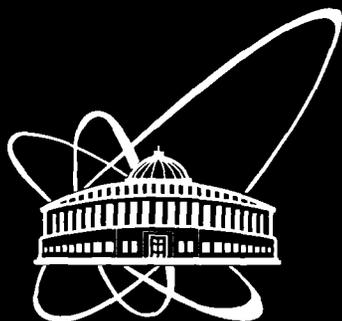




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STRUCTURE FACTOR
OF DIMYRISTOYLPHOSPHATIDYLCHOLINE
UNILAMELLAR VESICLES:
SMALL-ANGLE X-RAY SCATTERING STUDY

Submitted to «Поверхность. Рентгеновские, синхротронные
и нейтронные исследования»

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1. INTRODUCTION

Application of phospholipid vesicles as delivery agents of drugs, genetic materials and enzymes through living cell membrane and other hydrophobic barriers is the field of particular interest for pharmacology, medicine, genetic engineering, cosmetic and food industry [1,2]. Vesicle size appears to be a key factor in their permeation through tumor microvessels and residence in tumor tissues [3]. Thus, there is a strong driving force for the development of noninvasive and accurate methods for the characterization of vesicular dispersions [4].

A novel method of evaluating small-angle neutron scattering (SANS) curves in the range of scattering vector q from 0.006 \AA^{-1} to 0.382 \AA^{-1} was developed recently, it allows to determine important membrane parameters and to describe its geometry and hydration for the case of mixed lipid/detergent vesicles [5]. This method implied strip-function model to describe the neutron scattering length density across the membrane [6]. The similar model was applied for the calculation of internal membrane structure for three types of the phospholipid vesicles [7]. In this approach, the Guinier region of SANS curve ($0.039 \text{ \AA}^{-1} \leq q \leq 0.107 \text{ \AA}^{-1}$) was used for model calculations of membrane structure. The vesicle radius was a free parameter in the range of $200 \text{ \AA} - 500 \text{ \AA}$.

Another approach for calculation of the membrane thickness was developed on the basis of approximation of the neutron scattering length density across the membrane with a constant [8]. Due to the introduced form factor of lipid bilayer, this approximation can accurately describe SANS curves at large values of scattering vector, $q > 0.1 \text{ \AA}^{-1}$. Approximation of the neutron scattering length density across the membrane with a constant was used in the Guiner analysis to evaluate the membrane thickness from SANS experiment [9-12].

Newly proposed methods (Schmidel's model and Pencer's approach) of evaluation of the membrane thickness include the complementary techniques (freeze fraction cryomicroscopy and dynamic light scattering) to characterize the vesicle size and polydispersity [5,8].

The principal disadvantage of the neutron scattering is incoherent background from hydrogen nuclei of the lipid molecules [5]. X-ray scattering has a sufficiently low value of the incoherent background relative to the neutron scattering from biological objects. Application of small-angle X-ray scattering (SAXS) to the investigation of lipid vesicles is limited due to the small contrast between H₂O and lipid membrane. Aqueous sucrose solution increases the X-ray contrast between phospholipid membrane and bulk solvent sufficiently. The region of sucrose concentrations 30%-40% w/w exhibits the best experimental conditions for X-ray small-angle scattering experiments with phospholipid vesicles [13-15].

In the SANS study of vesicles, lipid concentrations in range of 1-10% w/w was used [5, 7-9, 11, 12, 15, 16]. Despite of the wide range of used lipid concentrations, the structure factor of vesicles was disregarded in these works. The purpose of present work is to study the DMPC vesicle structure factor and to characterize vesicular dispersions from SAXS experiment.

2. MATERIALS AND SAMPLE PREPARATION

Dimyristoylphosphatidylcholine (DMPC) - C₃₆H₇₂NO₈P and sucrose - C₁₂H₂₂O₁₁ were purchased from Sigma (France). H₂O was of Millipore standard (18MΩ·cm). 40% sucrose solution in water was used as a solvent medium. DMPC concentrations were 15mM, 30mM, 45mM, 60mM, 75mM (1%, 2%, 3%, 4%, 5% w/w). DMPC unilamellar vesicles were prepared by extrusion of multilamellar vesicles through polycarbonate filter with pore diameter 500 Å as described in details in [17].

3. METHODS

The spectra from unilamellar DMPC vesicles were collected at D22 small-angle X-ray spectrometer (LURE, France) with linear position sensitive detector. Acquisition time was 20 min per spectrum. The measured intensities were normalized to the scattering from water.

The model of separated form factors (SFF) was applied to interpret the SAXS curves [18]. In this model the macroscopic cross section of monodispersed population of vesicles is given by equation (1) for the case $R \gg d$,

$$\frac{d\Sigma}{d\Omega_{min}}(q) = n \cdot F_s(q, R) \cdot F_b(q, d) \cdot S(q, R), \quad (1)$$

where n is the number of vesicles per unit volume, $q = \frac{4\pi \cdot \sin(\Theta/2)}{\lambda}$ is the scattering vector, d is membrane thickness. $S(q, R)$ is the structure factor of vesicles. $F_s(q, R)$ is the form factor of a infinitely thin sphere with radius R

$$F_s(q, R) = \left(4\pi \cdot \frac{R^2}{qR} \cdot \text{Sin}(qR) \right)^2. \quad (2)$$

$F_b(q, d)$ is the form factor of symmetrical lipid bilayer of thickness d with scattering length density $\rho(x)$ in the direction perpendicular to the bilayer surface

$$F_b(q, d) = \left(\int_{-d/2}^{d/2} \rho(x) \cdot \text{Cos}(qx) \cdot dx \right)^2, \quad (3)$$

here $\rho(x)$ is the X-ray contrast between membrane and aqueous sucrose solution. The approximation of X-ray scattering length density across the membrane with a constant $\rho(x) \equiv \Delta\rho$ is far from being realistic, but can be used as first approximation similar to the neutron investigations [9-12]. The detailed characterization of the internal membrane structure is beyond the scope of present article, because SAXS curves were collected with good statistics in the short range of scattering vectors $0.005 \text{ \AA}^{-1} \leq q \leq 0.04 \text{ \AA}^{-1}$. In the approximation $\rho(x) \equiv \Delta\rho$, (3) is integrated to the expression

$$F_b(q, d) = \left(\frac{2\Delta\rho}{q} \cdot \text{Sin}\left(\frac{qd}{2}\right) \right)^2. \quad (4)$$

The intervesicle interaction can be simply taken into account through structure factor of sphere in Debye form [19]

$$S(qR) = 1 - \frac{8V_v}{v} \left(\frac{\text{Sin}(2qR)}{2qR} \right), \quad (5)$$

where V_v is the volume of vesicle $V_v = \frac{4}{3} \cdot \pi \cdot R^3$, $v=1/n$ is a volume of solution per one vesicle.

Vesicle polydispersity was described by nonsymmetrical Schulz distribution [20]

$$G(R) = \frac{R^m}{m!} \cdot \left(\frac{m+1}{\bar{R}} \right)^{m+1} \cdot \exp \left[-\frac{(m+1) \cdot R}{\bar{R}} \right], \quad (6)$$

where \bar{R} is the average vesicle radius. The polydispersity of vesicles was characterized as relative standard deviation of vesicle radius $\sigma = \sqrt{\frac{1}{(m+1)}}$.

The experimentally measured macroscopic cross section $d\Sigma(q)/d\Omega$ was calculated via convolution of (1) with the vesicle distribution function $G(R)$ by integration over the vesicle radius $R_{min}=70 \text{ \AA}$ to $R_{max}=2000 \text{ \AA}$:

$$\frac{d\Sigma}{d\Omega}(q) = \frac{\int_{R_{min}}^{R_{max}} \frac{d\Sigma}{d\Omega_{mon}}(q, R) \cdot G(R) \cdot dR}{\int_{R_{min}}^{R_{max}} G(R) \cdot dR} \quad (7)$$

Finally, the $d\Sigma(q)/d\Omega$ values were corrected for the resolution function of the D22 spectrometer as described in [21].

In order to calculate the model parameters \bar{R} , d , m , the experimentally measured macroscopic cross sections were fitted with the calculated macroscopic cross section via a least-square minimization with three independent parameters: \bar{R} , d , m .

The parameter

$$R_{factor} = \frac{1}{N} \cdot \sum_{i=1}^N \left(\frac{\frac{d\Sigma}{d\Omega}(q_i) - \frac{d\Sigma}{d\Omega_{exp}}(q_i)}{\frac{d\Sigma}{d\Omega_{exp}}(q_i)} \right)^2 \quad (8)$$

was used as a measure of a fit quality, where N is a number of experimental points. Quantities \bar{R} , d , m were found as a set of values minimizing the functional (8).

4. RESULTS AND DISCUSSION

The SAXS curve for the DMPC concentration 15 mM is fitted well with $S(q) \equiv 1$. Fitting with $S(q) \equiv 1$ of the SAXS curve with the DMPC concentration 30 mM gives overestimated results for the q range $0.005 \text{ \AA}^{-1} \leq q \leq 0.008 \text{ \AA}^{-1}$. The experimentally measured SAXS curves and the fitted curves with a structure factor in the Debye form are shown in Fig. 1, vesicle parameters are presented in Table 1. The experimental curves for the range of DMPC concentrations from 15 mM to 75 mM are fitted well with the introduced interaction as it is seen in Fig 1. The differences in the calculated values of average vesicle radius are in the range of system polydispersity of 26%. The calculated membrane thickness for the DMPC concentrations from 30 mM to 75 mM is within the accuracy of $\pm 2 \text{ \AA}$, which is good enough for the q range used. The large value of $d=40 \text{ \AA}$ obtained for the DMPC concentration 15 mM is a result of poor statistics at the far end of scattering curve. The increase of X-ray macroscopic scattering cross section due to the sucrose is substantially smaller than increasing cross section for the case of neutrons due to the isotopic substitution of H_2O with D_2O . The macroscopic cross sections for neutrons (D_2O solution) and X-rays (40% sucrose solution) were compared in the case of mixed DMPC/ C_{12}E_8 micelles measured at $T=10^\circ\text{C}$. The neutron macroscopic cross section is 33 times greater than cross section for X-rays [15]. It creates a necessity to use more concentrated samples for X-ray experiments relative to neutron experiments. For 20 min acquisition time, the DMPC concentrations above 30 mM appear to be more appropriate for SAXS experiment.

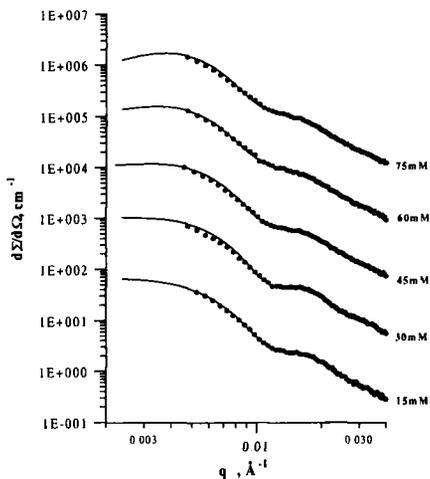


Fig. 1. The SAXS experimental spectra (points) and fitted curves for the 500 Å extruded DMPC vesicles, $T=30^{\circ}\text{C}$. The macroscopic cross section for the 15 mM DMPC vesicles in absolute units. The macroscopic cross sections for the 30 mM, 45 mM, 60 mM, and 75 mM DMPC are multiplied by 10, 100, 1000, 10000, respectively.

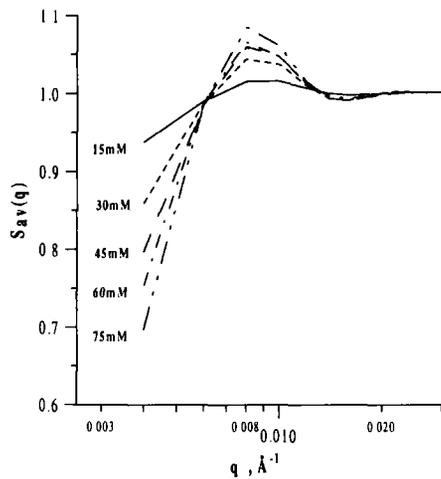


Fig. 2. The average structure factor of vesicles $S_{av}(q)$ calculated by the Debye model with the Schulz distribution for the 15 mM, 30 mM, 45 mM, 60 mM, and 75 mM DMPC concentrations at $T=30^{\circ}\text{C}$.

The results obtained for different DMPC concentrations give possibility to determine the average parameter value along with the accuracy of the parameter evaluation. The evaluation of the membrane thickness from the q range used ($q \leq 0.04 \text{ \AA}^{-1}$) creates a big uncertainty in its value because this range of q is just a beginning of the membrane Guinier region. There are no physical reasons for the differences in the values of R , σ , d for different DMPC concentrations we used (for 75 mM DMPC, $V_v/\nu = 0.106$). From the results presented in Table 1, the average SFF model parameters and errors were calculated for DMPC vesicles at $T=30^{\circ}\text{C}$: $\bar{R} = 254 \pm 16 \text{ \AA}$, $\sigma = 0.26 \pm 0.06$, $d = 37 \pm 4 \text{ \AA}$. The obtained value of DMPC membrane thickness is in a good

agreement with the value of $d=36.9 \text{ \AA}$ obtained from X-ray diffraction experiment on multilamellar vesicles at the same temperature [22].

Table 1. Vesicle parameters evaluated from the model calculations of SAXS curves, $T=30^\circ\text{C}$. C_{DMPC} – the DMPC concentration, \bar{R} – the average vesicle radius, σ – the relative standard deviation of radius, d – the membrane thickness, R_{factor} – the measure of fit quality. The accuracy in the calculations of \bar{R} and d is 1 \AA . The relative errors of σ are in the interval of 2-3%.

C_{DMPC} , mM	\bar{R} , \AA	σ ,	d , \AA	R_{factor} , %
15	242	0.25	40	0.2
30	252	0.22	35	0.2
45	255	0.25	35	0.2
60	261	0.29	36	0.3
75	261	0.28	37	0.2

Fig. 2 shows the calculated values of the structure factor $S_{\text{av}}(q)$ for different DMPC concentrations. The average $S_{\text{av}}(q)$ function is calculated from (5) via convolution with Schulz distribution function (6) and vesicle parameters from Table 1. As it is seen from Fig. 2, $|S_{\text{av}}(q)-1|<0.07$ for $q\geq 0.0042 \text{ \AA}^{-1}$, and $S_{\text{av}}(q)\approx 1$ for $q\geq 0.006 \text{ \AA}^{-1}$ in the case of the DMPC concentration 15 mM. $|S_{\text{av}}(q)-1|<0.05$ for 30 mM DMPC for $q\geq 0.006 \text{ \AA}^{-1}$. For DMPC concentrations ≥ 45 mM, $|S_{\text{av}}(q)-1|>0.05$ for $q=0.008 \text{ \AA}^{-1}$. For any concentration, the average structure factor $S_{\text{av}}(q)=1$ at $q\geq 0.02 \text{ \AA}^{-1}$ due to the system polydispersity. The influence of the structure factor is negligibly small for region of $q\geq 0.02 \text{ \AA}^{-1}$. This region is important for the determination of the membrane thickness.

5. CONCLUSIONS

For the first time DMPC vesicle average radius, polydispersity, structure factor and membrane thickness were calculated simultaneously from SAXS experiment. The

DMPC vesicles in 40% aqueous sucrose solution at $T=30^{\circ}\text{C}$ have average radius $254\pm 16\text{\AA}$, polydispersity $26\pm 6\%$, and membrane thickness $37\pm 4\text{\AA}$. The structure factor in the Debye form describes experimental results well.

Disregarding of structure factor calculations for the lipid concentrations $\geq 30\text{ mM}$ (2% w/w) leads to errors in the calculation of scattering curve. At the same time, the structure factor corrections are not important in the evaluation of the membrane thickness from the small-angle experiment. For the lipid concentrations about 15 mM (1% w/w), the structure factor correction is negligibly small. The use of samples with the lipid concentrations 5%-10% w/w is a simple way to improve statistics in the SAXS and SANS experiments, the correct evaluation of vesicles structure factor will be necessary in this case. This range of lipid concentrations appears to be prospective for the SAXS application to the study of the vesicle structure.

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Исследования структурного фактора однослойных везикул димиристоилфосфатидилхолина методом малоуглового рассеяния рентгеновских лучей

Методом малоуглового рассеяния рентгеновских лучей (МУРР) проведены измерения структуры однослойных везикул димиристоилфосфатидилхолина (ДМФХ) в 40 %-ном водном растворе сахарозы. Для описания экспериментальной кривой рассеяния была применена модель разделенных формфакторов для больших однослойных везикул. Измерения проводились при концентрации ДМФХ от 15 до 75 мМ (1–5 % по весу) и температуре образца 30 °С. Впервые по данным МУРР были рассчитаны: структурный фактор, полидисперсность популяции и средний радиус везикул, а также толщина мембраны. Показано, что коррекция малоугловой кривой рассеяния за счет учета структурного фактора пренебрежимо мала для концентрации ДМФХ 15 мМ (1 % по весу). Для концентраций липида более 30 мМ (2 % по весу) учет структурного фактора является необходимым для правильного описания кривой рассеяния.

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Structure Factor of Dimyristoylphosphatidylcholine Unilamellar Vesicles: Small-Angle X-Ray Scattering Study

Small-angle X-ray scattering (SAXS) experiments have been performed on dimyristoylphosphatidylcholine (DMPC) unilamellar vesicles in 40 % aqueous sucrose solution. Model of separated form factors was applied for the evaluation of SAXS curves from large unilamellar vesicles. For the first time vesicle structure factor, polydispersity, average radius and membrane thickness were calculated simultaneously from the SAXS curves at $T = 30^\circ\text{C}$ for DMPC concentrations in the range from 15 to 75 mM (1–5 % w/w). Structure factor correction to the scattering curve was shown to be negligibly small for the lipid concentration of 15 mM (1 % w/w). It was proved to be necessary to introduce structure factor correction to the scattering curves for lipid concentrations ≥ 30 mM (2 % w/w).

The investigation has been performed at the Frank Laboratory of Neutron Physics, JINR.

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