}$ is the mean outer radius of liposomes, and the polydispersity of liposome sizes is expressed in the size distribution σ_{R}. Finally, the scattering intensity of this system can be evaluated by summing over all individual intensities obtained from equation (7), weighted by the distribution function (8).

3 Results and discussion

3.1 Simulations

The first aim of our study was to test the method using the computer simulated data. We have thus simulated the neutron scattering intensity for different values of liposome parameters to determine their influences. We have used the values from 200 Å to 500 Å for the mean outer liposome radius $R_{2,mean}$ and the number of methylene CH_2 groups in hydrocarbon chain from 10 to 24. These values correspond to unilamellar liposomes prepared from saturated 1,2-diacylphosphatidylcholines by extrusion through polycarbonate filters. The polydispersity of extruded liposomes is described by the size distribution σ_{R}. We have supposed, that the lipid area on the phospholipid bilayer-aqueous phase interface may take values from 58 Å^2 to 64 Å^2 per one lipid molecule, as found recently by using the SANS [17] and SAXS [18], respectively, on the fluid lamellar phase of DPPC at about 50 °C. We suppose thus that the bilayers we simulate are in the fluid state. The number of water molecules penetrated into the polar bilayer shell has been varied between 0 and 30 molecules per one lipid molecule. The other values of simulation parameters needed are the neutron scattering length densities and volumes of different phospholipid fragments. The values published in the literature [18-31] are collected in the Tables I and II. We have used the values marked with bold letters in our simulations.

Table I. Volumetric data for phosphatidylcholine bilayers.

	V[Å^3]	Ref.
H_2O	29.9	[19]
D_2O	30.0	[20]
	30.4	[17]
Headgroup region	360	[21]
	321	[22]
	319	[23]
	325	[24]
	348	[25]
	326	[20]
CH_2	27.0	[21]
	28.1	[22]
	26.9	[19]
	28.0	[20]
CH_3	54.0	[21]
	52.7	[22]
	54.3	[19]
	53.6	[20]
DPPC	1212	[22]
	1218	[17]
	1218	[26]
	1232	[18]

Table II. Values of scattering amplitudes for 1,2-diacylphosphatidylcholine bilayers.

	b[10^{-4}Å]	Ref.
H	**-0.374**	[27]
	-0.3739	[28-30]
	-0.38	[31]
D	**0.667**	[27]
	0.6671	[28-30]
	0.65	[31]
C	**0.665**	[27]
	0.6646	[28-30]
	0.66	[31]
N	**0.940**	[27]
	0.9360	[28-30]
	0.94	[31]
O	**0.580**	[27]
	0.5803	[28-30]
	0.58	[31]
P	**0.517**	[27]
	0.5130	[28-30]
	0.51	[31]

In the first part of our simulations we have supposed that the liposomes are dispersed in the aqueous phase consisting of 100% D_2O. Scattering functions obtained by equation (7) for monodisperse liposomes are oscillating as shown in Fig. 3. It is seen in the curves of ln[$I(Q)Q^2$] vs. Q^2 convoluted by the Gaussian distribution function (8) that the oscillations due to liposome radius are smeared and that the curves can be approximated by linear functions in the region of small Q values. We have selected the region of 0.001 Å^{-2} $\leq$ Q^2 $\leq$0.006 Å^{-2} values for this approximation. By fitting the simulated curves in this region, we have obtained the slopes of ln[$I(Q)Q^2$] vs. Q^2 dependencies. In analogy to the equation (1) valid for planar sheets, the roots of the absolute values of these slopes are called radii of gyration R_g. However, it must be stressed that R_g is not the gyration radius of the bilayer, it is just the parameter of the linear function approximating the convoluted scattering curve in the selected range of Q values. The value of R_g obtained from simulated curves is sensitive to the polydispersity of liposomes and its uncertainty is large in case of small σ_{R} values. This is demonstrated in Fig. 4 which shows the dependence of R_g^2 on the ratio of σ_{R} used in simulations to the $\sigma_{\mathrm{R,P}}$ value calculated from the parabolic relation between the mean outer liposome radius and the distribution parameter obtained from experimental data in [4]. It is seen that this effect is insignificant for $\sigma_{\mathrm{R}} > 0.7\sigma_{\mathrm{R,P}}$ and we will use therefore $\sigma_{\mathrm{R}} = \sigma_{\mathrm{R,P}}$ corresponding to experiments in the following simulations. We have also studied the relation between the value of R_g obtained from simulated curves and the

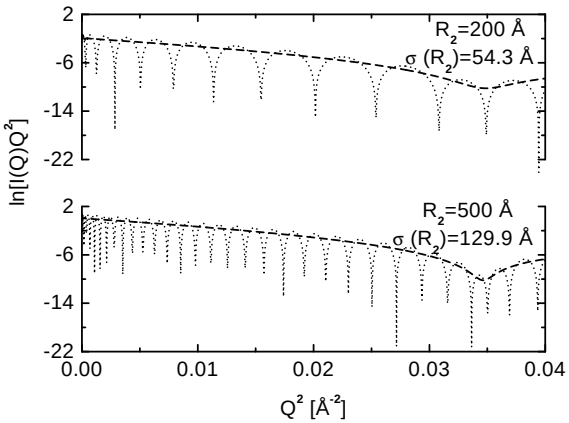

Fig. 3. Scattering curves of unilamellar liposomes without (full lines) and with (dashed lines) convolution by a Gaussian distribution function calculated for lipid area $A_{\mathrm{L}} = 62.9$ Å^2, number of water molecules $N_{\mathrm{L}} = 10$, the lipid bilayer thickness $d_{\mathrm{L}} = 48.4$ Å and mean outer liposome radii 200 and 500 Å.The values of σ_{R} were obtained from the parabolic dependence on $R_{2,mean}$ published in [4].

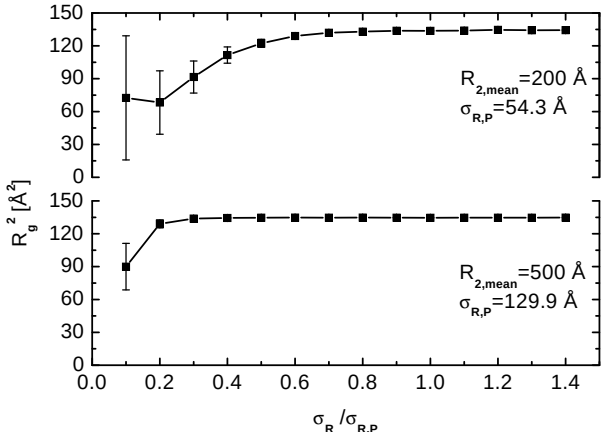

Fig. 4. Dependencies of R_g^2 values on the ratio $\sigma_{\mathrm{R}}/\sigma_{\mathrm{R,P}}$ obtained from simulated scattering curves calculated for lipid area $A_{\mathrm{L}} = 62.9$ Å^2, number of water molecules $N_{\mathrm{L}} = 10$, and mean outer liposome radii $R_{2,mean}$ 200 Å and 500 Å. The values of $\sigma_{\mathrm{R,P}}$ were obtained from the parabolic dependence on $R_{2,mean}$ published in [4].

mean value of outer liposome radius $R_{2,mean}$ for different combinations of A_{L} and N_{L} values. As expected, the R_g parameter does not depend on $R_{2,mean}$ for any A_{L} and N_{L} combination studied in the investigated 200 Å $\leq R_{2,mean} \leq$ 500 Å range (not shown). However, different

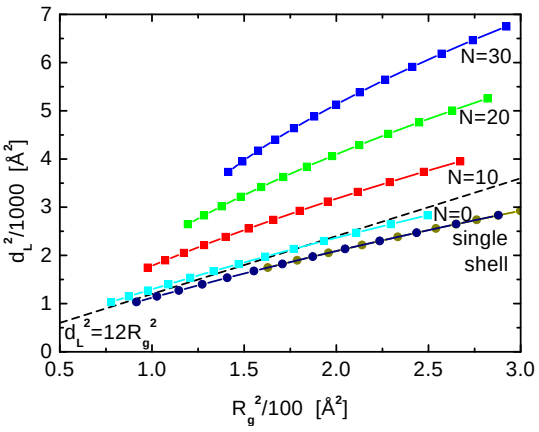

Fig. 5. Dependence of the lipid bilayer thickness d_{L} on the radius of gyration R_g evaluated from Kratky-Porod plots of scattering curves simulated by using the multishell liposome model, for constant lipid surface area $A_{\mathrm{L}} = 62.9$ Å^2 and different values of the number of water molecules N_{L} located in the bilayer polar region. The lipid bilayer thickness d_{L} was varied by changing the number of methylene groups in the acyl chains of phosphatidylcholine molecule between 12 and 24. Dashed line shows $d_{\mathrm{L}} = d_g = 12^{0.5} R_g$.

values of R_g were obtained for different A_{L} and N_{L} combinations. We have concluded thus that there is no effect of $R_{2,mean}$ on the R_g values in the studied range of $R_{2,mean}$ and Q values at given A_{L} and N_{L} combination, and have fixed therefore the liposomes mean radius to $R_{2,mean} = 300$ Å in the following simulations.

To see the effects of varying N_{L} values, which were input in the calculation of scattering curves, the dependencies of d_{L} vs. R_g were plotted in Fig. 5. In these calculations, the surface area A_{L} was constant and the bilayer thickness d_{L} was varied by changing the number of methylene groups in the bilayer hydrophobic region. It is clearly seen from these data, that the value of R_g obtained from the calculated convoluted scattering curves by using the shell model in Fig. 2 at the constant d_{L} value depends on the number of water molecules intercalated into the bilayer polar region N_{L}. The effect of surface area A_{L} supposed to be occupied by lipid at the bilayer –aqueous phase interface is relatively small in comparison to the effect of N_{L} (not shown), nevertheless it must be taken into account because A_{L} and d_{L} are closely related.

In analogy to equation (2), the bilayer thickness parameter d_g is frequently obtained from

$$d_g^2 \cong 12 \cdot R_g^2. \tag{9}$$

We have thus plotted in Fig. 5 also the values of d_{L} obtained supposing that $d_{\mathrm{L}} = d_g = 12^{0.5} R_g$. It is seen that these values approach the values of d_{L} obtained by the multishell model when N_{L}=0, i.e. when there is no penetration of water molecules into the polar part of bilayer. Physically, this situation is highly improbable. Included are also the values for a single shell model, which supposes a homogeneous scattering length density distribution within the bilayer. Such model was used in the scattering curve evaluation earlier [4, 9-12]. The deviation of d_{L} from d_g is observed also in this case. This deviation is practically the same when $N_{\mathrm{L}} >$0, e.g. there is

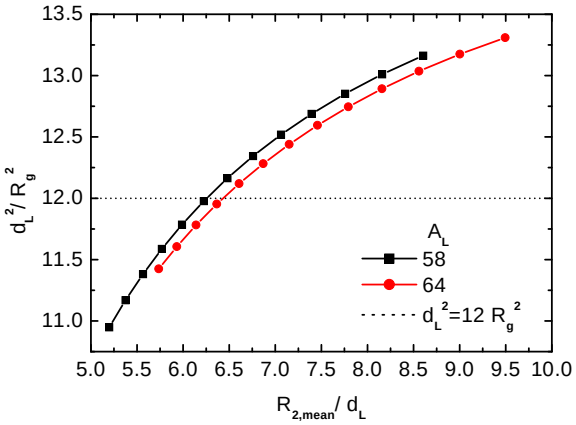

Fig. 6. Dependencies of d_{L}^2/R_g^2 values on the ratio $R_{2,mean}/d_{\mathrm{L}}$ obtained from Kratky-Porod plots of scattering curves simulated by using the multishell liposome model for different values of lipid surface area A_{L} , number of water molecules located in the bilayer polar region $N_{\mathrm{L}} = 10$. The lipid bilayer thickness d_{L} was varied by changing the number of methylene groups in the acyl chains of phosphatidylcholine molecule between 12 and 24. Dashed line is for $d_{\mathrm{L}} = d_g = 12^{0.5} R_g$.

practically no change in the corresponding curve when changing the bilayer homogeneous scattering length density. It is clear, that the single shell model is a rather crude approximation of the bilayer and deviates from the multishell model significantly. One would intuitively expect that the value of d_g should approach that of d_{L} when the ratio $R_{2,mean}/d_{\mathrm{L}}$ increases. However, it seems that this is not valid for liposome sizes and bilayer thicknesses expected in experiments with extruded liposomes prepared from phosphatidylcholines. As an illustration of this fact, we present in Fig. 6 the dependence of d_{L}^2/R_g^2 on $R_{2,mean}/d_{\mathrm{L}}$ obtained from scattering curves computer simulated with the fixed number of water molecules intercalated into the bilayer polar region N_{L}=10. In these simulations, the bilayer thickness d_{L} was varied by changing the number of methylene groups in the phospholipid acyl chains between 12 and 24 as well as by changing the lipid surface area A_{L} between 58 Å^2 and 64 Å^2. It is seen that $d_g \neq d_{\mathrm{L}}$ in the range of 200 Å $\leq R_{2,mean} \leq$ 500 Å expected for extruded liposomes. For example, $d_{\mathrm{L}}^2/R_g^2 < 12$ for $R_{2,mean}/d_{\mathrm{L}} < 6.4$ and $d_{\mathrm{L}}^2/R_g^2 > 12$ for $R_{2,mean}/d_{\mathrm{L}} > 6.4$ for liposomes with A_{L}=62 Å^2. A very important conclusion follows from this observation – the bilayer thickness parameter d_g can be used as a measure of bilayer thickness only in situations where the value of $R_{2,mean}$ is known.

In principle, both the bilayer thickness d_{L} (or the surface area A_{L}) and the number of water molecules N_{L} can be obtained from the scattering curves measured at different contrast values when the volumetric data are available from independent experiments. We illustrate this approach by using the data for DPPC published by Petrache *et al.* [18]. First, the convoluted scattering curve is calculated by using the multishell model in Fig. 2 for DPPC unilamellar liposomes (the mean radius $R_{2,mean} = 300$ Å, the size distribution $\sigma_{\mathrm{R}} = 91.15$ Å, the lipid surface area $A_{\mathrm{L}} = 62.9$ Å^2 and the number of intercalated water molecules per one lipid $N_{\mathrm{L}} = 6.3$) at

a given molar fraction of heavy and ordinary water $N_{D_2O}/(N_{D_2O} + N_{H_2O})$. This first step simulates the measuring of experimental curve. The following steps illustrate the evaluation of this experimental curve: The value of R_g(exp) is obtained from the simulated scattering curve by fitting the data in the region of 0.001 Å$^{-2} \leq Q^2 \leq 0.006$ Å^{-2}. Then, the d_L value is calculated for a given N_L value from the interval $0 \leq N_L \leq 20$. This is done by fixing the N_L value, calculating the scattering curves for different d_L values and fitting them by linear functions in the region of 0.001 Å$^{-2} \leq Q^2 \leq 0.006$ Å^{-2} till their R_g value fulfils the condition $|R_g - R_g(\mathrm{exp})| \leq 0.001$ Å. The set of paired d_L and N_L values is obtained at given contrast and this is plotted as a continuous curve by fitting the paired d_L and N_L points by a smooth polynomial function (Fig. 7). The whole procedure is repeated for another contrast. It is seen, that the continuous d_L vs. N_L curves obtained at two different contrasts intersect in one point. This intersection can be intuitively expected. From all intersections of curves calculated for $N_{D_2O}/(N_{D_2O} + N_{H_2O}) = 1.0$, 0.5 and 0.3 we have obtained average values $d_L = 44.8$ Å ($A_L = 62.9$ Å^2) and $N_L = 6.3$, the same as the input values.

3.2 Evaluation of experimental data

We have used the multishell model for evaluation of experimental SANS data obtained by Cherezov [14] with unilamellar DMPC liposomes at 30 °C and different contrasts $N_{D_2O}/(N_{D_2O} + N_{H_2O})$. The volumetric parameters used in the evaluation of these experimental data are collected in the Table III.

M_w [g/mol]	677.95
ν_L [ml/g]	0.978
V_L [Å^3]	1101
Headgroup region [Å^3]	319
CH_2 [Å^3]	27.929
CH_3 [Å^3]	55.858
H_2O [Å^3]	30.031
D_2O [Å^3]	30.131

Table III. Volumetric data for DMPC bilayers at 30 °C.

The molecular volume of DMPC was obtained from the absolute specific volume ν_L of DMPC at 30 °C measured by a neutral buoyancy method [32] as

$$V_L = \nu_L M_w / N_A, \tag{10}$$

where M_w is the DMPC molecular weight and N_A the Avogadro number. To obtain the hydrocarbon region volume, the volume of the head group $V_H = 319$ Å^3 [23] was subtracted from the DMPC molecular volume. It was then supposed that the molecular volume of the methylene group V_{CH_2} is independent of its position in the acyl chain and equal to the half of the molecular volume of the methyl group, i. e. $V_{CH_3} = 2V_{CH_2}$ [18, 33]. Finally, it was supposed that the volume of water molecules located in the head group region is the same as in the bulk aqueous phase [4, 34].

Table IV. Values of radii of gyration of unilamellar DMPC liposomes obtained at different contrasts in the region of 0.001 Å$^{-2} \leq Q^2 \leq 0.006$ Å^{-2} (adopted from [14]) and the values of $d_{\rm L}$ and $A_{\rm L}$ obtained by fitting the experimental data at fixed value of $N_{\rm L} = 7.3$.

$N_{\rm D_2O}/(N_{\rm D_2O} + N_{\rm H_2O})$	R_g^2(exp) [Å^2]	$d_{\rm L}$ [Å]	$A_{\rm L}$ [Å^2]
1.0	108.3	42.5	62.2
0.8	100.0	42.1	62.8
0.6	91.7	42.7	61.8
0.4	66.7	42.6	61.2

The experimental values of R_g^2(exp) obtained from the scattering curves in the region of 0.001 Å$^{-2} \leq Q^2 \leq 0.006$ Å^{-2} are collected in the Table IV. For each combination of two different R_g^2(exp) values, we have calculated dependencies like in plots in Fig. 7. From their intersections we have obtained $d_{\rm L}$ ($A_{\rm L}$) and $N_{\rm L}$ values shown in the Table V. In some cases, the intersection points were found outside the physically reasonable interval $0 < N_{\rm L} < 20$. It is evident that the precision of the R_g^2(exp) values is critical for obtaining the correct values of bilayer parameters. From the fitting of simulated curves we have found that for obtaining simultaneously the value of $A_{\rm L}$ with precision better than ± 1 Å^2 and that of $N_{\rm L}$ better than ± 1, the relative precision of the two different R_g^2(exp) values used must be better than 1.8%. This situation could be improved by using a constrained fit by fixing the value of $N_{\rm L}$ or $d_{\rm L}$ ($A_{\rm L}$). The values obtained at $N_{\rm L} = 7.3$ are shown in the Table IV. It is seen, that the scatter between $d_{\rm L}$ and $A_{\rm L}$ values obtained at different contrasts is now relatively small – the mean values ($\pm$ the standard deviation) are $d_{\rm L} = 42.6 \pm 0.5$

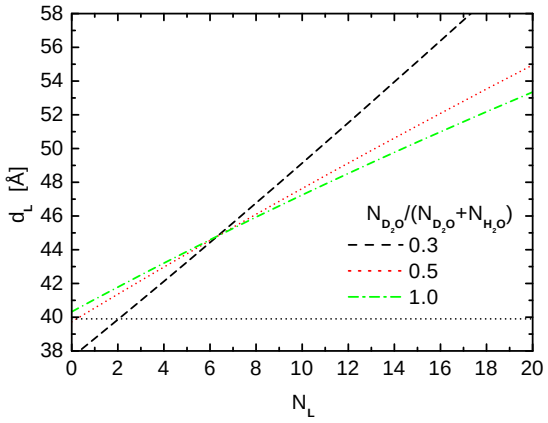

Fig. 7. Dependence of the lipid bilayer thickness $d_{\rm L}$ on the number of water molecules $N_{\rm L}$ located in the bilayer polar region for different contrasts. The data were obtained from the simulated scattering curves having the same radius of gyration R_g as the scattering curve of fluid DPPC unilamellar liposomes with the lipid surface area $A_{\rm L} = 62.9$ Å^2 and the number of water molecules located in the bilayer polar region $N_{\rm L} = 6.3$.

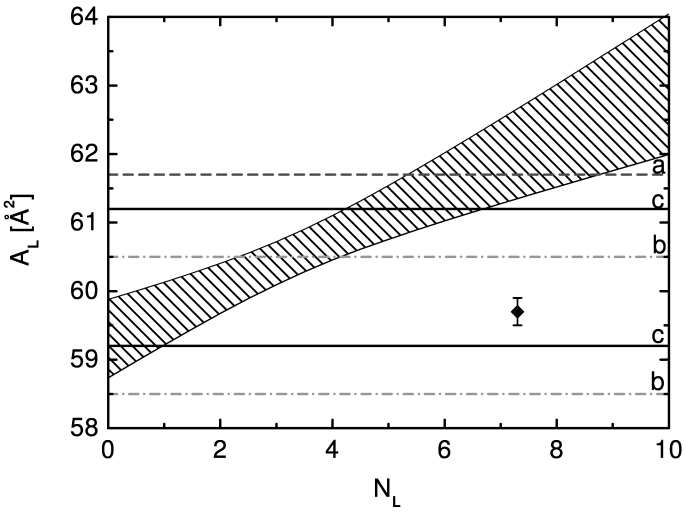

Fig. 8. Dependence of the DMPC surface area A_{L} on the number of water molecules N_{L} calculated from the experimental SANS data (shaded area).Horizontal lines and diamond – SAXD data. For details see text.

Å and $A_{\mathrm{L}} = 62.0 \pm 0.7$ Å^2. We have fitted the experimental data for different values of N_{L} and plotted the results as a function of A_{L} on N_{L} in Fig. 8. Since the fits provide also the hydrocarbon region thickness data ($2d_{\mathrm{N}}+2d_{\mathrm{S}}$), we have plotted them in Fig. 9 as a function of N_{L}. The shaded areas in Figs. 8 (9) represent all possible physically reasonable combinations of $A_{\mathrm{L}}(2d_{\mathrm{N}} + 2d_{\mathrm{S}})$ and N_{L} that can be obtained from SANS results of Cherezov [14] by using the multishell model. These can be compared with results of other authors.

Table V. Surface area A_{L}, bilayer thickness d_{L} and number of penetrated water molecules N_{L} obtained from experimental SANS data of Cherezov [14] by simulating simulateneously two scattering curves at indicated contrasts.

$N_{\mathrm{D_2O}}/(N_{\mathrm{D_2O}} + N_{\mathrm{H_2O}})$	$N_{\mathrm{D_2O}}/(N_{\mathrm{D_2O}} + N_{\mathrm{H_2O}})$	A_{L} [Å^2]	d_{L} [Å]	N_{L}
1.0	0.8	72.9	54.1	28.9
1.0	0.6	60.3	39.9	3.4
1.0	0.4	60.7	40.4	4.2
0.8	0.6	56.9	32.6	-5.8
0.8	0.4	60.4	38.5	2.0
0.6	0.4	60.8	40.7	4.5

Lis *et al.* [35] have studied the fluid lamellar phase of DMPC at 27 °C after addition of increasing amounts of water molecules per lipid N in the sample. By using the SAXD, they have measured the lamellar repeat period d, which remained constant in the presence of excess water after maximum swelling of lipid. They calculated from it the surface area A_{L} supposing that there is no penetration of water into the bilayer ($N_{\mathrm{L}} = 0$), i.e.

$$A_{\mathrm{L}} = 2(N_{\mathrm{w}}V_{\mathrm{w}} + V_{\mathrm{L}})/d, \tag{11}$$

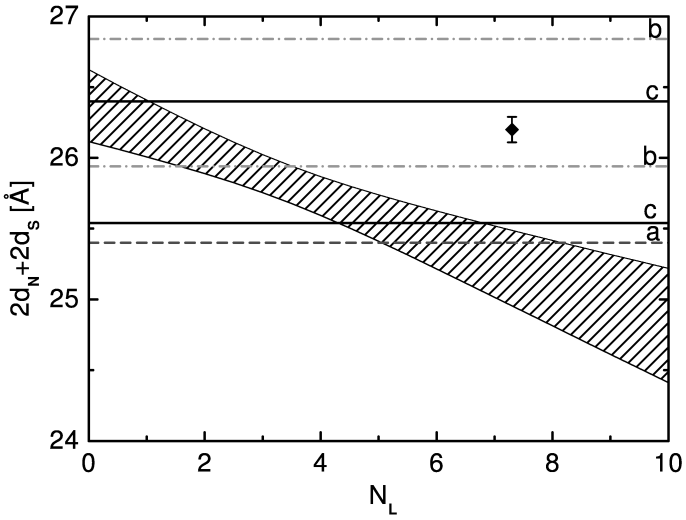

Fig. 9. Dependence of the DMPC hydrocarbon region thicknesss $2d_{\mathrm{N}} + 2d_{\mathrm{S}}$ on the number of water molecules N_{L} calculated from the SANS data (shaded area). Horizontal lines and diamond show the SAXD data. For details see text.

where N_{w} is the number of water molecules per lipid located between bilayers. One obtains the same A_{L} for $N_{\mathrm{L}} \neq 0$ under assumption that the molecular volume of penetrated water is the same as in the water located between bilayers. They supposed further that all water molecules in the sample (estimated by e.g. gravimetry) are located between bilayers (i.e. $N_{\mathrm{w}} = N$) below the limiting value of d at maximum swelling, and have obtained $A_{\mathrm{L}} = 65.2$ Å^2 at maximum swelling. However, the value of A_{L} thus obtained may be overestimated. At total number of water molecules per lipid in the sample $N > 15$ (which is less than this at the maximum swelling), the lamellar phase of phosphatidylcholines converts into multilamellar liposomes, and water fills-in structural packing defects otside the interbilayer space [3, 36], i.e. $N_{\mathrm{w}} < N$.

Using the values of d measured by Lis *et al.* [35] at various osmotic pressures, and the value of elastic area compressibility modulus of the bilayer $K_{\mathrm{A}} = 0.145 \pm 0.010$ N/m estimated by Evans and Needham [37], Rand and Parsegian [38] have obtained $A_{\mathrm{L}} = 61.7$ Å^2 for lamellar phase of DMPC at 27 °C as an extrapolated value at zero osmotic pressure. The hydrocarbon region thickness can be calculated from equation

$$2d_{\mathrm{N}} + 2d_{\mathrm{S}} = 2(V_{\mathrm{L}} - V_{\mathrm{H}})/A_{\mathrm{L}}. \tag{12}$$

One obtains then $2d_{\mathrm{N}} + 2d_{\mathrm{S}} = 25.4$ Å. The values of surface area and of the hydrocarbon region thickness are shown in Fig. 8 and Fig. 9 as horizontal dashed lines (lines a). Intersection with our results gives $N_{\mathrm{L}} = 7.1 \pm 2.3$.

Koenig *et al.* [36] have studied the fluid lamellar phase of DMPC with perdeuterated acyl chains at 30 °C after addition of known amounts of water N and at various osmotic pressures. By using the SAXD, they have measured the lamellar repeat period d. They calculated from it the surface area A_{L} by using equation (11), however, they have used only the data for $N < 15$

where the assumption $N_{\mathrm{w}} = N$ is valid. Extrapolating these data to the zero osmotic pressure, they obtained $K_{\mathrm{A}} = 0.141$ N/m (with asymmetric confidence interval from 0.091 to 0.260 N/m) and $A_{\mathrm{L}} = 59.5 \pm 1.0$ Å^2. Using equation (12), one obtains $2d_{\mathrm{N}} + 2d_{\mathrm{S}} = 26.39 \pm 0.45$ Å. The values of surface area and of the hydrocarbon region thickness are shown in Fig. 8 and Fig. 9 as horizontal dashed-dotted lines (lines b). Intersections with our region give $N_{\mathrm{L}} = 3.2 \pm 0.7$ on the top border and $N_{\mathrm{L}} < 0$ on the bottom border of their range.

Petrache *et al.* [32] have studied the fluid lamellar phase of DMPC at 30 °C by using the synchrotron SAXD. They have obtained the phosphate-phosphate distance d_{PP} across the bilayer from the bilayer electron density profiles at various osmotic pressures. Using this distance and taking the volume of head group V_{H} and of DMPC as in Table III, they have found the elastic area compressibility modulus of the bilayer $K_{\mathrm{A}} = 0.108 \pm 0.035$ N/m and the surface area $A_{\mathrm{L}} = 60.2 \pm 1.0$ Å^2 as an extrapolated value at zero osmotic pressure. Using this value and equation (12), one obtains $2d_{\mathrm{N}} + 2d_{\mathrm{S}} = 25.97 \pm 0.43$ Å. The values of surface area and of the hydrocarbon region thickness are shown in Fig. 8 and Fig. 9 as horizontal full lines (lines c). Intersections with our results gave $N_{\mathrm{L}} = 5.5 \pm 1.4$ on the top border and $N_{\mathrm{L}} < 0.7$ on the bottom border of their range. These values can be checked by using the measured repeat distance of the lamellar phase of DMPC $d = d_{\mathrm{L}} + d_{\mathrm{w}}$, where d_{w} is the thickness of the water layer between phospholipid bilayers. Petrache *et al.* [32] have observed $d = 62.7$ Å at the total number of water molecules located between bilayers $N = 25.7$. Using the SAND data of Büldt *et al.* [39], Petrache *et al.* [32] have concluded that the thickness of the polar head group region should be $d_{\mathrm{P}} = 9$ Å, in this case the bilayer thickness is $d_{\mathrm{L}} = 2(d_{\mathrm{N}} + d_{\mathrm{S}} + d_{\mathrm{P}}) = 43.97 \pm 0.43$ Å. The number of water molecules penetrated into the polar head group region can be thus calculated from the equation

$$N_{\mathrm{L}} = (dA_{\mathrm{L}} - 2V_{\mathrm{L}})/2V_{\mathrm{w}} - N/2. \tag{13}$$

Using this equation and the volumetric data in Table III, we have found $N_{\mathrm{L}} = 13.25 \pm 0.03$.

Koenig *et al.* [36] have obtained $K_{\mathrm{A}} = 0.136$ N/m (with asymmetric confidence interval from 0.123 to 0.152 N/m) and $A_{\mathrm{L}} = 67.5 \pm 0.2$ Å^2 from their ^{2}H-NMR data. They concluded that the surface area obtained from NMR data in their paper was overestimated. Petrache *et al.* [32] adopted the NMR K_{A} value from [36] and have found $A_{\mathrm{L}} = 59.7 \pm 0.2$ Å^2, $2d_{\mathrm{N}} + 2d_{\mathrm{S}} = 26.20 \pm 0.09$ Å and $N_{\mathrm{L}} = 7.3$. These values are plotted in Figs. 8 and 9 as diamonds.

Comparisons of our physically reasonable result space and results of other autors in Figs. 8, 9 show pleasingly similar values, but there are still discrepancies. Some of these discrepancies may be due to remaining imperfections in the SANS data evaluation method proposed in the present work, which must be further developed and tested. First, the simulated data resembling the structure of the real bilayer more closely than the multishell model have to be used as input data, such as atomic-scale molecular dynamics simulations of the bilayer [22, 33]. Second, the deviations of liposome shapes from spherical [40] must be taken into account. This work is in progress in our group.

Acknowledgments N. Kučerka thanks the staff of the Condensed Matter Division, Frank Laboratory of Neutron Physics, Joint Institute for Nuclear Research in Dubna, for the hospitality. This study was supported by the Slovak Ministry of Education grants to P. Balgavý and by the J. A. Comenius University grant to N. Kučerka. The experiments in Dubna were supported within the JINR project 07-4-1031-99/03.

References

[1] V. Luzzati, A. Tardieu: *Annu. Rev. Phys. Chem.* **25** (1974) 79

[2] V. Luzzati: *Curr. Opin. Struct. Biol.* **7** (1997) 661

[3] K. Gawrisch, W. Richter, A. Möpps, P. Balgavý, K. Arnold, G. Klose: *Stud. Biophys.* **108** (1985) 5

[4] P. Balgavý, M. Dubničková, D. Uhríková, S. Yaradaikin, M. Kiselev, V. Gordeliy: *Acta Physica Slovaca* **48** (1998) 509

[5] W. Knoll, J. Haas, H. B. Stuhrmann, H. H. Füldner, H. Vogel, E. Sackmann: *J. Appl. Cryst.* **14** (1981) 191

[6] O. Glatter, O. Kratky: *Small Angle X-Ray Scattering*, Academic Press, New York 1982

[7] L. A. Feigin, D. I. Svergun: *Structure Analysis by Small-Angle X-Ray and Neutron Scattering*, Plenum Publishing Corporation, New York 1987

[8] V. I. Gordeliy, L. V. Golubchikova, A. Kuklin, A. G. Syrykh, A. Watts: *Progr. Colloid Polym. Sci.* **93** (1993) 252

[9] S. Komura, Y. Toyoshima, T. Takeda: *Jpn. J. Appl. Phys.* **21** (1982) 1370

[10] P. C. Mason, B. D. Gaulin, R. M. Epand: *Phys. Rev. E* **59** (1999) 3361

[11] R. J. Gilbert, R. K. Heenan, P. A. Timmins, N. A. Gingles, T. J. Mitchell, A. J. Rowe, J. Rossjohn, M. W. Parker, P. W. Andrew, O. Byron: *J. Mol. Biol.* **293** (1999) 1145

[12] J. Pencer, F. R. Hallett: *Phys. Rev. E* **61** (2000) 3003

[13] V. I. Gordeliy, M. A. Kiselev: *Biophys. J.* **69** (1995) 1424

[14] V. G. Cherezov: *PhD. Thesis*, Moscow Physico-Technical Institute 1997

[15] T. Nawroth, H. Conrad, K. Dose: *Physica B* **156 & 157** (1989) 477

[16] M. Gradzielski, D. Langevin, L. Magid, R. Strey: *J. Phys. Chem.* **99** (1995) 13232

[17] J. Lemmich, K. Mortensen, J. H. Ipsen, T. Honger, R. Bauer, O. G. Mouritsen: *Phys. Rev. E* **53** (1996) 5169

[18] J. F. Nagle, R. Zhang, S. Tristram-Nagle, W. Sun, H. I. Petrache, R. M. Suter: *Biophys. J.* **70** (1996) 1419

[19] S. S. Berr, M. J. Coleman, R. R. M. Jones, J. S. Johnson: *J. Phys. Chem.* **90** (1986) 6492

[20] H. I. Petrache, S. E. Feller, J. F. Nagle: *Biophys. J.* **72** (1997) 2237

[21] A. Tardieu, V. Luzzati, F. C. Reman: *J. Mol. Biol.* **75** (1973) 711

[22] R. S. Armen, O. D. Uitto, S. E. Feller: *Biophys. J.* **75** (1998) 734

[23] W. J. Sun, R. M. Suter, M. A. Knewtson, C. R. Worthington, S. T. Nagle, R. Zhang, J. F. Nagle: *Phys. Rev. E* **49** (1994) 4665

[24] D. M. Small: *J. Lipid Res.* **8** (1967) 551

[25] B. W. Koenig, S. Krueger, W. J. Orts, C. F. Majkrzak, N. F. Berk, J. V. Silverton, K. Gawrisch: *Langmuir* **12** (1996) 1343

[26] J. F. Nagle, M. C. Wiener: *Biochim. Biophys. Acta* **942** (1988) 1

[27] D. L. Worcester, N. P. Franks: *J. Mol. Biol.* **100** (1976) 359

[28] V. F. Sears: *Methods of Experimental Physics* (Eds. K. Skold, D. L. Price), Academic Press, New York 1986 pp. 521-550

[29] V. F. Sears: *Neutron News* **3** (1992) 29

[30] http://ne43.ne.uiuc.edu/n-scatter/n-lengths/list.html

[31] B. D. Gaulin, J. Katsaras: *Physics Can.* (1997) 247

[32] H. I. Petrache, S. Tristram-Nagle, J. F. Nagle: *Chem. Phys. Lipids* **95** (1998) 83
[33] K. Tu, J. Tobias, M. L. Klein: *Biophys. J.* **69** (1995) 2558
[34] M. C. Wiener, S. Tristram-Nagle, D. A. Wilkinson, L. E. Campbell, J. F. Nagle: *Biochim. Biophys. Acta* **938** (1988) 135
[35] L. J. Lis, M. McAlister, N. Fuller, R. P. Rand, V. A. Parsegian: *Biophys. J.* **37** (1982) 657
[36] B. W. Koenig, H. H. Strey, K. Gawrisch: *Biophys. J.* **73** (1997) 1954
[37] E. A. Evans, D. Needham: *J. Phys. Chem.* **91** (1987) 4219
[38] R. P. Rand, V. A. Parsegian: *Biochim. Biophys. Acta* **988** (1989) 351
[39] G. Buldt, H. U. Gally, J. Seelig, G. Zaccai: *J. Mol. Biol.* **134** (1979) 673
[40] A. J. Jin, D. Huster, K. Gawrisch, R. Nossal: *Eur. Biophys. J.* **28** (1999) 187