Feasibility of complementary use of neutron and X-ray scattering techniques in research of lipid mixtures

Mitsuhiro Hirai

Department of Physics, Gunma University, Maebashi 371-8510, Japan

mhirai@fs.aramaki.gunma-u.ac.jp

Abstract. It is well recognized that the complementary use of X-ray and neutron small-angle scattering methods serve us fruitful information on nano-scale structures of materials at different phases, especially for systems composed of different components in solutions. This report briefly reviews some recent applications of X-ray and neutron scattering methods of the solutions of lipid mixtures composed of glycosphingolipid, cholesterol and phospholipid. The applications presented here would be very useful and feasible for studies of membrane interfaces in many cases. One of the most promising methods, called "spin contrast variation", is also introduced in comparison with other conventional methods.

1. Introduction

On the application of X-ray and neutron scattering techniques to the structural studies of biological materials, the complementary information obtained by X-ray and neutron is quite useful. The scattering amplitude of an atom depends on the number of the electrons within the atom for X-ray. On the other hand that for neutron depends on the nuclear potential, magnetic moment and nuclear spin of the atom. Alternatively the scattering amplitude of a particle depends on the electron density for X-ray and on the scattering length density for neutron. Most of biological materials are not composed of elements with magnetic moments. Table 1 lists the values of the neutron scattering amplitudes for relevant nuclei relating to biological systems, in comparison with those for X-ray. The effective scattering amplitude (the average excess scattering amplitude) of a particle occluded in a matrix, such as in water solvents, is called "contrast" that depends on the difference between the average scattering amplitudes of the particle and the matrix. Therefore, by the complementary use of X-ray and neutron we are able to observe the structures of the particle at different two phases, one for X-ray and the other for neutron, which is called "source contrast variation". In the following sections the concept of "contrast" and a few of the prominent contrast variation methods are reviewed briefly, and some of the recent applications to the lipid membrane studies are introduced.

2. Concept of contrast variation methods

Stuhrmann et al. [1, 2] established the concept of "contrast". In the neutron solution scattering method of biological materials different types of "contrast variation method" were developed by using an isotope effect between hydrogen (H) and deuterium (D) in scattering amplitude [3-5]. As shown in Table 1, the nuclear scattering amplitudes and the spin-dependent scattering amplitudes for H and D are quite different. When polarized neutron and dynamic nuclear-spin polarization (DNP) instruments are available, we are able to vary the "contrast" by using the term of the spin-dependent scattering...
amplitude and to get a large gain of the measurable scattering intensity. This method is called "spin contrast variation" [6]. Figure 1 shows the simulated values of the average scattering amplitudes of relevant biological components depending on the neutron-proton spin polarization. The neutron-proton spin polarization $P_N = \{ P_N \}$ is assumed to be 1. In the calculations the following equation of the coherent scattering length $F$ of proton depending on proton spin polarization is used.

$$F = \left\{ b_{coh}^2 + B^2 I^2 P_H^2 + 2b_{coh} B P_H \cdot P_N \right\}^{1/2}$$

where the parameters in the above equation are given in Table 1. In Fig. 1 the scattering densities of protein and DNA are calculated based on the chemical compositions of bovine albumin and A-T-G-C, respectively. For example, at $P_H \cdot P_N = -1$ the average scattering amplitude of the protein is about three times higher than that at $P_H \cdot P_N = 0$ (for the ordinary measurements using unpolarized neutron and proton), namely the gain factor of the scattering intensity is around 10 times higher than that for the ordinary measurements. This method needs a polarized neutron scattering optics and a dynamic nuclear spin polarization technique. Although there are only a few experiments using the spin contrast variation [7-10], this method would become a key method at a future neutron source to analyze complex systems composed of various elements such as protein, lipid and so on.

| nucleus | spin $I$ | $b_{coh}$ (10^{-12} cm) | $B$ (10^{-12} cm) | $\alpha_{inc}$ (10^{-24} cm^2) | $f_{x-ray}$ (10^{-12} cm) |
|---------|---------|-----------------|-----------------|----------------|-----------------|
| $^1$H   | 1/2     | -0.374          | 2.912           | 79.9           | 0.28            |
| $^2$H (D) | 1      | 0.667           | 0.285           | 2.04           | 0.28            |
| $^{12}$C | 1/2     | 0.665           | 0               | 0              | 1.69            |
| $^{14}$N | 0       | 0.937           | 0.14            | 0.49           | 1.97            |
| $^{16}$O | 1/2     | 0.580           | 0               | 0              | 2.25            |
| $^{31}$P | 1/2     | 0.517           | 0.026           | 0.006          | 4.23            |
| $^{32}$S | 0       | 0.285           | 0               | 0              | 4.5             |

Table 1. Neutron and X-ray scattering amplitudes of some relevant atoms, where $b_{coh}$ isotropic nuclear coherent scattering length; $B$ spin-dependent scattering length; $\alpha_{inc}$ incoherent scattering cross section; $f_{x-ray}$ scattering amplitude for X-ray (1 Å).

Fig. 1. Average scattering densities of relevant components of biological materials depending on proton-spin polarization $P_H = \{ P_H \}$ when the neutron-proton spin polarization $P_N = \{ P_N \}$ is 1. The arrow at $P_H = 0$ indicates the contrast at $P_H \cdot P_N = 0$ corresponding to the ordinary measurements using unpolarized neutron and proton; the arrow at $P_H = -1$, the contrast at $P_H \cdot P_N = -1$ corresponding to the polarized neutron scattering measurements using polarized protons whose spin directions are antiparallel to the neutron-proton ones. In "spin contrast variation method", the contrast is varied by proton-spin polarization using a dynamic nuclear-spin polarization technique.
As is well known, the use of the difference between the nuclear scattering amplitudes of H and D is much feasible to vary the contrast of a solute particle by changing the H$_2$O-D$_2$O ratio in a solvent (so-called "solvent contrast variation method" [2]) or by varying the ratio between the H and D atoms within the solute particle, namely, by the deuteration of the particle (so-called "inverse contrast variation method" [4]). The former and latter methods are summarized in Fig. 2, where (A) shows the average scattering densities of relevant biological molecules and (B) shows the concepts of both methods in the case of lipid bilayer systems. The merit of the former method is easiness in sample preparations, and the demerit is an unavoidable change of signal-to-noise ratio of observed scattering data depending on H-D ratio in a solvent, which results from the large value of the incoherent scattering cross section of H as shown in Table 1. On the other hand, although the deuteration of some components and the reconstitution of the sample with deuterated and undeuterated components are necessary, the inverse contrast variation method is more useful to avoid such an artifact and to obtain high-statistic scattering data. The merit of the later method is important for analyzing scattering data by using model scattering functions. We have successfully applied this method to lipid mixtures as shown below.

![Fig. 2. (A), Average scattering densities $\rho$ of relevant biological components for neutron and X-ray, where the thick arrows are indicate how to change the contrast of alkyl chain (CH$_2$) of fatty acid in solvent contrast variation and inverse contrast variation methods; (B), schematic difference between these methods for a model structure of lipid bilayer composed of deuterated and undeuterated alkyl chains.]

**3. Application of inverse contrast variation and source contrast variation**

By use of density gradient centrifugation and immunoprecipitation methods, lipid microdomains formed in mammalian plasma membrane were found to be associated with various molecules involved in signal transduction and pathology [11-13], and are now considered to be crucial for regulating signal cascade [13]. However, the physicochemical mechanism of the formation of lipid microdomains, so-called "lipid rafts" [15, 16], is still at issue. A common feature of the microdomains is their peculiar lipid composition, being rich in glycosphingolipids (GSLs), sphingomyelin and cholesterol. Gangliosides treated in our studies are major components of GSLs, which are acidic lipids composed of a ceramide linked to an oligosaccharide chain containing one or more sialic acid residues. The function of GSL microdomains is assumed to be attributable to the peculiar features of the ganglioside molecules both in their ceramide and oligosaccharide portions that act as hydrogen bond donor and acceptor [17]. In spite of a rapid expanding of biochemical and immunological studies of lipid rafts, the physicochemical properties of GSL-molecules and their complex with other lipids have been still ambiguous. By using X-ray and neutron scattering methods (small-angle neutron scattering, SANS; small-angle X-ray scattering, SAXS; neutron spin-echo, NSE) we have been studying the structure and dynamics of gangliosides and those aggregates with other lipids [18-28]. Due to the presence of the huge hydrophilic head portion, gangliosides and those mixtures with cholesterol and phospholipid show notable characteristics and phase behaviors as shown previously [17-28].
the form factor of the are modified from those in the reference [26].

M1 - phosholipid ternary mixtures, where we used the inverse contrast variation method and the source contrast variation, complementary [26, 27]. Fig. 3A shows the SANS profile of the small uni-lamellar vesicle (SUV) of monosialoganglioside (G₄₃) - dipalmitoylphosphocholine (DPPC) mixture (molar ratio of [G₄₃]/[DPPC] = 0.1/1) depending on the change of the inverse contrast, where the contrast was varied by changing the molar ratio between deuterated and undeuterated DPPC as [d-DPPC]/[h-DPPC] = 1/0, 0.7/0.3, 0.3/0.7, 0/1. As shown in the insert of Fig. 3A, the above four samples with different inverse contrasts for neutron give the same SANS profile, indicating the completeness of the isotopic substitution of d-DPPC and h-DPPC, namely, the successful preparation of the deuterated samples. In other words the scattering profiles in Fig. 3 correspond to those of the same SUV at five different phases (one for X-ray, four for neutron). Then we were able to fit all experimental SANS and SAXS profiles by use of the model scattering function with detail internal structures as shown below.

The scattering function \( I(q) \) of the SUV solution with a size distribution is given by

\[
I(q) \propto \int_{R_{\text{min}}}^{\infty} I_s(q,R)D(R)dR
\]

where \( D(R) \), the size distribution function of the SUVs with radius \( R \); \( I_s(q,R) \), the form factor of the SUV with radius \( R \); \( R_{\text{min}} \), a minimum particle radius defined by the SUV bilayer thickness, namely the lengths of the head and tail portions of lipid molecules. When the structure of the SUV is simplified as a particle consisting of spherical shells with different scattering densities, the form factor \( I_s(q,R) \) of the SUV is given by

\[
I_s(q,R) = 9\left[ \bar{p}_1 V_1 j_1(qR_1)/(qR_1) + \sum_{i=2}^{n} (\bar{p}_i - \bar{p}_{i-1}) V_i j_i(qR_i)/(qR_i) \right]^2
\]

In many cases of SUV systems [30], the \( D(R) \) function is given by a Gaussian distribution function as

\[
D(R) = \frac{1}{\sqrt{2\pi}\sigma} \exp\left( -\frac{(R - \bar{R})^2}{2\sigma^2} \right)
\]

Fig. 3. (A), Experimental SANS profile of \([G_{43}] / [\text{DPPC}] = 0.1/1\) SUV depending on inverse contrast variation, namely on the change of the molar ratio between deuterated and undeuterated DPPC as [d-DPPC]/[h-DPPC] = 1/0, 0.7/0.3, 0.3/0.7, 0/1, insert: experimental SAXS profiles of the above four SUVs; (B), best-fitted theoretical scattering functions superposed the experimental data in Fig. 3A, where the upper insert shows the theoretical and experimental X-ray ones. The size distribution functions obtained by the DLS measurement and the modelling analysis are also shown in the lower insert. These figures are modified from those in the reference [26].
where $\bar{R}$ and $\sigma$ are the average radius and its standard deviation, respectively. Based on the above equations and the SUV model structure as shown in Fig. 4A, we were able to fit all SANS and SAXS profiles in Fig. 3A by a unique model as shown in Fig. 3B. The size distribution obtained by the fitting is also in good agreement with that obtained by the dynamic light scattering (DLS) measurement. As shown in Fig. 4, the initial SUV model possessed a symmetric bilayer consisting of six shells that consider the protrusion of the hydrophilic heads of $\text{GM}_{1}$ molecules from both sides of the bilayer surfaces. However, such a symmetric bilayer model hardly explained all scattering profiles consistently. The final model optimized by the least-square fitting, shown in Fig. 4B, clearly shows an asymmetric bilayer structure in which $\text{GM}_{1}$ molecules preferentially locate at the outer-leaflet of the bilayer [26].

![Diagram](image)

**Fig. 4.** (A), Initial SUV model consisting of six shells adopted for the model fitting analysis; (B), asymmetric bilayer structure determined by the modelling analysis shown in Fig. 3B, where the obtained structural parameters within the bilayer (thickness and contrast of each shell) are given. These figures are modified from the reference [26].

The above results evidently demonstrate that the scattering data of a sample at various phases obtained by the complementary use of X-ray and neutron scattering methods are able to avoid some ambiguity in modeling analyses and to refrain from difficulties in so-called "phase problems" for determining the structure in real space. The complementary use of the SANS inverse contrast variation and the source contrast variation of SANS and SAXS was also applicable to observe the microdomain formation of $\text{GM}_{1}$-cholesterol-rich region within the phospholipid bilayer [27].

As an example of simple applications of the source contrast variation, the permeability of water through the SUV bilayer of the lipid mixtures (dissialoganglioside ($\text{GD}_{3}$), cholesterol, L-α-phosphocholine (PC) = 0.1/0.1/1 [mol/mol]) was determined by using time-resolved SAXS and SANS measurements [29]. Figure 5 schematically shows the method we employed. After preparing the SUV sample in H$_2$O solvent, the SUV solution was mixed with D$_2$O solvent at an appropriate volume ratio. Just after the mixing, the time-resolved measurements were started. The H-D exchange reaction in the solvent is much faster than the H$_2$O-D$_2$O permeation through the SUV bilayer. Thus, it can be regarded that the core of the SUV contained only H$_2$O at the initial state. In addition the volume fraction of the SUVs in the solution was negligibly small, therefore, the H$_2$O-D$_2$O ratio was considered to be constant while the time-resolved measurements. Clearly, SAXS cannot monitor the H$_2$O-D$_2$O permeation through the SUV bilayer, whereas SANS can detect the change of H$_2$O-D$_2$O ratio within the water core of the SUV. It should be mentioned that the complementary use of X-ray and neutron is essentially important to distinguish between the permeation of water through the bilayer and some deformation of the SUV structure caused by an artifact. In our study using the scheme in Fig. 5 [29] the SANS profile of the SUV varied gradually and was asymptotic to the final one in ~12 hours.
whereas the SAXS profile held the initial one. Due to the SAXS data the possibility of some deformation of the SUV structure was eliminated. The permeability of water through the bilayer was determined directly to be \((9.1 \pm 0.9) \times 10^{-4} \text{ cm s}^{-1}\) by the time course of radius of gyration that is sensitive to a change of the contrast of the SUV for neutron.

![Diagram of change of contrast for neutron and X-ray](image)

Fig. 5. Scheme of complementary use of time-resolved SANS and SAXS for measuring the permeability of water through the lipid bilayer of SUV. This method was applied to ternary lipid mixture as a model of lipid raft [29].

4. Conclusion

In the above sections we have shown some feasibility and importance of the complementary use of SAXS and SANS methods in lipid membrane studies. To clarify functional properties of plasma membrane composed of various constituents such as lipids and proteins, direct observations of membrane interfaces in situ would be quite important. Both X-ray and neutron scattering measurements should be employed simultaneously especially for a complex system, which would allow us to observe experimental data of the same system at different phases and to determine the detailed structure by using a model with fine structures.

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