**Recent Progress in Biophysical Research of Biological Membrane Systems**

**Review**

Evaluation of Interbilayer and Transbilayer Transfer Dynamics of Phospholipids Using Time-Resolved Small-Angle Neutron Scattering

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The bilayer structure of biomembranes consists of thousands of lipids, the composition of which is different for each organelle. Since most lipids are synthesized in the endoplasmic reticulum, subsequent distribution to each organelle determines the composition and function of the biomembranes. Thus, interbilayer transfer and transbilayer movement (flip-flop) of phospholipids play important roles in maintaining homeostasis. A crucial task in biophysics and cell biology is to understand how rapidly lipids migrate between bilayers spontaneously or through proteins and to control these lipid dynamics. Time-resolved small-angle neutron scattering (TR-SANS) is a powerful technique to determine the intervesicular exchange and flip-flop rates of lipids in situ and real time. In this review, I explain how TR-SANS detects the interbilayer and transbilayer transfer of phospholipids and introduce recent progress of my group on the evaluation of spontaneous and protein- (or peptide-) mediated lipid transfer in several phospholipid dispersion systems.

**Key words** phospholipid; lipid transfer; flip-flop; neutron scattering

1. Introduction

Most intracellular lipids are synthesized in the endoplasmic reticulum (ER) and transported to the plasma membrane (PM) and other organelles via lipid transfer proteins and transport vesicles. In the PM several phospholipids are distributed asymmetrically between the cytoplasmic side (for phosphatidylycerine and phosphatidylethanolamine) and extracellular side (for sphingolipids) of the bilayer. Several groups of membrane proteins in the PM control the transbilayer movement (flip-flop) of phospholipids: the asymmetric lipid composition of the PM is achieved via energy-dependent processes by the activities of P4ATPases (flippases) and ATP binding cassette (ABC) transporters (flopases). Disruption of the asymmetry by phospholipid scramblases is involved in apoptotic cell death or platelet-dependent coagulation, and resultant exposure of phosphatidylserine to the extracellular leaflet of apoptotic cells is recognized by macrophages. On the contrary, the ER membranes are believed to have symmetric lipid composition between two leaflets in normal state. Phospholipids in eukaryotic cells are mainly synthesized at the cytoplasmic leaflet of the ER membrane. Newly synthesized phospholipids must be promptly transferred to the luminal leaflet of the membrane so as to maintain membrane integrity. This is done conceivably by the action of unidentified ER scramblases. In this way, control of the membrane lipid dynamics plays an important role in maintaining homeostasis of living cells.

However, quantitative comprehension of the lipid dynamics remains obscure. This is largely due to a lack of techniques that reasonably and easily measure the interbilayer and transbilayer lipid transfers. For example, Wimley and Thompson measured phospholipid transfer from donor vesicles to acceptor vesicles using a radioisotope-labeled lipid, but this method requires separation of the donor/acceptor particles by a procedure such as gel filtration or ultracentrifugation, and hence lipid transfer between particles identical in composition and size cannot be determined. Lipid transfer can be detected by means of a fluorescence quenching or fluorescence resonance energy transfer without the procedure of donor/acceptor particle separation. However, these methods only provide information on the dynamics of fluorescent lipids, which is largely dependent on the properties of the fluorophore. Anglin and Conboy have detected phospholipid flip-flop in a supported lipid bilayer by sum frequency generation spectroscopy; however, their observed flip-flop rate is remarkably faster than that obtained by other methods, suggesting that the dynamic properties of phospholipid bilayers may differ between the supported bilayers and free-standing vesicles.

Small-angle neutron scattering (SANS) is a method to extract a static structure on submicron scale. However, transient structural changes (due to temperature jump etc.) can be also tracked by time-resolved SANS (TR-SANS) technique. My group has succeeded in evaluating the dynamics of phospholipids in vesicles by TR-SANS. This method extracts dynamic information in equilibrium (steady) state by utilizing the difference in scattering between isotopes. This is unique to neutron scattering and cannot be done with X-rays. Although this method requires a relatively high concentration of sample, it requires neither the procedure of particle separation, nor fluorescence-labeling into lipids that changes physicochemical properties. In addition, it can determine the rates of both intervesicular exchange and flip-flop of phospholipids in situ and in real time. Therefore this method can measure these
dynamics of any lipid systems as far as deuterated lipids are available. In this review, I introduce several examples of phospholipid dispersion systems to which the TR-SANS methods were applied.

2. Principle of Lipid Dynamics Evaluation by SANS

Scattering of neutrons is caused by density fluctuation of the scattering length, which is inherent in each atom. The scattering length density (SLD) in neutron scattering corresponds to the dielectric constant (or refractive index) in light scattering and the electron density in X-ray scattering, but it is noteworthy that the SLD is greatly different between isotopes, especially, hydrogen (H) and deuterium (D). When vesicles consisting of normal (hydrogenated) lipids and of deuterated lipids (H-vesicle and D-vesicle, respectively) are mixed in a solvent (D₂O/H₂O) having an intermediate SLD, each particle initially has different SLD from that of the solvent and causes strong scattering. However, when these lipids migrate between vesicles and between leaflets within vesicles, exchange of D- and H-lipids in vesicles diminishes the difference in the SLD between particles and solvent, resulting in reduction of scattering intensity. In other words, by lipid exchange two kinds of “white” and “black” vesicles change to “gray,” which is the same color as the solvent, and become invisible when viewed by the eyes of neutrons (Fig. 1). In the absence of flip-flop, only the outer leaflet of the vesicles becomes invisible. Because the scattering intensity is proportional to the square of the contrast, i.e., the difference in the SLDs between vesicle and solvent, the contrast is given by the following equation using scattering intensity I(t):

\[
\frac{\Delta I(t)}{\Delta I(0)} = \frac{I(t) - I(\infty)}{I(0) - I(\infty)}
\]

(1)

where, the value is normalized by the initial contrast. The initial intensity, I(0), agrees with the average intensity of H-vesicle and D-vesicle, and the residual intensity at infinite time, I(∞), corresponds to the intensity of vesicles prepared from equimolar amounts of hydrogenated and deuterated lipids (D/H-vesicle). The temporal change in the contrast can be expressed by the following equation using the rate constants of the intervesicular exchange \(k_{ex}\) and the flip-flop \(k_f\) of lipids:

\[
\frac{\Delta \rho(t)}{\Delta \rho(0)} = \left\{ \frac{1}{2} - \frac{k_e}{X} \right\} \exp\left(-\frac{k_{ex} + 2k_f + X}{2} t\right) + \left\{ \frac{1}{2} + \frac{k_f}{X} \right\} \exp\left(-\frac{k_{ex} + 2k_f - X}{2} t\right)
\]

(2)

where, \(X = \sqrt{k_{ex}^2 + 4k_f^2}\). Although Eq. (2) is somewhat complicated, it is a double-exponential function consisting only of \(k_{ex}\) and \(k_f\). Therefore by tracing the SANS intensity change after mixing of D- and H-vesicles, two rate constants can be accurately calculated.

3. Dynamics of Various Phospholipids

1,2-Dimyristoylphosphatidylcholine (DMPC, Fig. 2A) has two 14-carbon acyl chains, which are slightly shorter than the acyl chain of the biological membrane lipids. DMPC vesicles (D-, H-, and D/H-vesicles) with a particle size approximately 120 nm were prepared by extrusion method using DMPC and DMPC-d₄, whose two acyl chains were fully deuterated, and SANS measurements were carried out in buffer containing 50% D₂O, which has an intermediate SLD (SLD\(\text{DMPC-d}_{4}=2.9 \times 10^{10} \text{ cm}^{-2}\)) between the SLDs of D-vesicle (SLD\(\text{DMPC-d}_{4}=5.4 \times 10^{10} \text{ cm}^{-2}\)) and H-vesicle (SLD\(\text{DMPC}=0.28 \times 10^{10} \text{ cm}^{-2}\)). As expected, D- and H-vesicle had strong and equivalent scattering intensity in 50% D₂O, and D/H-vesicle (prepared from 1:1 mixture of DMPC and DMPC-d₄) showed little scattering. The intensity profile against the scattering vector q, which is a function of the scattering angle 2θ and the neutron wavelength λ (q = 4π sin θ/λ), showed that the scattering intensity at lower angles (0.007 Å⁻¹ ≤ q ≤ 0.07 Å⁻¹) is proportional to \(q^2\), which is characteristic of the planar (bilayer) structure. Next, equimolar amount of D- and H-vesicles were mixed and subsequent SANS intensity change was followed by time-resolved measurement. The scattering intensity gradually decreased while the shape of the scattering curve remained unchanged, indicating spontaneous lipid exchange between the particles. From the scattering intensity at each time, the contrast was calculated using equation (1). First, since the contrast decay

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Fig. 1. Principle of the Detection of Lipid Exchange Dynamics by SANS

Vesicles consisting of deuterated lipid with high SLD (white) and of hydrogenated lipid with low SLD (black) are visible, whereas exchange between white and black lipids (intervesicular lipid exchange) alters the SLD of the outer leaflet of vesicles, which approaches the SLD of solvent (gray). Therefore, only inner leaflet of vesicles remains visible. Flip-flop further exchanges white and black lipids in the inner leaflet and changes its SLD, so that the whole vesicle becomes gray and invisible, which can be detected as a decrease in the SANS intensity.

Fig. 2. Chemical Structures of DMPC (A), POPC (B), POPA (C), and POPG (D)

Deuterated phospholipids with fully deuterated myristoyl groups (A) or palmitoyl group (B–D) are also used in this study.
profiles did not depend on the phospholipid concentration, this suggests that spontaneous phospholipid exchange between particles is governed not by collision of vesicles but by diffusion of lipid molecules into aqueous phase. Second, since intervesicular lipid exchange takes place only on the outer leaflet of the vesicles, it reduces the contrast by half. However, the observed contrast decayed well below 0.5, suggesting that DMPC does flip-flop spontaneously. The fitting curves according to equation (2) considering both intervesicular transfer and flip-flop, satisfactorily reproduced the measured decay profiles, and the rate constants were obtained. The half-lives of the intervesicular transfer and the flip-flop of DMPC at 37°C were determined approximately 150 and 510 min, respectively.

Cholesterol is known to increase the packing (decrease the fluidity) of the membrane by its hard sterol skeleton. DMPC/cholesterol vesicles containing 20 or 40 mol% cholesterol were evaluated by TR-SANS. As a result, the flip-flop frequency of DMPC was greatly reduced with increasing the mole fraction of cholesterol, and was almost completely inhibited in the presence of 40 mol% cholesterol. A membrane containing 40 mol% cholesterol forms a liquid-ordered phase, in which the lipid mobility is markedly suppressed. This suggests that phospholipids cannot rotate in such hard membranes. On the other hand, the rate constant of the intervesicular exchange was not greatly decreased, suggesting that the increased membrane packing does not affect the transition state of the intervesicular transfer, where the membrane phospholipid dissociates into aqueous phase.

The cholesterol content in biological membranes varies widely depending on organelles; it is low (5%) in the ER membrane but comprises approximately 40% in the PM. Cholesterol's inhibitory action for the phospholipid flip-flop should be involved in maintaining high asymmetry on the PM. Unfortunately, dynamics of cholesterol itself cannot be evaluated by this method because highly deuterated cholesterol is not commercially available so far. It has been reported that intervesicular exchange of cholesterol is on the order of several hours, comparable to DMPC exchange, but the flip-flop occurs much more frequently on the order of milliseconds. Two hydrophobic chains of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC, Fig. 2B) are those (C16:0, C18:1) most frequently seen among the biological membrane lipids. Using vesicles (diameter, 120 nm) consisting of POPC or POPC-\(\beta\)CD, in which the palmitoyl chain was deuterated, TR-SANS measurements were carried out in buffer containing 30% D\(_2\)O, which has an intermediate SLD (SLD\(_{\text{POPC,}}\beta\text{-CD} = 1.5 \times 10^{-10}\text{cm}^{-2}\) between them (SLD\(_{\text{POPC,}}\beta\text{-CD} = 2.8 \times 10^{-10}\text{cm}^{-2}\) and SLD\(_{\text{POPC}} = 0.26 \times 10^{-10}\text{cm}^{-2}\)). As shown in Fig. 3, the intervesicular exchange of POPC was very slow, with a half-life of approximately 90 h, suggesting that longer hydrophobic chains (as compared with DMPC) increase the dissociation energy from the membrane into water. When methyl-\(\beta\)-cyclodextrin (M/\(\beta\)CD) was added to this system, intervesicular movement of lipids was promoted depending on M/\(\beta\)CD concentration. This indicates that M/\(\beta\)CD forms clathrate with hydrophobic chain(s) of the phospholipid, thereby promoting its intervesicular movement. After long-time incubation the contrast reached a constant value of about 0.55, but it did not decrease further. This clearly indicates that POPC does not exhibit flip-flop. When a phospholipid flips, its polar headgroup has to pass through the hydrophobic region of the bilayer, which is energetically unfavorable. Because POPC has a longer acyl chain than DMPC and thereby, forms bilayers with higher hydrophobic thickness, POPC's headgroup can hardly cross this region. Therefore it is considered that phosphatidylcholine in biological membranes does not flip-flop spontaneously.

1-Palmitoyl-2-oleoylphosphatidic acid (POPA, Fig. 2C) and 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG, Fig. 2D) have the same acyl chain composition as POPC and different headgroup. While POPC has a large zwitterionic headgroup, POPA and POPG have smaller headgroup containing only negative charge of phosphate group. These differences in the headgroup structure are expected to suppress the intervesicular transfer and conversely promote the flip-flop of POPA and POPG. TR-SANS measurements were performed using POPA vesicles and POPG vesicles. Results for POPG vesicles are shown in Fig. 3. These lipids showed no intervesicular exchange in the absence of M/\(\beta\)CD. Meanwhile, flip-flop was observed in the presence of M/\(\beta\)CD, judging from the contrast decay toward less than 0.5. The half-lives of flip-flop for POPA and POPG at 37°C were approximately 420 and 230 min, respectively. Thus the dynamic characteristics of phospholipids were found to vary greatly depending on the type of headgroup and length of the acyl chains.

4. Dynamics of Phospholipid Bilayer Nanodisc

Nanodisc is a phospholipid–protein complex that is relevant to nascent high-density lipoproteins and is applicable as a drug carrier and tool to immobilize membrane proteins. Although nanodisc consists of a lipid bilayer, the physicochemical properties of the bilayer surrounded by amphipathic \(\alpha\)-helices of apolipoproteins should be different from those of lipid bilayer of vesicles. Indeed, nanodiscs consisting of DMPC and apolipoprotein A-I had tighter lipid packing state than DMPC vesicles, as suggested by fluorescent anisotropy. TR-SANS was conducted for 1:1 mixture of nanodiscs consisting of DMPC and of DMPC-\(d_{14}\). Unlike for vesicles, flip-flops cannot be detected because of no distinction between

Fig. 3. Time–Course of the Contrast for POPC Vesicles (Circles) and POPG Vesicles (Squares) after Mixing D- and H-Vesicles (15 + 15 mM) at 37°C in the Absence (Open Symbols) and Presence (Closed Symbols) of 1 mM M/\(\beta\)CD.

Data for POPC were reproduced from ref. 11.
inner and outer leaflets of the bilayer in nanodisc. Indeed, the time-course of the contrast was decreased with single exponential that reflects only lipid exchange. Importantly, DMPC nanodiscs represented approximately 20-fold higher lipid exchange than DMPC vesicles. Kinetic analysis at different temperatures revealed that while the activation entropy of lipid exchange was negative for DMPC vesicles, it was positive for nanodiscs. This suggests that the entropic constraints of the closer packed bilayer are responsible for the accelerated dissociation of phospholipids from nanodiscs as compared with vesicles. The same is observable of the dissociation of cholesterol, i.e., cholesterol in nanodiscs exhibits faster dissociation than that in vesicles because tighter membrane packing in nanodiscs destabilizes lipids entropically.\(^{17}\)

5. Effects of Transmembrane Helices on Phospholipid Flip-Flop

As described earlier, half of the phospholipids (and their precursors) synthesized on the cytoplasmic leaflet of the ER membrane should be translocated to the luminal leaflet rapidly to maintain mass balance between two leaflets. It is believed that this process is accomplished by ER scramblases, which mediate the transbilayer lipid transfer bidirectionally and energy-independently. However, the ER scramblases have not yet been identified\(^{16}\) and the detailed mechanisms remain to be clarified. It has been reported that peptides that mimic α-helices of transmembrane proteins can stimulate flip-flop of fluorescence-labeled phospholipids in vesicles,\(^{29}\) which indicates that the ability to catalyze flip-flop in the ER is not necessarily restricted to one specific protein. Therefore based on a hypothesis that fast flip-flop in the ER is achieved by a mere presence of transmembrane helices of several membrane proteins, TR-SANS was applied to POPC membranes with model transmembrane peptides to discover whether the peptides induce flip-flop.\(^{20}\) Vesicles were prepared with POPC (H-vesicle) and POPC-d\(^{13}\) (D-vesicle) in the absence and presence of 0.4 mol% transmembrane peptides. TR-SANS measurement was started immediately after mixing Sec14 and equivalent volume of D- and H-vesicles (15 ± 15 mM) in buffer with 30% D\(_2\)O. The contrast decayed toward less than 0.5. This result suggests that simple insertion of transmembrane helices of short peptides into the membrane bilayer leads to fast flip-flop in the ER. Importantly, the hydrophobic (polarity) of the central residue of the transmembrane peptides plays an important role in the presentation of the scramblase activity. Recent study with fluorescence-labeled phospholipids also demonstrated flip-flop promotion by peptides depending on the central residues and positive correlation between the flip-flop promotion ability and the polarity around the center of the peptide in the bilayer.\(^{21}\)

6. Determination of Protein-Mediated Intervesicular Phospholipid Transfer

As described above, POPC represents very slow dynamics, \(i.e.,\) the half-life of the intervesicular transfer is ca. 90 h and this lipid does not flip-flop spontaneously, and MβCD can mediate and accelerate the lipid exchange.\(^{11}\) Thus using proteins instead of MβCD, TR-SANS can determine phospholipid transfer activity of the proteins.

Sec14, a yeast phosphatidylinositol/phosphatidylinositol transfer protein, shows transport activity of phosphatidylinositol and phosphatidylcholine in vitro.\(^{22}\) This protein is involved in regulation of lipid metabolism and vesicle transport of lipids and proteins from the trans-Golgi network.\(^{22,23}\) However, the details of the lipid transport mechanism of Sec14 and the relation between lipid transport activity and intracellular function are not clear. Hence TR-SANS experiments were carried out for quantitative analysis of Sec14-mediated transfer of phospholipids.\(^{24}\)

D- and H-vesicles (d~120 nm) were prepared using POPC and POPC-d\(^{13}\), and TR-SANS measurement was started immediately after mixing Sec14 and equivalent volume of D- and H-vesicles (15 ± 15 mM) in buffer with 30% D\(_2\)O. The contrast decayed toward less than 0.5. This result suggests that simple insertion of transmembrane helices of short peptides into the membrane bilayer leads to fast flip-flop in the ER. Similar measurements were carried out using smaller POPC vesicles with a particle size of approximately 40 nm, and remarkably rapid decay in the SANS intensity (contrast) was observed. Combination with the vesicle floatation assay demonstrated that Sec14 recognizes the membrane curvature and selectively binds on and exchanges lipids in smaller vesicles.

7. Conclusion

I introduced the method and examples of evaluating phospholipid transfer using the unique features of SANS. This is a powerful tool to determine the rates of intervesicular exchange and flip-flop of lipids \emph{in situ} and real time. This technique is expected in the future to be applied for elucidation of the function of proteins involved in lipid transfer, such as lipid transfer proteins (as introduced in this review), flippases, floppases, and scramblases.

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