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# Application of small-angle X-ray scattering to the characterization and quantification of the drug transport nanosystem based on the soybean phosphatidylcholine

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ABSTRACT

Phospholipid transport nanosystem (PTNS) for drug delivery is based on soybean phosphatidylcholine. The morphology of PTNS is investigated by means of small-angle X-ray scattering. The obtained results allow one to answer the key question from the viewpoint of organization of drug incorporation whether the PTNS nanoparticles have a structure of micelles or vesicles. It is demonstrated that PTNS is a vesicular system with an average vesicle radius of 160 ± 2 Å.



## 1. Introduction

The development of drug delivery systems allows one to essentially improve the efficiency of drug application. For today, a number of ready drug forms based on different delivery systems have already been introduced in medicine. These drugs are highly efficient and much in demand on the drug market. In this respect, phospholipid-based carriers are of sufficient interest as biodegradable, biologically inactive systems, without allergic, antigenic and pyrogenous reactions [1]. Phospholipids are highly specialized lipids which are the main component of cellular membranes of all variety of living organisms [2]. Their main function consists in the formation of a double lipid layer (bilayer) in the membranes [3]. It is known that the phospholipids contained in a large number in some food (eggs, liver, meat, sunflower seeds, corn, soybeans, etc.) cannot be considered as medical food sources of phospholipids as they contain other components (cholesterol, oils, etc.). Specially developed "essential" phospholipids (EPL) cleared of oils and undesirable impurities are applied to ensure the therapeutic effect in medicine [4]. For the first time, EPL (as a medicine to treat toxic liver damages) was extracted from soybeans by developing and using high purification technologies [5]. During 50 years which passed from the moment of receiving a dosage form of EPL, they were carefully investigated; their pharmacological properties and therapeutic effects were studied in the experiments, numerous clinical trials, and in broad medical practice. Soybean phospholipids are coproducts of soybean oil processing, the production of soybean phospholipids rises with the continuous increase of soybean oil yield. New technologies for isolation and purification of certain phospholids can enormously improve the development of medicine (biomembrane bionics, liposomes, intracellular drug carriers, etc.) and chemical industry (aggregation and dispersion of nanomaterials, etc.) [17].

The most popular phospholipid delivery nanosystems are unilamellar vesicles with a diameter in the range of 300–2000 Å [6]. In recent years, the technology of obtaining phospholipid delivery nanosystems (PTNS) with an extremely small diameter from soybean phosphatidylcholine has been developed in the V.N. Orekhovich Research Institute of Biomedical Chemistry. PTNS is

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produced as a lyophilized powder, which is stable in time [7]. The incorporation of some drugs into PTNS sufficiently increases their bioavailability and therapeutic effectiveness [8].

The carrier morphology is the key characteristic from the viewpoint of the drug incorporation technology. However, nothing is known about the PTNS morphology so far. Dynamic light scattering makes it possible to measure the average radius of PTNS as 136 ± 18 Å, but this technique cannot give a direct answer to the key question whether the PTNS nanoparticles have a structure of micelles or vesicles [7].

To clarify this point, it is necessary to apply more complex methods like small-angle X-ray scattering (SAXS). The SAXS technique is an effective method to characterize both micelles and vesicles [9]. The main difficulty in the SAXS application to characterize small unilamellar vesicles (ULV) is a weak contrast between ULVs and surrounding water. The density of electrons and hence contrast (difference between the electron density in the lipid bilayer and the solvent) can be increased by using solutions of disaccharides [10–13]. Say, the scattering intensity from dimyristoylphosphatidylcholine (DMPC) ULVs in the 40% (w/w) sucrose solution in water exceeds the intensity from the same ULVs in pure water about 100 times [10].

PTNS contains soybean phosphatidylcholine (95–97% purity) from Lipoid® and maltose, the weight ratio of phosphatidylcholine to maltose is 1:4. PTNS nanoparticles are formed in the maltose solution after dilution of lyophilized powder in water. The concentration of maltose in the sample depends on the degree of the PTNS dilution in water. This makes it possible to vary contrast for X-rays. The methodology of contrast variation by disaccharides in the X-ray scattering experiment on ULVs opens up an opportunity to investigate the PTNS structure using the SAXS technique at synchrotron facilities [10–13].

The purpose of communication is to present the first result on the characterization of the PTNS structure at the Kurchatov Synchrotron Radiation Source of the National Research Center «Kurchatov Institute» in Moscow. The analysis of the PTNS SAXS spectrum in comparison with the SAXS data from the DMPC unilamellar vesicles allows one to make conclusion about the vesicular structure of PTNS nanoparticles.

## 2. Materials and methods

### 2.1. Materials

Lipoid S100 (purchased from Lipoid GmbH, Germany) was used for obtaining nanoparticles with a content of phosphatidylcholine of at least 95%. Maltose monohydrate (purchased from company MERCK, Germany) was used for the lyophilization of nanoparticles.

### 2.2. Obtaining of phospholipid nanoparticle emulsion

The phospholipid nanoparticle emulsion was obtained in two stages: (1) obtaining of a crude emulsion using a mechanical stirrer (RW 20.N, IKA, Germany) and (2) obtaining of a thin (nano) emulsion using microfluidizer M110EH30K, Microfluidics, USA.

*Obtaining of crude emulsion*: 5 g of S100 Lipoid was dispersed at room temperature ($T$ = 25 °C) in 200 ml of deionized water obtained in Milli-Q (Millipore, USA) using a mechanical stirrer at a speed of 500–700 rpm/min for 5 min until a homogeneous emulsion with no visible agglomerates was formed.

*Obtaining of thin emulsion*: The previously obtained crude emulsion was poured into the homogenizer or microfluidizer receiving tanks. The emulsion was subjected to homogenization at a pressure of 1000 atm at a temperature of 45 °C and the number of cycles from 1–8. The temperature of the resulting emulsion was maintained using the homogenizer or microfluidizer built-in cooling system. Sampling for control measurements was carried out after each cycle of homogenization.

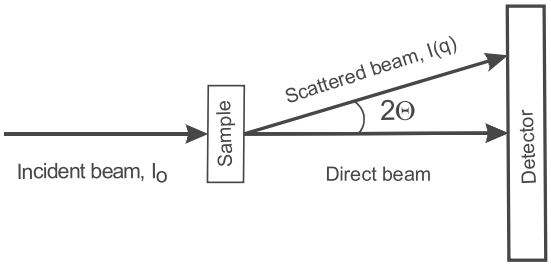


**Fig. 1.** The layout of the small-angle scattering experiment. The incident photon beam with the intensity $I_o$ is scattered from the sample at an angle $2\theta$. The intensity of the scattered beam $I(q)$ is measured by a position-sensitive detector.

### 2.3. Method of data analysis

The scattering intensity of photons $I(q)$ is measured in the SAXS experiment as a function of the scattering vector $q = \frac{4\pi \times \sin(\theta)}{\lambda}$, where $2\theta$ is the scattering angle of photons, and $\lambda$ is the photon wavelength. The layout of the SAXS experiment is presented in Fig. 1. The scattering intensity $I(q)$ is measured by a position-sensitive detector. For the case of monodisperse vesicle population within the framework of the separated form factor model [15,16], $I(q)$ is given by the following expression

$$I(q) = I_0 \times n \times F_s(q, R) \times F_b(q, d_m), \quad (1)$$

where $I_o$ is the intensity of the incident beam, $n$ – number of vesicles in $cm^3$, $F_s(q,R)$ – form factor of the spherical surface with radius $R$, and $F_b(q,d_m)$ – form factor of the symmetrical lipid bilayer, $d_m$ – thickness of the lipid bilayer of ULVs. $F_s(q,R)$ and $F_b(q,d_m)$ are determined as follows:

$$F_s(q, R) = \left(4\pi \times \frac{R^2}{qR} \times \sin(qR)\right)^2, \quad (2)$$

$$F_b(q, d_m) = \left(\int_{-d_{m/2}}^{d_{m/2}} \rho_c(x) \times \cos(qx) \times dx\right)^2. \quad (3)$$

Here $\rho_c(x)$ is the contrast (difference) between the scattering length density of the lipid bilayer and the solvent [16]. For the case of X-rays, the scattering length density is proportional to the density of electrons in the lipids or the solvent. Respectively, the contrast is proportional to the difference between the electron density of the lipid bilayer and the surrounding solvent.

Eq. (3) can be simplified in the case of a homogeneous lipid bilayer $\rho_c(x) \equiv \Delta\rho$ = const:

$$F_b(q, d_m) = \left(\frac{2\Delta\rho}{q} \times \sin\left(\frac{qd_m}{2}\right)\right)^2. \quad (4)$$

The homogeneous approximation $\rho_C(x) \equiv \Delta\rho$ = const is widely used due to the simplicity of calculations.

It follows from the Eq. (2) that the first minimum in the form factor $F_s$ of the spherical surface with radius $R$ is calculated as

$$q_R = \frac{\pi}{R}. \quad (5)$$

The SAXS curves from PTNS and DMPC describe the size of vesicles in the range of small values of $q$, see the left parts of the curves in Fig. 2. The oscillations in the experimental SAXS spectra are smeared by vesicle polydispersity and spectrometer resolution.

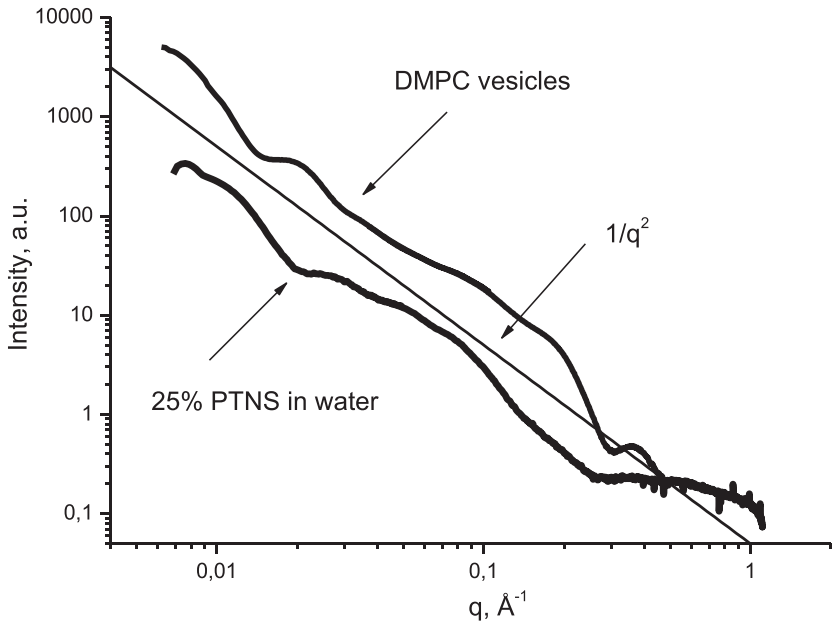


**Fig. 2.** Small-angle X-ray scattering spectra. Unilamellar vesicles from DMPC in 40% (w/w) sucrose buffer (DMPC vesicles) and 25% (w/w) solution of phospholipid transport nanosystem in water (25% PTNS in water). The straight line shows the function $1/q^2$.

From the Eq. (4), one can also determine the position of the minimum in the form factor $F_b$ of the lipid bilayer (right parts of the scattering curves at large values of $q$)

$$q_m = \frac{2\pi}{d_m}. \tag{6}$$

In the experiment, the clearness of the minimum is also smeared due to the internal structure of the lipid bilayer ($\rho_C(x) \neq$ const), solvent penetration to the region of polar head groups of phospholipids and spectrometer resolution [16].

### 2.4. Experiment

A PTNS sample for the SAXS measurement was prepared via dilution of lyophilized PTNS (25% w/w) in water. The sample composition after dilution (w/w): 5% of phospholipid, 20% of maltose, 75% of water. ULVs from dimyristoylphosphatidylcholine (DMPC) were prepared via extrusion through pores with a diameter of 500 Å [14]. A 40% sucrose solution was used as a solvent for ULVs. The DMPC concentration in the sample was 3% (w/w).

The measurements of PTNS particles were carried out at room temperature at the DICSI station of the Kurchatov Synchrotron Radiation Source at the NRC «Kurchatov Institute», Moscow, Russia. The measurements of DMPC vesicles were performed at $T = 30\,^{\circ}C$ on the A2 station of the synchrotron DORIS at DESY, Hamburg, Germany [15]. Both PTNS (soybean phosphatidylcholine) at room temperature and DMPC at $T = 30\,^{\circ}C$ were in the liquid crystalline phase. The measurements at the DICSI station were carried out at two sample-to-detector distances of $L_{sd} = 30$ and 243.5 cm with a photon wavelength of $\lambda = 1.625$ Å. The measurements at the A2 station were carried out at two values of $L_{sd} = 68.3$ and 295.8 cm, $\lambda = 1.5$ Å.

## 3. Results and discussion

The experimental SAXS curves from 25% PTNS solution in water and from DMPC vesicles in a 40% sucrose solution are presented in Fig. 2. For comparison, the line

$$I(q) = \text{Const} \times \frac{1}{q^2} \tag{7}$$

is also presented. One can see from the Eq. (4) that the scattering intensity as a function of $q$ should behave as $1/q^2$. Indeed, the PTNS and DMPC curves in Fig. 2 are in good agreement with the law $I \sim 1/q^2$. By contrast, in the case of micelles the scattering curves decay proportional to $I \sim 1/q^0$ [9]. This fact, together with the similarity of scattering curves for both PTNS particles and DMPC vesicles, argue that PTNS nanoparticles have a structure of vesicles as presented in Fig. 3.

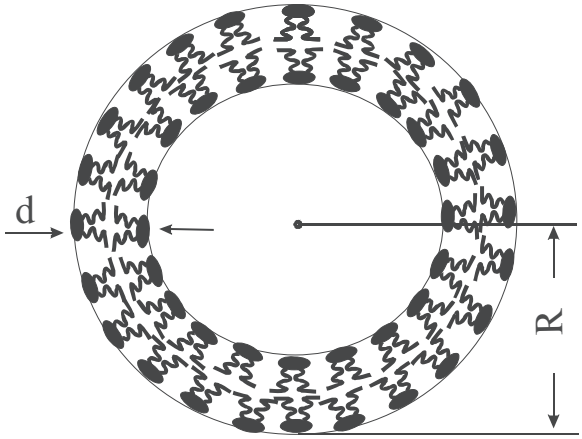


**Fig. 3.** Phospholipid unilamellar vesicle with radius $R$ and thickness of the lipid bilayer $d$. For the PTNS vesicle (25% (w/w) of FTNS in water): $R_{PTNS} = 160 \pm 2$ Å, $d_{PTNS} = 27.2 \pm 0.1$ Å. For the DMPC vesicles in 40% (w/w) sucrose buffer: $R_{DMPC} = 210 \pm 2$ Å, $d_{DMPC} = 20.5 \pm 0.1$ Å.

The PTNS vesicle radius and lipid bilayer thickness can be estimated using Formulas (5) and (6), respectively. For the PTNS spectrum the minimum position in the form factor of the spherical surface (2) is equal to $q_R = 0.0196 \pm 0.0002$ Å$^{-1}$, which corresponds according to the Eq. (5) to the average radius $R_{PTNS} = 160 \pm 2$ Å. The same calculation for DMPC vesicles gives $R_{DMPC} = 210 \pm 2$ Å. Hence, the average radius of the extruded DMPC vesicles exceeds the average PTNS vesicle radius by 50 Å.

For the PTNS spectrum the minimum position in the form factor of the lipid bilayer (4) is equal to $q_m = 0.231 \pm 0.001$ Å$^{-1}$. Hence, from the Eq. (6), one can obtain the thickness of the lipid bilayer $d_{PTNS} = 27.2 \pm 0.1$ Å. For DMPC vesicles the same calculation gives the bilayer thickness $d_{DMPC} = 20.5 \pm 0.1$ Å, i.e. the DMPC bilayer thickness is less than the PTNS bilayer thickness by 7 Å.

Note that the employed approach gives underestimated values for the lipid bilayer thickness [16]. In the case of DMPC vesicles at a 40% sucrose concentration in water, the value of the bilayer thickness (numerically adjusted to SAXS data) is equal to $34.8 \pm 0.6$ Å [15]. In the case of PTNS particles, the fitting procedure gives the value of the bilayer thickness to be equal to $43.5 \pm 0.5$ Å (this is only the preliminary result which is required to be confirmed by further calculations).

Nevertheless, the relation between the above estimations of $d_{DMPC}$ and $d_{PTNS}$ looks realistic and can be explained by longer fatty chains of PTNS phospholipids. Indeed, in the PTNS case, the phospholipid composition of soybean phosphatidylcholine is as follows: 13% C16:0 (palmitic), 4% C18:0 (stearic), 10% C18:1 (oleic), 64% C18:2 (linoleic), and 6% C18:3 (linolenic), while DMPC consists of 99% C14:0 (myristic).

## 4. Conclusions

On the basis of the SAXS data analysis and comparison of the DMPC and PTNS spectra, one can conclude that the phospholid transport nanosystem (PTNS) represents a vesicular system with the average radius of $R_{PTNS} = 160 \pm 2$ Å, i.e. the average size of vesicles is about 320 Å. This first insight into the morphology of PTNS nanoparticles allows one to carry out more detailed synchrotron investigations of PTNS and the respective numerical analysis.

Full SAXS analysis by fitting the scattering curves is complicated because of a complex mixture of different components of soybean phospholipids. The paper represents a preliminary step to a wider program of future investigations.

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