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NEUTRONS IN STUDIES OF PHOSPHOLIPID BILAYERS AND BILAYER–DRUG INTERACTION. II. SMALL-ANGLE SCATTERING*

NEUTRÓNY V ŠTÚDIU INTERAKCIÍ LIEČIVS FOSFOLIPIDOVÝMI DVOJVRSTVAMI. II. MALOUHLOVÝ ROZPTYL

Original research article

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Abstract In this paper, we demonstrate several possibilities of using neutron scattering in pharmaceutical research based on examples of scientific results achieved at our University. In this second part, elementary principles of small-angle neutron scattering and simple methods of scattering data evaluation are described. Results of scattering on bilayers in unilamellar liposomes with intercalated general anaesthetics, antimicrobials or cholesterol demonstrate the potential of this method at determination of structural properties of bilayers.

Slovak abstract V tomto článku poukazujeme na viacero možností využitia neutrónov vo farmaceutickom výskume na príkladoch vedeckých výsledkov, ktoré sme získali na našej univerzite. V tejto druhej časti popisujeme elementárne princípy malouhlového rozptylu neutrónov a jednoduché metódy vyhodnocovania rozptylových údajov. Pomocou výsledkov rozptylu na dvojvrstvách v unilamelárnych lipozómoch s interkalovanými všeobecnými anestetikami, antimikróbnymi tenzidmi alebo cholesterolom, demonštrujeme potenciál tejto metódy pri určovaní štruktúrnych vlastností dvojvrstiev.

Keywords *neutron scattering, phospholipid liposomes, lipid bilayer, alkanes, alkan-1-ol, cholesterol*

Kľúčové slová: *neutrónový rozptyl, fosfolipidové lipozómy, lipidová dvojvrstva, alkány, alkán-1-oly, cholesterol*

INTRODUCTION

Small-angle neutron scattering (SANS), in general, refers to measuring the intensity of scattered neutrons, where q is close to zero. Although neutron scattering with small transferred momentum q is observed also for organised objects exhibiting symmetry in at least one direction, it is particularly important due to its sensitivity to the presence of symmetrically disordered structures in the studied system. The fact that waves scattered at small angles carry information about the internal structure of also disordered systems was found first by Guinier (1937), who thus laid the foundations of the small-angle scattering method.

BASIC PRINCIPLES

We strongly suggest to the readers not familiar with the neutron scattering, to see the first part of our paper (Belička *et al.*, 2014a) where the elementary principles and the terminology used are explained. Here we extend this explanation to SANS. The intensity of scattered neutrons at small angles is dominantly dependent on the size of inhomogeneities in the irradiated sample (Svergun and Feigin, 1987). These inhomogeneities are any areas, the size of which is larger than the wavelength of the neutrons, but also sufficiently small that the scattered waves from the different places of one particle are still coherent. Such inhomogeneities are, in our case, cells,

** Dedicated to Doc. Dr. Martin Bútora, PhD. on the occasion of his 70th birthday.*

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their organelles, various artificially prepared lipid complexes (uni- and multilamellar liposomes), biomacromolecules (e.g. proteins), or complexes of lipids with other biomolecules (e.g. DNA). The size of the objects themselves also affects the range of values of transferred momentum *q*, which contains information about their internal structure and needs to be measured as accurately as possible. In general, if one wants to explore objects with typical size *d*, then one has to measure the intensity of scattered neutrons in the interval $(0,q_{max})$, where

$$q_{max} = \frac{2\pi}{d}. \tag{1}$$

For example, to investigate structures with dimensions in the range of 1 – 1000 nm, it is necessary to measure the values of *I(q)* in the *q* range from 0.006 to 6 nm.

In principle, the SANS method is identical to the neutron diffraction method, that is, one observes single elastic neutron scattering on the nuclei of the irradiated atoms, which implies a description of the superposition of waves in the form of the Fourier transform. However, due to the sizes of the examined objects, the condition of coherence of scattered waves is satisfied only for small values of transferred momentum and, for elastic scattering, therefore only for small values of scattering angles. At low concentrations of the examined objects, the above-mentioned fact has the effect that the scattered neutron waves are coherently combined to form minima and maxima of intensity only when they come from one and the same object, however, not when they come from different objects. Each irradiated object thus adds its separate share to the resulting detected intensity; this share is independent from other objects. Neutrons are, of course, scattered on all the atomic nuclei, regardless of whether they are in the examined objects or in the surrounding solution. But as has already been mentioned in the first part of our review (Bellička *et al.*, 2014a), the resulting wave scattered by the object is dependent on the distribution of the *contrast* density of the scattering length, which is, in the studied areas, zero apart from the studied structure, and therefore, when counting/integrating the scattered waves, it is not necessary to take account of the areas filled with an aqueous medium. For scattering intensity attributable to a single irradiated object, we have (similarly as with neutron diffraction) $I_1(\vec{q})=\|F(\vec{q})\|^2$, where $F(\vec{q})$ is the *form factor* of the given object equal to

$$F(\vec{q}) = \int \Delta\rho(\vec{r}) e^{-i\vec{q}\cdot\vec{r}} d^3\vec{r}, \tag{2}$$

where $\Delta\rho(\vec{r})$ is the scattering length contrast density. In the irradiated volume, there are usually a large number of examined objects, since $I_1(\vec{r})$ is not practically detectable at objects investigated. Although the objects have basically the same structure, their spatial distribution of $\Delta p(\vec{r})$ can be different only due to different orientation as a result of free motion. They can also differ in size, although they still have substantially the same structure (e.g. discs, spheres of different sizes).

According to the size distribution of the present objects, the samples are divided into *monodisperse* (all objects are the same size) and *polydisperse* (there are objects of different sizes in the sample). The distribution of sizes *R* in polydisperse samples is described by means of a distribution function, which is most often the Gaussian distribution function

$$f(R;\bar{R},\sigma) = \frac{1}{\sqrt{2\pi\sigma^2}} \exp\left(-\frac{(R-\bar{R})^2}{2\sigma^2}\right), \tag{3}$$

where $\bar{R}$ is the mean size of objects and σ its standard deviation, or the Schulz–Flory distribution function

$$f_{Schulz}(R;\bar{R},\sigma) = \left(\frac{t+1}{\bar{R}}\right)^{t+1} \frac{R^t}{\Gamma(t+1)} \exp\left(-\frac{R}{\bar{R}}(t+1)\right),$$
$$t = \left(\frac{\bar{R}}{\sigma}\right)^2 - 1. \tag{4}$$

The total intensity of scattering from a set of several objects is thus expressed through the mean intensity of scattering from a single object:

$$I(q) = K \cdot \langle I_1(\vec{q}) \rangle = K \cdot \langle \backslash F(\vec{q}) \backslash^2 \rangle, \tag{5}$$

where *K* is the scaling constant directly proportional to the number of examined objects present in the irradiated volume and the notation ⟨.⟩ represents the mean over all present objects. The resulting scattering intensity, due to averaging over the orientations of the object, depends only on the size of the transferred momentum *q*. Summation or integration over all possible orientations and sizes directly implies combination of scattering intensities of individual objects.

The above considerations apply in full only if the examined systems are *diluted*, that is., if the irradiated objects are sufficiently distant from each other and the neutron waves scattered from two adjacent objects do not combine. For concentrated samples, the above argument does not apply and the scattering curves clearly show the effect of concentration. Moreover, the increased concentration involves not only interference of waves from neighbouring objects, but often interactions between more or less distant objects, which generally has an impact on their spatial orientation and arrangement. The above-mentioned phenomenon can sometimes be simply prevented by diluting the sample at the cost of reducing the scattering intensity, but it is not always possible to do, for example, because of reasons associated with the preparation or nature of the sample itself, or narrowing the interval of the investigated transferred momentum *q*, which implies a limitation on structural information which can be obtained from the measured data. In these cases, it is therefore necessary to include, in the expression for total scattering intensity (5), a factor describing the effect of concentration and interaction of the examined objects, the so-called *structure factor S(q)*, which generally depends on the spatial arrangement of the particles, their size and orientation, but also on the properties of the environment among the irradi-

ated particles. For the total intensity of scattered neutrons, it then holds that:

$$I(q) = K \cdot \langle I_1(\vec{q}) \rangle = K \cdot \langle \| F(\vec{q}) \|^2 \rangle, \quad (6)$$

Due to the complicated interaction between the particles, the expression of an explicit form of the structural factor is complex and, in practice, is therefore used in various approximate forms. Most often, however, samples are prepared in diluted form, in which case, it is not necessary to consider the spatial distribution and interaction of the particles, and for the structure factor, we have $S(q) \equiv 1$ for the whole interval of the measured q.

SANS intensity is characterised by the presence of multiple model-independent characteristics (so-called invariants) of the irradiated objects dependent on the scattering length density distribution in their volume, which can be obtained from the scattering curves without additional assumptions on their structure (Svergun and Feigin, 1987). In terms of the object structure, one of the most important is the so-called *radius of gyration* of the irradiated object defined similarly as in mechanics by the relationship:

$$R_g^2 = \frac{\int_V r^2 \rho(\vec{r}) d^3\vec{r}}{\int_V \rho(\vec{r}) d^3\vec{r}}, \quad (7)$$

where integration takes place over the entire object's volume V. It can be obtained from the scattering curve by fitting it in the region of low values of q by the so-called *Guinier approximation*

$$I(q) = I(0) q^{r-3} \exp(-q^2 R_g^2 / r), \quad (8)$$

where $I(0)$ is the (extrapolated) scattering intensity in the direction of the beam and r is the geometric factor of the objects which, at constant $p(\vec{r})$ in the case of infinite planar objects, has a value of approximately 1; in the case of objects in the form of infinite cylinders, it has a value of approximately 2; and for spherically symmetrical objects, it ranges about 3. As shown by Balgavý *et al.* (1998), the value of r ≈ 1 is, in the region of values of q^2 between 0.1 and 0.6 nm^{-2}, at the same time a good approximation for the case of polydisperse system of hollow spheres with constant wall thicknesses, approximating unilamellar lipid liposomes. An example of evaluating neutron scattering curves only through the model-independent invariant Rg can be found in the paper of Uhríková *et al.* (2001) investigating structural changes of dioleoylphosphatidylcholine (DOPC) bilayers in the form of unilamellar liposomes due to intercalation of *N*-dodecyl-*N*,*N*-dimethylamine-*N*-oxide (C12NO). The molar ratio of C12NO : DOPC in the homogenised and extruded mixture gradually increased from 0.0 to 5.0. By fitting the intensity of SANS in the range of validity of the approximation of a liposome system using a system of unoriented planar sheets with identical thickness by (8), they obtained dependences of R_g^2 and r on the molar ratio of C12NO : DOPC (Fig. 1). As can be seen, approximately up to the *molar* ratio of C12NO:DOPC ~ 1.5, the value of $r \approx 1$ indicates that there are unilamellar liposomes or discoid mixed micelles present in the sample; in the interval between 1.5 and 2.5 molar ratios, the value of r increases; and in the interval between 2.5 and 3.5, its value $r \approx 2$ indicates the presence of rod-shaped micelles. In molar ratios above 4.0, a transition from rod-shaped objects to spherical objects can be identified, which most likely are spherical mixed micelles. These results suggest a molecular mechanism for the disintegration of biomembranes as an effect of this bactericidal drug. In the papers of Balgavý *et al.* (1998, 2001) and Kučerka *et al.* (2003) it has been shown, that the radius of gyration obtained from fitting of SANS curves of unilamellar phospholipid liposomes can be used for the characterization of lipid bilayer thickness. The parameter $d_g \cong 12^{0.5} R_g$ has been found to correlate linearly with the bilayer thickness estimated using X-ray diffraction. This method was used later to measure effect of *n*-decane on the bilayer thickness (Uhríková *et al.* 2000, 2003). Earlier the differences in capacitance between the phospholipid black lipid films formed using different chain length *n*-alkanes have been interpreted as indicating that

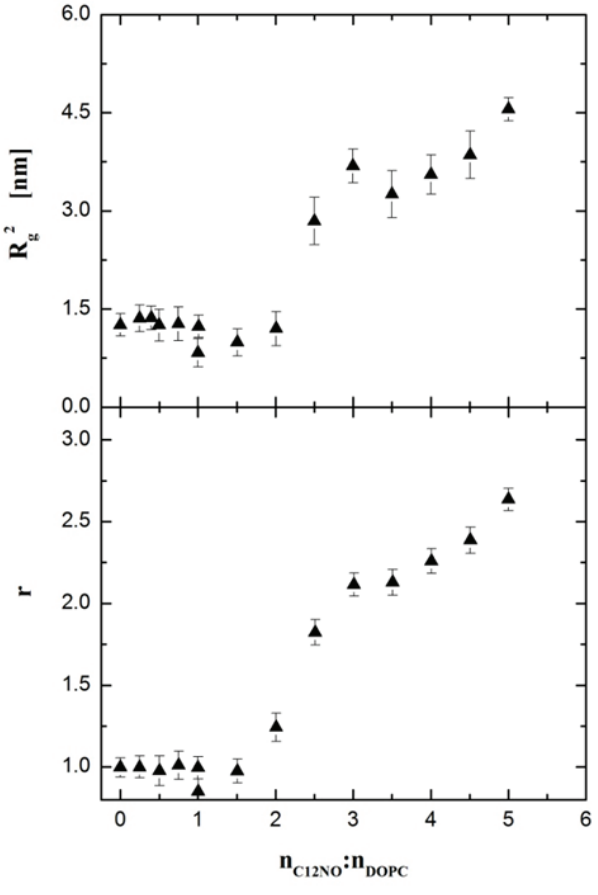

Fig. 1. Dependence of the square of the radius of gyration R_g *and the geometric factor* r *of a mixture of DOPC and C12NO on the molar ratio* $n_{C12NO}:n_{DOPC}$ *obtained by Guinier approximation. Adapted from Uhríková et al (2001).)*

short *n*-alkanes (6 10 carbons) give rise to thicker bilayers and this effect has been correlated with their general anesthetic potency (Hendry *et al.* 1978). Uhríková *et al.* (2000, 2003) demonstrated that the n decane effect on the thickness is rather small and that one has to take into account other effects but not solely the bilayer thickness.

Another way of processing curves of the SANS is their evaluation through models, which to a greater or lesser extent describe our perceptions of the studied systems. The choice of an appropriate model used to evaluate scattering data is determined, on the one hand, by the level of our knowledge about the internal structure of lipid bilayers, but on the other hand, it limits the number of free parameters of the model, which is the given amount of data that can be used, and, of course, is also determined by the ability of the experimental method to capture the required details. Lipid bilayers investigated by SANS usually are in the form of unilamellar liposomes prepared by extrusion through membrane filters. The advantage of unilamellar liposomes compared to multilamellar ones lies mainly in the simplification of the system without significantly affecting the state of the studied bilayer. When studying the effects of various drugs on the structure and physical properties of the lipid bilayers, a so-called three-layer model proved useful in the practice (Kučerka *et al.*, 2004a, 2004b). In this model, the bilayer is divided into three mutually separated areas – two outside, in contact with the aqueous environment, constituting the hydrophilic region of the bilayer, and the centre, which is a hydrophobic area of hydrocarbon chains (Fig. 2). In the basic 3-layer model, there are only hydrophobic parts of lipid and doping molecules without water molecules present in the hydrophobic area. In the hydrophilic areas, there are analogously only hydrophilic parts of the molecules present, the space between them being filled with water molecules or molecules of the aqueous solution. The form factor of a unilamellar liposome, whose bilayer is described by the 3-layer model, is expressed as follows:

$$F(q) = \frac{4\pi}{q^3}\sum_{i=1}^{3}\Delta\rho_i\left(A(q,R_i) - A(q,R_{i-1})\right),$$
$$A(x,y) = xy\cos(xy) - \sin(xy), \qquad (9)$$

where index *i* marks and runs through individual areas of the unilamellar liposome from the inside out, $\Delta\rho_i$ is the scattering length contrast density of the ith area, and R_i is the inner radius of the i^{th} area. Based on the model understanding of the separation of hydrophilic and hydrophobic parts of the molecules in the three-layer model, for the scattering length contrast densities we get:

$$\Delta\rho_C = \frac{(n_{CH} + r_{mix}n'_{CH})b_{CH} + (n_{CH_2} + r_{mix}n'_{CH_2})b_{CH_2} +}{(n_{CH} + r_{mix}n'_{CH})V_{CH} + (n_{CH_2} + r_{mix}n'_{CH_2})V_{CH_2} +}$$
$$\frac{(n_{CH_3} + r_{mix}n'_{CH_3})b_{CH_3}}{(n_{CH_3} + r_{mix}n'_{CH_3})V_{CH_3}} - \frac{b_W}{V_W} = \frac{b_C}{V_C} - \frac{b_W}{V_W} \qquad (10)$$

and

$$\Delta\rho_h = \frac{b_H + r_{mix}b'_H + n_W b_W}{V_H + r_{mix}V'_H + n_W V_W} - \frac{b_W}{V_W}, \qquad (11)$$

where $\Delta\rho_c$ and $\Delta\rho_h$ are the scattering length contrast densities of the hydrophobic area and of the hydrophilic area, respectively; n_a is the abundance of molecular group *a* in the molecule; subscript *H* marks the polar part and *W* water molecule; b_a is the scattering length of molecular group *a*

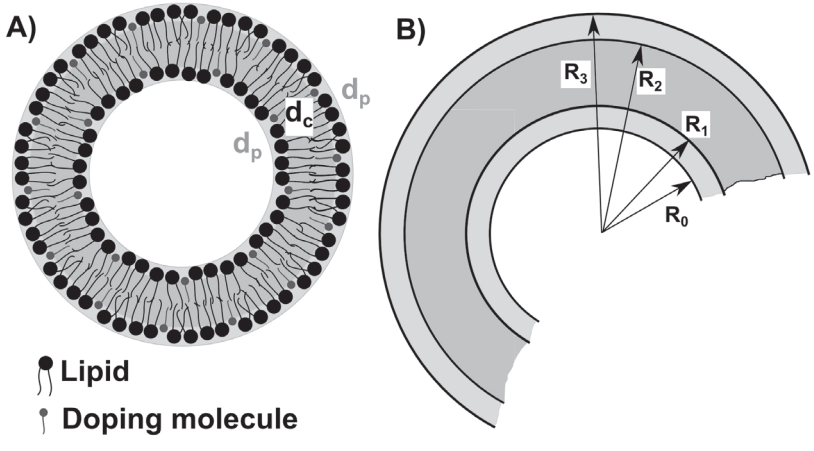

Fig. 2. Schematic drawing of a unilamellar liposome represented by the 3-layer model. A) Large black dots indicate lipid molecules, small black dots indicate intercalated drug molecules. d_P *– thickness of the polar areas,* d_C *– thickness of the hydrophobic area. B)* R_0 *to* R_3 *represent the radii of the interfaces of individual areas in the direction away from the centre.*

and V_a is its molecular volume at the given conditions; and r_{mix} refers to the molar ratio of doping drug molecules with respect to the amount of the lipid present. Variables without the apostrophe refer to lipid molecules and those with the apostrophe are variables describing the doping drug molecules. The molecular volumes, which represent the input internal parameters of the model, are most frequently obtained by densitometry. Based on their results, we also consider the volumes of the methylene groups to be identical regardless of their position. n_w is the number of water molecules located in the hydrophilic area per lipid molecule and its value is determined from the structural parameters of the bilayer.

Within the bilayer, we define a primitive cell, which consists of one molecule of the present lipid, of a portion of the doping molecule determined by r_{mix}, if present, and of the corresponding number of molecules of water n_w in the hydrophilic region, which holds the polar part of the lipid molecule. Since the bilayer can be regarded locally as planar, the given primitive cell may be imagined as a prism with base area A and height $d = dC/2+d_h$. The distribution of hydrophobic and hydrophilic parts of the molecules in the bilayer together with molecular volumes then determines the relationships between structural parameters A, d_C, and d_h:

$$A = \frac{V_C}{d_C / 2} = \frac{V_H + r_{mix} V'_H + n_W V_W}{d_h} \qquad (9)$$

The above relationship implies that to characterise a three-layer model of a bilayer, one of the parameters A and d_C and the thickness of the polar area dh are sufficient. These then determine the last parameter n_w. Along with the liposome radius $R = R_2$ in the case of a monodisperse system, or its mean value $\bar{R}=\langle R_2 \rangle$ and standard deviation σ in the case of a polydisperse system; the chosen pair of structural parameters completely determines the model scattering intensity (6) on a diluted system of unilamellar liposomes described by the three-layer model.

However, the thickness d_h turned out to be a specific parameter, since its size not only determines the thickness of the hydrophilic areas, but, in contrast to d_C, also their scattering length contrast density $\Delta\rho_h$ (11), as it determines n_w (12). To take this effect into account, d_h for a specific system is determined separately using the contrast variation method, in which only the scattering length density of the water medium is varied, and thus of water molecules in the hydrophilic areas, while the structural parameters of the system remain constant. Simultaneous fitting of neutron scattering curves from structurally identical samples differing in the ratio of $^2H2O/H_2O$ thus allows determining the desired parameter d_h.

General anaesthetics

A practical example of use of the above described model is, for example, the paper of Klacsová *et al.* (2011), in which it was applied to studying the effect of intercalation of a homologous series of simple aliphatic alcohols (abbreviation CnOH, n is the number of alkyl carbon atoms) on the structure of a bilayer in unilamellar liposomes. As described extensively by Bělička *et al.* (2014a), CnOHs are general anaesthetics. The theory predicted that at a constant CnOH concentration in the phospholipid bilayer, the bilayer thickness should be smallest for the shortest CnOH and that should increase with the alkyl chain length n (Cibula *et al.*, 1994). Neutron diffraction experiments confirmed this prediction (Petrenko et al, 2010), but it was interesting to investigate effects of CnOHs on other bilayer structural parameters.

Klacsová *et al.* (2011) studied unilamellar liposomes prepared by extrusion of DOPC multilamellar liposomes. To avoid aggregation of unilamellar liposomes after their preparation, lipid bilayers contained also 4 mole percent of negatively charged dioleoylphosphatidylserine (DOPS). The aqueous suspensions of the lipid–alcohol mixtures had concentrations of 10 mg/ml, making the resulting samples sufficiently diluted, and were extruded 51 times through 50 nm pores of polymer filters, which provided the formation of a polydisperse system of unilamellar liposomes. The measurements were carried out in two steps: in the first one, at a constant molar ratio of CnOH : lipid = 0.4, contrast variation was measured for all the examined alcohols at 20°C (Fig. 3) and, in the second one, a series of samples was measured for each alcohol with its increasing content in the lipid at different temperatures in the range of 20–51°C. The first step served to find d_h and the possible effect of the length of the alcohol alkyl chain on its value, which was confirmed; therefore, in the next step, the value of d_h was set and fixed at the value corresponding to the given alcohol. Evaluation of scattering curves from the second step provided dependences of the remaining parameters n_w, A and $D_L = d_C + 2d_h$ on the number of carbons in the alcohol alkyl chain and on the temperature. Selected results are shown in Fig. 4. Dependences of A and D_L (or d_C) were linear in the examined intervals of CnOH : lipid molar ratios. As can be seen in Fig. 4, the number of water molecules per molecule of lipid, as well as the area of the primitive cell, increase faster if longer chain alcohols are inserted in the bilayer. Conversely, the total thickness of the membrane was found to decrease for all the investigated alcohols, the decrease being the greater, the shorter the chain of the inserted alcohol. The experimental findings in this study were confirmed by the results of molecular dynamics simulation of the bilayers. The given effect can be explained by way of illustration as a result of elimination of the free volume under the shorter chains of alcohols by surrounding lipid chains, resulting in a reduction of the primitive cell height. The experimental A values were further evaluated to find the mean partial surface area of the CnOH molecule A_{CnOH} at the lipid bilayer – aqueous phase interface; the values of A_{CnOH} increase with the alkyl chain length n. Surprisingly, the value of A_{CnOH} for the alkyl chain lengths $n \leq 12$ were found to be ≤ 0.2 nm^2 – a value specific for the crystalline or solid rotator phase of alkanes. This has been also reproduced in molecular dynamics (MD) simulations of lipid bilayers. The MD simulations demonstrated that the cause is the location of short-chain CnOHs in bilayers – the OH group of C8OH is located below choline and phosphate groups of DOPC on the level of carbonyl groups.

Antimicrobials

N,N-Dimethyl-*N*-alkylamine-*N*-oxides (CnNO, where n is the number of carbon atoms in the alkyl chain) are widely used in different areas of industry (Devínsky, 1986) and have been studied extensively over recent decades. A wide variety of biological activities have been discovered for these compounds, including antimicrobial effects (Devínsky *et al.*, 1990; Balgavý and Devínsky, 1996). Due to their amphiphilic character, CnNOs easily insert into lipid bilayers. Depending on their alkyl chain length, a hydrophobic mismatch in the hydrophobic region may occur, which is then compensated by a *trans-gauche* isomerization of hydrocarbon chains or by their mutual interdigitation. This should lead to structural changes of the bilayer. Similarly to other homologous series of amphiphilic compounds with linear hydrophobic substituents, the cut-off effect has been observed not only in antimicrobial activity but also in other biological activities (Balgavý and Devínsky, 1996). As suggested by Balgavý & Devínsky (1996), the cut-off effects – a quasi-parabolic dependence are primarily caused by a combination of partition equilibria and elimination of free volume below the hydrophobic substituents, which are usually shorter than phospholipid hydrocarbon chains in biomembranes.

This hypothesis was recently confirmed in the paper by Belička *et al.* (2014b). As in the above-described publication with alkanols, it was demonstrated by means of SANS on unilamellar liposomes of DOPC that at the same concentration of amphiphilic CnNOs, the size of a defect underlying the changes in the bilayer varies in accordance with the trends predicted in the paper by Balgavý & Devínsky (1996). As can be seen in the selected results in Fig. 5, the number of water molecules per molecule of lipid, the area of the primitive cell and the total thickness of the membrane does vary with the length of the CnNO alkyl in accordance with the free volume model.

Sterols

The introduced basic three-layer model is relatively simple. Further improvement was inspired by molecular dynamics simulations of lipid bilayers. The obvious was the introduction of non-homogeneous distribution of water molecules in the bilayer polar region and their partial penetration into hydrophobic region accompanied by a simultaneous partial penetration of phospholipid hydrocarbon methylene groups into polar regions (Kučerka *et al.*, 2004b). This improved model was used in interpretation of SANS data obtained with various saturated and unsaturated phospholipid bilayers containing cholesterol or beta-sitosterol (Gallová *et al.*, 2004a, 2004b, 2008, 2010, 2011). Both sterols were found to increase the lipid bilayer thickness in unilamellar liposomes prepared from phosphatidylcholines with saturated or monounsaturated acyl chains, as well as from phosphatidylcholines isolated from hen eggs. Surprisingly, very small differences were found in effects of these two sterols on bilayers, though the influence of sitosterol on humans spans rather a broad range of activities (see Gallová *et al.* (2011) for references).

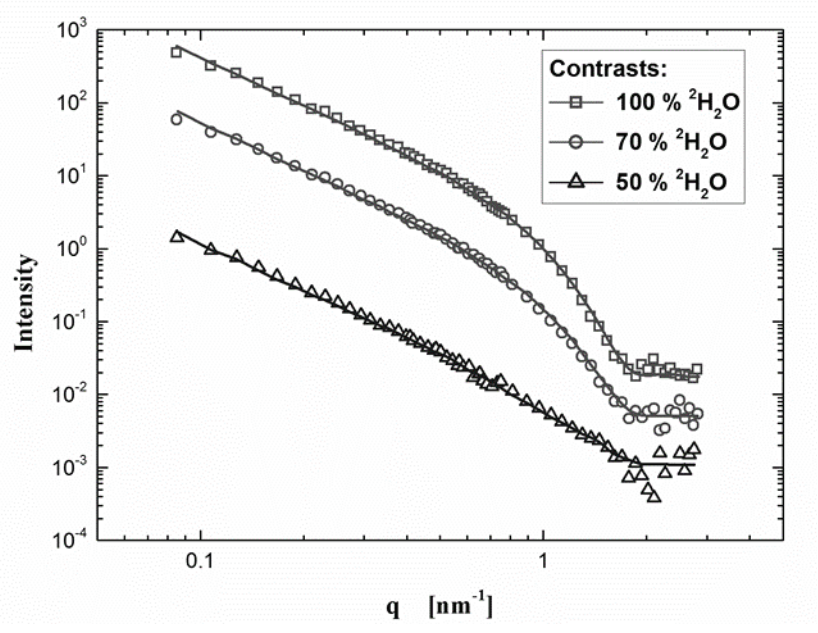

Fig. 3. Curves of small-angle scattering of neutrons on unilamellar liposomes formed by a mixture of PCPS (96 % DOPC + 4 % DOPS) and C10OH in a molar ratio of $n_{C10OH}:n_{PCPS}$ *= 0.4 at a temperature of 20°C. The corresponding contrast values are as follows: 100 % (squares), 70 % (circles), and 50 % (triangles). The respective solid lines represent the result of the evaluation of the contrast variation method using the three-layer model. The scattering curves are mutually shifted vertically for better visibility. Adapted from Klacsová* et al. *(2011). DOPC: dioleoylphosphatidylcholine; DOPS: dioleoylphosphatidylserine; PCPS:*

Advanced models

By adding additional layers, the models described above can be further modified to reflect the desired structure in the lipid bilayer (methyl or methine groups, etc.). However, as stated in the paper of Kučerka *et al.* (2004b), such refinements will no longer bring fundamentally new information. In recent years, new models have been developed, in which the distributions of individual functional molecular groups such as glycerol, phosphate, etc., are directly parameterised (Kučerka *et al.*, 2008). Combination of the experimental data of neutron scattering with scattering and diffraction of synchrotron radiation and their simultaneous evaluation thus provide very detailed and accurate data on lipid bilayers. One of the first systematic studies in this direction has been published in paper by Kučerka *et al.* (2009). The authors have studied the structural properties of monounsaturated diacylphosphatidylcholine lipid bilayers (diCn:1PC, where n = 14, 16, 18, 20, 22, and 24 is the number of acyl chain carbons). High-resolution x-ray scattering data were analyzed in conjunction with contrast-varied SANS data using techniques developed by Kučerka *et al.* (2008). Analyses of the data show that the manner by which bilayer thickness increases with increasing acyl chain length in diCn:1PC bilayers is dependent on the double bond's position. For commonly available monounsaturated diCn:1PCs, this results in the nonlinear behaviour of both bilayer thickness and lipid interface area, whereas for diC18:1PC bilayers, lipid area assumes a maximum value. These experimental results were supported by MD simulations of bilayers. Further results are described in a review paper by Heberle *et al.* (2012).

CONCLUSION

The use of neutrons in pharmaceutical research described in this article represents only a small portion of possible applications, which are now commonly used. The limited scope of the publication allowed only a brief presentation of some of the experimental results obtained at the Faculty of Pharmacy, Comenius University in Bratislava. Important are studies of raft and domain formation in bilayers per-

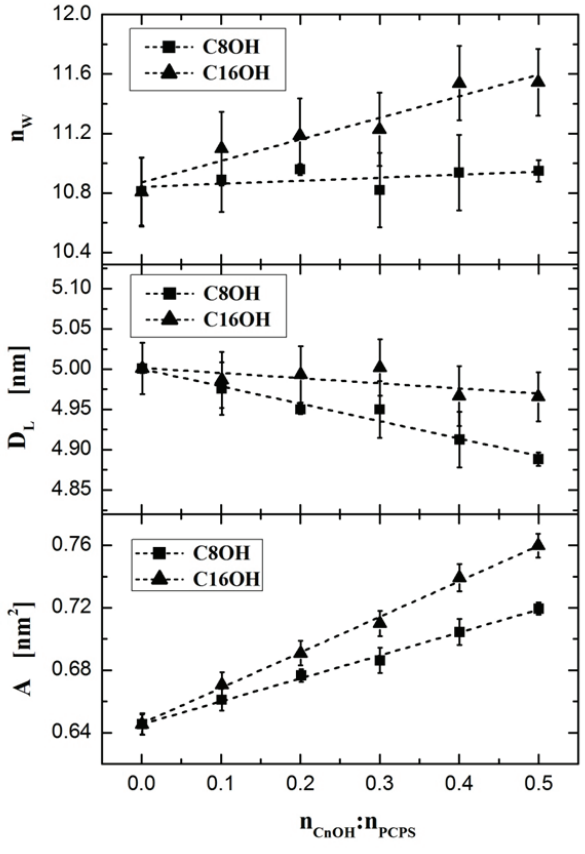

Fig. 4. Dependences of the surface area of the primitive cell (A)*, the total thickness of the bilayer* (D_L)*, and the number of water molecules in the hydrophilic area of bilayers per lipid molecule* (n_W) *on the molar ratio* of CnOH : PCPS *for* n = 8 *(black symbols) and n = 16 (red symbols) at 20°C. The dashed lines represent weighted linear fits. Adapted from Klacsová et al. (2011).*

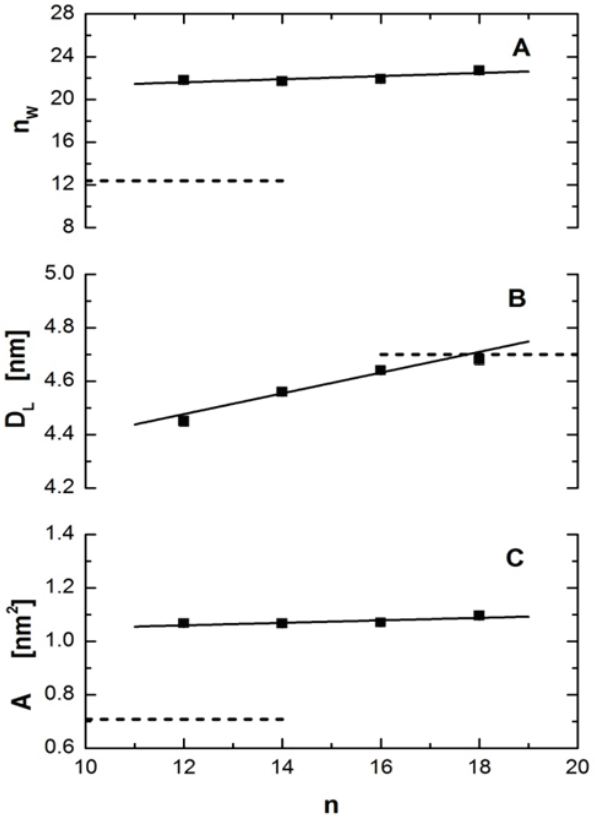

Fig. 5. Dependences of the surface area of the primitive cell (A)*, the total thickness of the bilayer* (D_L)*, and the number of water molecules in the hydrophilic area of bilayers per lipid molecule* (n_W) *on the alkyl length n of CnNO molecules at CnNO : DOPC = 1 : 1 molar ratio and 20°C. The solid lines represent weighted linear fits, the dashed lines the control sample. DOPC: dioleoylphosphatidylcholine*

formed by N. Kučerka in cooperation with groups in USA and Canada (Pencer *et al.* 2007, Armstrong *et al.* 2013, Heberle *et al.* 2013), studies of DNA interaction with lipid bilayers (Uhríková *et al.*, 2009, 2012a, 2012b, Uhríková, 2014, Uhríková & Pullmannová, 2014), interaction of metal ions with bilayers (Uhríková *et al.*, 2008, Hubčík *et al.*, 2012, Kučerka *et al.*, 2013, 2014) and neutron specular reflectometry of floating bilayers (Belička *et al.* 2014). Important are studies of molecular drug structure and dynamics using quasielastic neutron scattering in combination with other experimental techniques and theoretical calculations performed by the group of Prof. J. Wasicki in Poznan in Poland (see Pajzderska *et al.* (2014) for references). High quality neutron scattering research is also conducted at the Faculty of Pharmacy of Charles University in Hradec Králové, Czech Republic. The reader is therefore recommended to become familiar with its results published in a recent review article on drug–skin interaction (Zbytovská & Vávrová, 2011). In the near future, we plan to publish a tutorial review paper, focusing on issues concerning the use of neutron reflectometry and neutron spin echo in pharmaceutical research based on results obtained in our laboratories.

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