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A detailed analysis of partial molecular volumes in DPPC/cholesterol binary bilayers

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ABSTRACT

We examined the volumetric behavior of the dipalmitoylphosphatidylcholine (DPPC)/cholesterol binary bilayer system with high accuracy and more cholesterol concentrations to reveal the detailed molecular states in the liquid-disordered (L_d) phase, the liquid-ordered (L_o) phase and the gel phase. We measured the average specific volume of the binary bilayer at several temperatures by the neutral flotation method and calculated the average volume per molecule to estimate the partial molecular volumes of DPPC and cholesterol in each phase. As a result, we found that the region with intermediate cholesterol concentrations showed a more complicated behavior than expected from simple coexistence of L_d and L_o domains. We also measured fluorescence decay of *trans*-parinaric acid (tPA) added into the binary bilayer with more cholesterol concentrations to get further insight into the cholesterol-induced formation of the L_o phase. On the basis of these results we discuss the molecular interaction between DPPC and cholesterol molecule in the L_o phase and the manner of L_d/L_o phase coexistence.

1. Introduction

Cholesterol, one of the major components in biological membranes, is known to be a modulator of their physicochemical properties, and its membrane effects have been intensively studied using various experimental techniques such as differential scanning calorimetry [1–3], X-ray diffraction [4–8], nuclear magnetic resonance [9–12], fluorescence spectroscopy [13–16] and computer simulations [17–21]. Since the recognition of the existence of cholesterol-containing functional microdomains called 'lipid rafts' [22–24], lipid researchers have focused on clarifying the role of cholesterol in the raft formation using binary or ternary lipid systems [25–27]. In these artificial lipid bilayer systems, addition of cholesterol induces formation of the liquid-ordered (L_o) phase, which is thought to represent the physicochemical state of the lipid raft [28–30].

It has been reported that the L_o phase has intermediate properties between the liquid-disordered (L_d) phase and the gel phase [31]. Phase diagrams of phospholipid/sterol binary systems, which give fundamental information on the effect of cholesterol on the properties of the L_o phase, have been proposed by several researchers [26,32–35]. However, the detailed mechanism of the cholesterol-induced L_o phase formation in the molecular level is still unclear.

Simple volumetric measurements are useful because they are able to give quantitative information on the volume of each molecule in a binary lipid bilayer. Greenwood et al. [36] examined the effect of cholesterol on the molecular packing in phospholipid/cholesterol mixed bilayers by the neutral flotation method. They estimated the partial molecular volumes of phospholipid and cholesterol in the L_d and L_o phases on the basis of the dependence of the average molecular volume on cholesterol mole fraction. However, perhaps due to not measuring enough concentrations and temperatures, they did not report an L_d/L_o coexistence region, which has been reported to exist [26,32–35].

In this study, we re-examined the volumetric behavior of dipalmitoylphosphatidylcholine (DPPC)/cholesterol binary membranes with more cholesterol concentrations and temperatures and with similar accuracies of the measured density, the cholesterol concentration, and the temperature to those in the previous work by Greenwood et al. [36]. As a result, some regions were clearly discernible in the average molecular volume vs. cholesterol mole fraction plot, especially at temperatures above the main transition temperature of the pure DPPC bilayer. The partial molecular volumes of DPPC and cholesterol were obtained and found to be in agreement with those obtained for fluid phases by Greenwood et al. [36], but in disagreement with older gel phase data [37] that were also compiled [36]. In addition, the dependence of the average molecular volume on cholesterol mole fraction deviated from linearity expected from simple coexistence of the L_d and L_o domains, giving a new insight into coexistence properties.

Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; L_d, liquid-disordered; L_o, liquid-ordered; D_2O, deuterium oxide; tPA, *trans*-parinaric acid

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2. Materials and methods

2.1. Materials

1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Deuterium oxide (D_2O) and *trans*-parinaric acid (tPA) were obtained from Cambridge Isotope Laboratories (Andover, MA) and Cayman Chemical (Ann Arbor, MI), respectively.

2.2. Buoyant density measurement

The specific volume of the DPPC/cholesterol binary bilayer $\overline{v}(x_c)$ was determined according to the neutral flotation method described by Greenwood et al. [36], where x_c is the cholesterol mole fraction. Briefly, DPPC and cholesterol were separately dissolved in chloroform/methanol (4 : 1, v/v) and mixed at an appropriate mole ratio. The obtained solution was dried at 60 °C under nitrogen flow and in vacuum, and the resulting lipid film was dissolved into an H_2O/D_2O solvent, the density of which was adjusted to be as near as possible to that of the bilayer. After centrifugation (19, 800$g \times 10 - 20$ min) in a temperature-controlled centrifuge (Kubota, Model 5922), an aliquot of an H_2O/D_2O solvent with either higher or lower density than that of the solvent in the centrifugation tube was added according to whether the bilayers sedimented or floated. This procedure was repeated until the change in density by the addition of the solvent became less than 5×10^{-4}g/cm^3. As the bilayers were distributed as a fairly narrow band after the final centrifugation, the density difference between vesicles must be smaller than 5×10^{-4}g/cm^3. We repeated the measurement at least three times by using the data obtained in the last measurement as an initial density in the next measurement to eliminate the error caused by the difference in solvent density between inside and outside of the multi-lamellar vesicle.

In order to obtain the data with high accuracy, we added more than 10μL of the solvent for the density adjustment and used the D_2O solution as fresh as possible to prevent contamination of H_2O. Moreover, we checked the temperature of the sample solution after centrifugation and adopted the data for the density analysis only when the measured temperature was within ±0.5 °C of the desired temperature.

Fig. 1 shows how to estimate the partial molecular volume of each molecule in the binary system. The procedure of the analysis is based on that described by Greenwood et al. [36]. We calculated the average volume per molecule $\overline{V}(x_c)$ from the obtained specific volume of the binary bilayer $\overline{v}(x_c)$ by:

$$\overline{V}(x_c) = \frac{\overline{v}(x_c)}{N_A}\{(1-x_c)M_L + x_c M_c\}, \tag{1}$$

where N_A, M_L and M_c are Avogadro's number, the molecular weight of DPPC and the molecular weight of cholesterol, respectively (Fig. 1B). In order to make the deviation from linearity clearly visible, we subtracted a straight line from $\overline{V}(x_c)$:

$$\overline{V}^*(x_c) = \overline{V}(x_c) - Cx_c, \tag{2}$$

where C is the gradient of the straight line fitted to the data at lower x_c region (Fig. 1C). Assuming that the partial molecular volumes of DPPC (V_{DPPC}) and cholesterol (V_{chol}) are constant in a phase, $\overline{V}^*(x_c)$ gives a straight line as

$$\begin{aligned}\overline{V}^*(x_c) &= (1-x_c)V_{DPPC} + x_c V_{chol} - Cx_c \\ &= -(V_{DPPC} - V_{chol} + C)x_c + V_{DPPC}.\end{aligned} \tag{3}$$

Therefore, we can estimate the partial molecular volumes in the phase as

$$V_{DPPC} = \overline{V}^*(0) \tag{4}$$

and

$$V_{chol} = \overline{V}^*(1) + C. \tag{5}$$

2.3. Fluorescence experiment

DPPC and cholesterol were dissolved in methanol and in hexane/2-propanol (3 : 2, v/v), respectively and mixed at an appropriate mole ratio. The fluorescence probe tPA in methanol was added at the DPPC/tPA mole ratio of 200:1 [16]. The obtained solution was dried under nitrogen flow and in vacuum, and the resulting lipid film was dissolved into distilled and deionized water. The final concentration of DPPC was 200 μM. The fluorescence experiments were performed with a FluoTime 200 spectrometer (PicoQuant). The excitation and emission wavelengths were 298 nm and 405 nm, respectively, and the temperature was adjusted to be 45 °C. The fluorescence decay curve $I(t)$ of tPA was analyzed by using a stretched exponential function derived based on a continuous distribution of lifetimes [38–40] as follows:

$$I(t) = \int_{-\infty}^{t} IRF(t) \sum_{i=1}^{n} A_i \exp\left\{-\left(\frac{t-t'}{\tau_i}\right)\right\}^{1/h_i} dt', \tag{6}$$

where $IRF(t)$ and τ_i are the impulse response function and the characteristic time scale of the decay, respectively. When the heterogeneity parameter h_i is 1, the decay is a simple exponential process.

When two stretched exponential functions ($n = 2$) were used to analyze the decay profile in the L_d/L_o coexistence region, the fractional fluorescence intensity of component 1 (domain fraction R_1) was numerically calculated by the following equation:

$$R_1 = \frac{\int_0^\infty A_1 \exp\{-(t/\tau_1)\}^{1/h_1} dt}{\int_0^\infty A_1 \exp\{-(t/\tau_1)\}^{1/h_1} dt + \int_0^\infty A_2 \exp\{-(t/\tau_2)\}^{1/h_2} dt}. \tag{7}$$

3. Results

3.1. Detailed volumetric behavior of DPPC/cholesterol binary bilayers

In order to examine the molecular interaction in DPPC/cholesterol binary bilayers, we scrutinized the dependence of their specific volume on cholesterol mole fraction x_c at constant temperature by the neutral flotation method (Fig. 2). We measured the specific volume of the binary bilayer $\overline{v}(x_c)$ with more cholesterol concentrations (the x_c interval of about 0.03) than in previous studies to quantitatively analyze the molecular volume behavior (Fig. 2A and B), and calculated the molecular volume deviating from an appropriate straight line $\overline{V}^*(x_c)$ as described in Materials and methods (Fig. 2C and D). Although in principle, we had better use the molecular volume for the analysis of the phase behavior rather than the specific volume of the bilayer, the latter can be helpful especially in the assignment of the phase boundary. We divided the obtained specific volume profiles into three regions based on the break points and the linearity.

At temperatures above the main transition temperature of the pure DPPC bilayer ($T_m = 41.5$ °C [1–3]), regions I ($x_c < x_1$) and III ($x_c > x_2$) are the regions where $\overline{V}^*(x_c)$ showed linearity at the lower and higher x_c ends, respectively (Fig. 2) and region II is in between ($x_1 < x_c < x_2$). These three regions correspond to the L_d phase, the L_d to L_o transition region and the L_o phase, respectively [26,32–35]. The volumetric behaviors shared the fundamental characteristics, irrespective of the temperature; (1) $\overline{v}(x_c)$ in the region I (the L_d phase) decreased with increasing x_c, (2) $\overline{v}(x_c)$ in the region III (L_o phase) increased with increasing x_c and (3) $\overline{V}^*(x_c)$ in the region II was located below the straight line connecting the data points at x_1 and x_2, indicating the volumetric behavior in the