Location of Cholesterol in Liposomes by Using Small-angle X-ray Scattering (SAXS) Data and the Generalized Indirect Fourier Transformation (GIFT) Method

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Abstract: We investigated the location of cholesterol (Chol) in liposomes and its interaction with phospholipids using small-angle x-ray scattering (SAXS) data and applying the generalized indirect Fourier transformation (GIFT) method. The GIFT method has been applied to lamellar liquid crystal systems and it gives quantitative data on bilayer thickness, electron density profile, and membrane flexibility (Caillé parameter). When the GIFT method is applied to the SAXS data of dipalmitoylphosphatidylcholine (DPPC) alone (Chol [−]) or a DPPC/Chol = 7/3 mixed system (Chol [+] molar ratio), change in the bilayer thickness was insignificant in both systems. However, the electron density for the Chol (+) system was higher than that for the Chol (−) system at the location of hydrophilic groups of phospholipids, and whereas Caillé parameter value increased with temperature for the Chol (−) system, no significant change with temperature was observed in the Caillé parameter for the Chol (+) system. These results indicated that Chol is located in the vicinity of the hydrophilic group of the phospholipids and constricts the packing of the acyl chain of phospholipids in the bilayer.

Key words: small angle x-ray scattering (SAXS), generalized indirect Fourier transformation (GIFT), liposome, cholesterol
determine the structures of liquid crystal phases such as lamellar and hexagonal structures formed by amphiphilic molecules and to estimate the distance between pores in inorganic porous materials. Recently, the validity of the generalized indirect Fourier transformation (GIFT) method in analyses of scattering curves of colloidal dispersions has been reported. Information can be calculated about particles inside of micelles—for example, core-shell structure (form factor) and particle-particle interaction (structure factor)—in a model-free way. Furthermore, the GIFT method is reportedly applicable to both dilute and concentrated dispersion systems and it allows analysis of scattering curves obtained with a laboratory-scale SAXS camera without using large-scale apparatus for small-angle neutron scattering. This method has recently been applied to lamellar liquid crystal systems from which quantitative data on the number of lamellar layers in a pile, the thickness of the bilayer, and parameters concerning membrane flexibility in addition to bilayer spacing could be obtained from ordinary X-ray scattering data. The present study aimed to examine the location of Chol in liposomes and its interaction with phospholipids by extending the applicability of the GIFT method.

2 EXPERIMENTAL

2.1 Materials

The phospholipid used was phosphatidyl choline with a C₄₀ hydrocarbon chain (DPPC, 99% up, from NOF Co., Ltd., Tokyo, Japan). Chol (99% up, purchased from Wako Co., Ltd., Tokyo, Japan) and ultra-pure water were used as the additive and solvent, respectively.

2.2 Methods

DPPC alone (Chol[-]) or a mixture of DPPC and Chol (7:3 in molar ratio, Chol[+]) was dissolved in chloroform (99% up, Wako Co., Ltd.) in a round-bottom flask. The solvent was removed using a rotary evaporator, and the residue was obtained as a thin lipid film on the flask wall after being vacuum dried for 24 hr. Ultra-pure water was then added to the lipid film to give a lipid suspension at a concentration of 30 mM under stirring with a magnetic stirrer for more than 3 hr. The lipid suspension was then vortex-treated to hydrate the lipid thoroughly, yielding a liposome suspension.

X-ray scattering data were recorded on a SAXSess camera (Anton Paar Co., Ltd., Graz, Austria). The measuring system consisted of a W3830-sealed glass bulb X-ray source (PANalytical Co., Ltd., Almelo, Netherland; Cu-Kα [wavelength = 0.154 nm]), a multilayer film Goebel mirror, a block collimator, a semi-transparent beam stop, a vacuum-proof glass capillary cell, a TCS120 temperature controller, a vacuum housing, and an imaging plate detector. The measurable range of scattering vector $q$ was 0.06 < $q$/nm$^{-1}$ < 27. Samples were enclosed in a vacuum-tight thin quartz capillary and measured at several temperatures for 1 hr. The 2-dimensional scattering pattern was collected on an image plate detection system (Cyclone, PerkinElmer Inc., Massachusetts, USA) and was finally integrated into 1-dimensional scattering curves using SAXS Quant software (Anton Paar Co., Ltd.). All data were normalized to the same incident primary beam intensity for the transmission calibration and were corrected for background scattering from the capillary and the water for absolute intensity calibration. The analyses of scattering data were performed with a GIFT software package developed by Otto Glatter. This method is based on the simultaneous determination of the form factor and structure factor.

3 RESULTS and DISCUSSION

3.1 Theory

Experimentally obtained scattering curves can be expressed by the following equation:

$$I(q) = nP(q)S(q)$$  \hspace{1cm} Eq.1

where $n$ is the number density of particles, and $P(q)$ and $S(q)$ are, respectively, the form factor and structure factor. These factors provide information on the internal structure of the particles and particle-particle interaction, respectively. The value of $q$ provides the length of scattering vector and is given by Eq. 2.

$$q = (4\pi/\lambda)\sin(\Theta/2)$$  \hspace{1cm} Eq.2

where $\lambda$ is the wavelength of the X-ray (CuKα line = 0.154 nm) and $\Theta$ is the detection angle of scattered X-rays. When the target of measurement is a lamellar phase, the form factor $P(q)$ is given by Eq. 3 as a scattering function, $P_1(q)$, of the Lorentz factor in the direction of lamellar phase thickness $\Delta t$.

$$P(q) = (2\pi A / q^2)P_1(q)$$  \hspace{1cm} Eq.3

$A$ is the area of planar lamella and $P_1(q)$ is given by Eq. 4 as a Fourier transform of the normal bilayer pair distance distribution function (PDDP), $p_1(r)$. The function $p_1(r)$ in Eq. 4 is given by Eq. 5 as the self-correlation function of electron density fluctuation that is a function of distance, $r$, if the fluctuation at $r_1$ is $\Delta \rho(r_1)$.

$$P_1(q) = 2\int_0^\infty p_1(r)\cos(qr)dr$$  \hspace{1cm} Eq.4

$$p_1(r) = 2\int_0^\infty \Delta \rho(r)\Delta \rho(r_1+r)dr_1$$  \hspace{1cm} Eq.5

The structure factor, $S(q)$, can be expressed as Eq. 6 according to the modified Caillé theory.$^{22}$
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\[ S(q) = N + 2 \sum_{m=1}^{N-1} (N-m) \cos(mqd) \exp \left[ - \left( \frac{d}{2\pi} q^2 \eta \right) \right] \times (\pi n m)^{-d/(2\pi q^2 \eta)} \]

Eq. 6

Here, \( N \) is the average number of lamellar layers in a pile, \( \eta \) is the Caillé parameter that gives membrane flexibility, \( d \) is the spacing of bilayers, and \( \gamma \) is the Euler constant \( \gamma \approx 0.5772 \). The Caillé parameter can be written using the elasticity module, \( K_c \), and bulk module, \( B \), as follows:

\[ \eta_1 = q_1^2 k_B T / 8 \pi (K_c B)^{1/2} \]

Eq. 7

where \( q_1 = (2\pi / d) \).

3.2 SAXS results

Figure 1 shows the scattering curves of the single system DPPC (Chol(−)) and DPPC/Chol = 7/3 mixed system (molar ratio, Chol(+)). SAXS peaks of both samples were positioned at a \( q \) ratio of 1:2:3 owing to the lamellar structure with the identical face-to-face distance in the small-angle region. By contrast, the Chol(−) system also showed a narrow peak \( (q = 14.7 \text{ nm}^{-1}) \) and a broad peak \( (q = 15.2 \text{ nm}^{-1}) \) in the wide-angle region, which are characteristic of the \( L_a \) phase\(^{25,26} \), whereas the Chol(+) system showed a single broader peak. The addition of Chol to phospholipids compacts the packing of the phospholipid acyl chain, which increases the freedom of acyl chains because the range of the phase transition temperature is broadened and \( T_c \) starting point shifts toward lower temperatures\(^{11-13} \). In other words, the addition of Chol raises the freedom of the acyl chains, and the peak assigned to the gel disappears\(^{20} \).

3.3 Lipid bilayer thickness (PDDF)

Figure 2 shows the PDDF \((\rho_r)\) for the Chol(−)/Chol (+) systems. The PDDF for lamellar systems represents the distance between lipid bilayers as indicated in Fig. 2B. Bilayer thickness, \( D_{\text{max}} \), was determined using the convergence point of \( \rho_r(r) \) and was approximately 5.2 nm for both samples, irrespective of the presence or absence of Chol. This value is identical to that obtained by Frühwirth \textit{et al.}\(^{20} \).

3.4 Electron density profile (DECON profile)

PDDF \((\rho_r)\) corresponds to the convolution of the electron density profile of the mono-bilayer (Eq. 5). Hence, electron density profile—that is, DECON profile, \( \rho_t(r) \)—can be obtained via deconvolution of the PDDF. Figure 3 shows the results of calculations of the electron density profile for both samples. The DECON profile is the electron density profile for the lipid bilayer from its hydrophobic group terminal to the hydrophilic group terminal with the electron density of water as the zero ordinate, \( \rho_t(r) = 0 \), as shown in Fig. 3B. The DECON profile rose slowly with increasing \( r \)—namely, toward the hydrophilic part from the

Fig. 1 SAXS and WAXS curves of Chol(−) (solid line) and Chol(+) (dashed line) at 25°C.

Fig. 2 Profiles of lipid bilayer thickness for Chol(−) (solid line) and Chol(+) (dashed line) at 25°C (A) and schematic membrane model (B).

Fig. 3 Electron density profiles, \( \rho_t(r) \), (A) for Chol(−) (solid line) and Chol(+) (dashed line) at 25°C (B) and schematic membrane model.
hydrophobic part—and converged on the zero value at $r = \text{ca. 2.7 nm}$ (Fig. 3A). The electron density for the Chol (+) system was higher than that for the Chol (−) system at distances 1.0–1.5 nm from the hydrophobic group terminal. The electron density for Chol is reportedly higher than that for water. Hence, Chol is suggested to be localized in the region from the middle part of the hydrophobic groups of phospholipids to the vicinity of their hydrophilic groups (Fig. 3B).

Furthermore, the DECON profile in Fig. 3 obtained using the GIFT method is not quantitative—that is, the integration value of $d_r(r)$ is incomparable between Chol (+) and Chol (−). In addition, the $q$ range of Chol (+), which was used to calculate $d_r(r)$, is slightly different from that of Chol (−). Hence, the difference in whole electron density cannot be discussed.

3.5 Structure factor ($S(q)$) and modified Caillé theory (MCT) parameters

Figure 4 shows the values of $S(q)$ that give information on the interaction between bilayers, and Table 1 lists the values of the MCT parameters, respectively, at various temperatures for the Chol(−)/(+) system. Bilayer $N$ in Table 1 depends on the $q$ range for the GIFT method and does not indicate the actual bilayer number. In this study, $q$ ranging from 0.01 to 6 was used with the GIFT method. Hence, the bilayer number in Table 1 is appropriate.

The peak of $S(q)$ for the Chol (−) system attenuated and broadened with increasing temperature (left side of the figure). This outcome is attributed to the weakened interaction between bilayers caused by increased lipid fluidity at higher temperatures. The bilayer spacing, $d$, in Table 1 for the Chol(−) system was 7.4 nm at 40°C. Takeda et al. have reported that $d$-spacings in the rippled gel phase is larger than those in gel and liquid crystal. Furthermore, the Caillé parameter, $\eta_1$, which is the fluidity of bilayers increased with rising temperature, especially above the $T_c$ (41°C) of DPPC. These results were in good agreement with those in Fig. 4.

No significant change with temperature was observed in $S(q)$ or the values of the MCT parameters, $d$ and $\eta_1$, for the Chol(+) system (right side of Fig. 4 and Table 1). Specifically, the results obtained here are consistent with those reported previously that although the Chol in liposomes makes the acyl chains of phospholipids flexible, it gives strength to the lipid membranes owing to increased acyl chain packing. Thus, the results in Fig. 4 and Table 1 obtained using GIFT analysis fully reflect the role of Chol in phospholipid membranes. These combined results lead to the conclusion that membrane properties (interaction of Chol with phospholipids) can be determined by examining the $S(q)$ obtained using the GIFT analysis.

4 CONCLUSIONS

The location of cholesterol and its interaction with phospholipids in liposomes were examined with SAXS data using the GIFT analysis. The results indicated that cholesterol is located in the vicinity of the hydrophilic groups of the phospholipids instead of in the hydrophobic part of the lipid bilayers in liposomes. In addition, cholesterol in liposomes was suggested to tighten the packing of the acyl chains of phospholipids in the bilayers through its interaction with the chains. Recently, form factor and structure factor have been calculated by fitting some models—i.e., strip, hybrid Gaussian, and Gaussian-Lorentzian—to evaluate scattering data obtained using small-angle X-ray/neutron scattering. However, these profiles were obtained with the assumption of lamellar stacks and the form and structure factors could not be obtained simultaneously. Conversely, the GIFT method is based on a completely model-free form factor and a parameterized structure.
factor, and these parameters can be obtained simultaneously. Hence, the GIFT method is a powerful technique for analyzing bilayer membrane properties, and this study can be applied to the evaluation of solubilization location of drugs in vesicles or lamellar structures.

References


