Cholesterol-Phospholipid Interactions: New Insights from Surface X-Ray Scattering Data

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We report a structural study of cholesterol-DPPC (1,2-dipalmitoyl-sn-glycero-3-phophocholine) monolayers using x-ray reflectivity and grazing incidence x-ray diffraction. Reflectivity reveals that the vertical position of cholesterol relative to phospholipids strongly depends on its mole fraction (χ_{CHOL}). Moreover, we find that at a broad range of χ_{CHOL} cholesterol and DPPC form alloylike mixed domains of short-range order and the same stoichiometry as that of the film. Based on the data presented, we propose a new model of cholesterol-phospholipid organization in mixed monolayers.

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Cholesterol plays a crucial role in determining the physicochemical properties of biomembranes [1]. The current concept of the cell membrane structure envisions the presence of relatively ordered, cholesterol-enriched assemblies, so-called lipid rafts, in a disordered lipid sea that are supposedly involved in many cellular processes including protein sorting, signal transduction, etc. [2,3]. Because of the intrinsic limitations of contemporary techniques, the existence of lipid rafts *in vivo* remains unproven [4]. Understanding cholesterol-phospholipid interactions could provide new insight into the nature of lipid rafts as well as to the mechanism of their formation.

Today, despite the intensive research in the field, consensus is still not achieved on the structural organization of cholesterol-phospholipid mixtures. One of the important questions that has attracted significant attention and controversy is whether cholesterol forms condensed complexes with phospholipids in biological membranes [5]. McConnell *et al.* [6,7] and most recently Ratajczak *et al.* [8] believe that mixtures of cholesterol and phospholipids form relatively ordered condensed complexes of a fixed stoichiometry which is independent of the overall cholesterol content. Existence of such complexes would mean that there is only one membrane lipid composition at which cholesterol and lipids would mix without forming individual phases. By contrast, other researchers assert that cholesterol molecules adopt regular distribution in a phospholipid matrix forming superlattices [9,10]. This research attempts to resolve the controversy using highly sensitive synchrotron surface x-ray scattering methods. Our results strongly suggest that in mixed monolayers cholesterol molecules neither form ordered complexes with phospholipids of a fixed stoichiometry [6] nor arrange in superlattice structures [9,10].

Grazing incidence x-ray diffraction (GIXD) can provide information on the in-plane lateral molecular organization in the ordered phase of a cholesterol-phospholipid film that can be related to its molecular composition. Knowledge of a vertical relative position of cholesterol within a phospholipid membrane can be gained using x-ray reflectivity (XR) [11,12]. In this Letter, we report structural investigation of cholesterol-DPPC (1,2-dipalmitoyl-sn-glycero-3-phophocholine) monolayers at the air-liquid interface with GIXD and XR. In order to account for variations in local cholesterol concentrations within the cell membrane, monolayer mixtures with a broad range of cholesterol mole fractions (χ_{CHOL}) were characterized.

The experiments were performed at the 9-ID beam line at the Advanced Photon Source, Argonne National Laboratory (Argonne, IL), at the wavelength of $\lambda = 0.920\,17$ Å. The liquid surface spectrometer and Langmuir trough chamber have been previously described [13]. Pure DPPC and cholesterol (Avanti Polar Lipids, Inc.) monolayers as well as their mixtures with 13, 25, 46, 70, and 85 mol% of cholesterol compressed to the surface pressure of 20 mN/m were probed. The measurements were carried out on Dulbecco's phosphate buffered saline without calcium and magnesium (D-PBS) (Invitrogen) at $23 \pm 0.2\,^{\circ}\mathrm{C}$. The resolution for the in-plane scattering angle $2\theta_{XY}$ was set to 1.4 mrad (9.56×10^{-3} Å^{-1}) by a Soller collimator. XR data were analyzed using both model-dependent (MD) "slab" model refinement [14,15] and model-independent (MI) stochastic fitting [16] routines employing RFIT2000 (Oleg Konovalov, ESRF) and STOCHFIT [16] software, respectively. Both MD and MI approaches yielded very similar results.

Diffraction patterns for pure DPPC and cholesterol monolayers and for their mixtures are presented in Figs. 1(a)–1(c), while corresponding repeat distances and unit cell parameters are summarized in Table I. Analysis of Bragg rod profiles is presented in the supplementary material [17]. GIXD spectra obtained for pure DPPC and cholesterol are consistent with our previously published data [14,18] and are shown in Figs. 1(a) and 1(c).

The mixture with χ_{CHOL} 0.13 mostly preserves the packing motif of DPPC in the liquid-ordered (LO) phase with a centered rectangular unit cell [Fig. 1(a)]. The

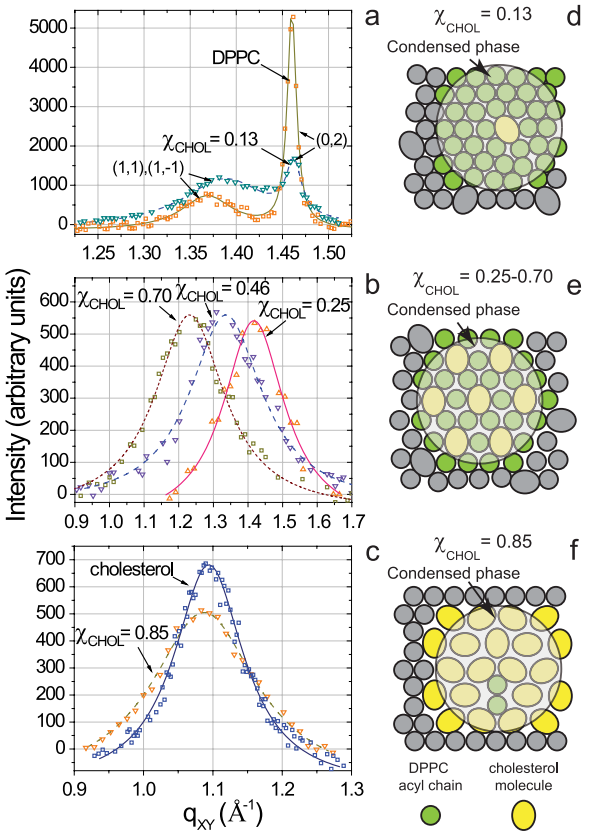

FIG. 1 (color online). Grazing incidence x-ray diffraction data: scattering intensity, integrated over q_z range, against scattering vector q_{xy} of (a) pure DPPC and the cholesterol-DPPC mixture with $\chi_{CHOL} = 0.13$; (b) cholesterol-DPPC mixture with $\chi_{CHOL} = 0.25$, $\chi_{CHOL} = 0.46$, and $\chi_{CHOL} = 0.7$; (c) pure cholesterol and the cholesterol-DPPC mixture with $\chi_{CHOL} = 0.85$. The cartoon schematics of the in-plane lateral molecular distribution in the mixtures with (d) $\chi_{CHOL} = 0.13$ (the condensed phase is mainly DPPC), (e) $\chi_{CHOL} = 0.25$–0.7 (the condensed phase is the mixture of cholesterol and DPPC with the stoichiometry identical to that of the film), and (f) $\chi_{CHOL} = 0.85$ (the condensed phase is mainly cholesterol).

calculated tilt of the acyl chains is reduced from 32° for DPPC to 26.4° for the mixture. Broadening of the diffraction peaks for the mixed monolayer reflects reduction in the size of the crystalline domains due to the presence of cholesterol. The diffraction peak position of cholesterol-DPPC mixture with χ_{CHOL} 0.85 is identical to that of pure cholesterol [Fig. 1(c)], but an increased full width at half maximum (FWHM) of the mixed monolayer peak indicates a reduced size of the LO domains, as compared to cholesterol. These results suggest that cholesterol neither forms ordered complexes with DPPC nor participates in any superlattice structures in the mixtures with χ_{CHOL} below 0.13 and above 0.85. Instead, at these molar ratios the dominant lipid species form an individual LO phase with crystalline domains smaller than those observed in monolayers of pure compounds, as illustrated schematically in Figs. 1(d) and 1(f).

Mixtures with χ_{CHOL} between 0.25 and 0.7 are characterized by a broad diffraction peak [Fig. 1(b)] with the maximum position shifting gradually from $q_{xy} = 1.42$ Å^{-1} to $q_{xy} = 1.23$ Å^{-1} with increase of cholesterol content. The symmetrical intensity distribution along q_{xy} was successfully fitted with a single Lorentzian function. Considered together with the intensity distribution along q_z (Bragg rod) with maximum at $q_z \approx 0$ Å^{-1}, this diffraction pattern suggests that on average the DPPC and cholesterol molecules form hexagonal lattice in the LO phase with molecular axes oriented normal to the surface (see supplementary material [17]). It is most probable that locally molecules pack in a distorted structure with the size of correlated regions of only a few molecules [12], rather than in superlattice structures. The d-spacing values for these mixtures are in a range of 4.43–5.09 Å and are larger than 4.27 Å repeat distance for pure DPPC and smaller than 5.75 Å d-spacing for pure cholesterol (Table I). This provides solid evidence that, indeed, cholesterol forms ordered complexes with DPPC [Fig. 1(e)]. However, should the stoichiometry of these complexes be fixed, as suggested by McConnell [6,7] and Ratajczak *et al.* [8] the diffraction peak position and consequently the average intermolecular distance in the LO phase would be invariant for the entire range of χ_{CHOL}. Most interestingly, our GIXD results suggest quite an opposite, a strong correlation of the intermolecular distance and, thus, the stoichiometry of the ordered cholesterol-DPPC complexes and χ_{CHOL}.

Ratajczak *et al.* have recently reported a GIXD study of dihydrocholesterol-sphingomyelin (DChol-SM) monolayers [8]. The authors hypothesized that DChol and SM form ordered complexes of a fixed stoichiometry which coexist with the ordered domains of pure DChol. The domains of both kinds are believed to form hexagonal structures as indicated by a single Bragg peak in the GIXD pattern. The observed single diffraction peak for the mixtures is explained as a superposition of these two peaks. The shift of the peak position with corresponding

TABLE I. Grazing incidence x-ray diffraction data.

Experiment	d spacing (Å)	a, b[a] (Å)	A_{uc}[b] (Å^2)
DPPC[c]	$d_{(1,1),(1,-1)} = 4.60$ $d_{(0,2)} = 4.27$	$a = 5.46$ $b = 8.54$	46.6
Cholesterol	5.75	6.64	38.2
Cholesterol-DPPC mixture			
13:87[c]	$d_{(1,1),(1,-1)} = 4.55$ $d_{(0,2)} = 4.30$	$a = 5.37$ $b = 8.6$	46.2
25:75	4.43	5.11	22.6
46:54	4.69	5.41	25.3
70:30	5.09	5.88	29.9
85:15	5.75	6.64	38.2

[a]For a centered rectangular unit cell dimensions $a \neq b$; for a hexagonal unit cell $a = b$.
[b]A_{uc} denotes the unit cell area.
[c]Please note that A_{uc} for DPPC and cholesterol-DPPC 13:87 mixture contains two acyl chains.

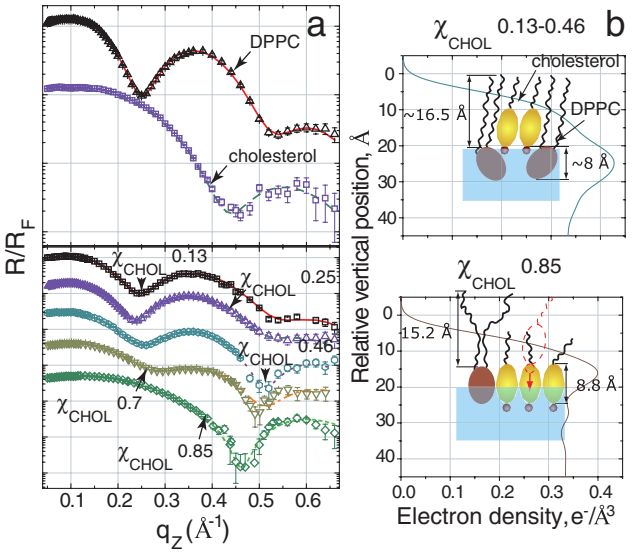

FIG. 2 (color online). (a) X-ray reflectivity data (symbols) and corresponding fits (lines) normalized by Fresnel reflectivity plotted against scattering vector q_z of (top panel) pure DPPC (triangles) and pure cholesterol (squares); (bottom panel) mixed cholesterol-DPPC monolayers with 13 (squares), 25 (triangles), 46 (circles), 70 (inverted triangles), and 85 (diamonds) mol% of cholesterol. For clarity the data have been offset vertically. (b) A representative electron density distribution in cholesterol-DPPC mixed monolayer perpendicular to the aqueous interface together with the cartoon schematics of the corresponding out-of-plane molecular arrangement in the mixtures with (top panel) $\chi_{\mathrm{CHOL}} = 0.13$–$0.46$ and (bottom panel) $\chi_{\mathrm{CHOL}} = 0.85$.

increase of the DChol content in the mixture is further explained by the change in a relative intensity and width of the complex and pure DChol diffraction peaks. This reasoning, however, is not applicable to the cholesterol-DPPC system, where single diffraction peak from the mixed monolayer (χ_{CHOL}0.25–0.7) cannot be fitted with two Lorentzian peaks at q_{xy} values, corresponding to d spacing of the fixed stoichiometry ordered complexes and that of pure cholesterol. The resulting fit has either a significant asymmetry of the peak shape, or a considerable variation in the peak intensity depending on χ_{CHOL} in the mixture; neither was observed in our GIXD data.

To elucidate the average molecular composition of observed cholesterol-DPPC complexes we recall Vegard's law, initially formulated for alloys. This law states that in a mixture of alloys at constant temperature a linear relation exists between the crystal lattice parameter and concentration [19]. A special case of Vegard's law is when the crystal lattice parameter of a binary mixture of alloys is the stoichiometric average of the crystal lattice parameters of individual alloys.

Applying the Vegard's law to our system, the unit cell area of the mixed monolayer should be the stoichiometric average of the unit cell areas of DPPC acyl chains and cholesterol molecules. To eliminate the effect of molecular tilt, the cross-sectional unit cell areas must be used in calculations. For instance, area per DPPC acyl chain $A_{\mathrm{AC}} = A_{\mathrm{uc}} \times \cos(32)/2 = 19.8$ Å^2 and that per cholesterol molecule is simply 38.2 Å^2 (Table I). Since the number of DPPC acyl chains is twice of that of DPPC molecules, then in the χ_{CHOL} 0.46 mixture calculated $A_{\mathrm{uc}} = (19.8 \times 0.54 \times 2 + 38.2 \times 0.46)/(0.46 + 0.54 \times 2) = 25.3$ Å^2. Accordingly, the calculated A_{uc} values of 22.4, 25.3, and 29.7 Å^2 for the mixtures with χ_{CHOL} 0.25, 0.46, and 0.70, respectively, are in a good agreement with our experimental results of 22.6, 25.3, and 29.9 Å^2 (Table I). Therefore, the average molecular composition of the LO phase in cholesterol-DPPC monolayers is the same as the overall composition of the film. Moreover, if the stoichiometry of the LO phase is the same as that of the entire monolayer, and there are only LO and liquid-disordered (LD) phases present, the stoichiometry of the LD phase also equals that of the entire film.

In order to assess whether cholesterol-DPPC mixtures exhibit the same structural organization at biologically relevant conditions, the diffraction pattern for the mixed monolayer with χ_{CHOL} 0.66 at 23 °C and 20 mN/m was compared to that at 37 °C and 32 mN/m, the surface pressure consistent with molecular areas found in biological membranes [20]. The position of the peak remains the same signifying that at $\chi_{\mathrm{CHOL}} = 0.66$ an average intermolecular distance, and, consequently, the molecular composition of the LO phase in the mixed monolayer at 37 °C and 32 mN/m is identical to that at 23 °C and 20 mN/m (see supplementary material [17]).

It is important to note that results of Ratajczak *et al.* [8] can be successfully interpreted within our model where intermolecular distances of the ordered DChol-SM complexes depend on χ_{DCHOL}. Cross-sectional areas of DChol molecule and SM acyl chain are 37.8 and 21.4 Å^2 [8]. The calculated A_{uc} values for the mixtures with χ_{DCHOL} 0.45, 0.5, 0.6, and 0.65 are then 26.2, 26.9, 28.4, and 29.3 Å^2, respectively, which is in good agreement with reported experimental values of 26.4, 27.0, 29.1, and 29.9 Å^2 [8].

Analysis of XR data [Fig. 2(a), Table II, supplementary material [17]] demonstrates that for the mixtures with χ_{CHOL} 0.13–0.46 cholesterol is located within the hydrophobic region of DPPC and its relative vertical position is almost invariant [Fig. 2(b)]. In this range of χ_{CHOL}, cholesterol-DPPC mixtures show gradual increase in the thickness of the acyl chain portion, due to the corresponding decrease of the DPPC molecular tilt. This thickening of the hydrophobic region correlates with concomitant reduction in the thickness of the head group region in a way that the overall thickness of the monolayer remains unaltered within experimental error.

At higher concentrations χ_{CHOL} 0.70 and 0.85 cholesterol molecules descend into the head group region of DPPC, shifting towards the aqueous interface, as suggested by the considerably different electron density profiles (Fig. 2, Table II, supplementary material [17]). This downward shift of cholesterol molecules results in reduced

TABLE II. X-ray reflectivity data fitting parameters for cholesterol-DPPC mixed monolayers.

χ_{CHOL}	L_{CW} [a] (Å)	L_{AC} [b] (Å)	L [c] (Å)
0.13	8.9	15.4	24.3
0.25	8.7	16.5	25.2
0.46	7.1	17.4	24.5
0.70	5.8	NA	23.6
0.85	0	15.2	24.0

[a] L_{CW}—distance between cholesterol hydroxyl group and lowest part of DPPC choline group.
[b] L_{AC}—thickness of DPPC acyl chains region.
[c] L—overall thickness of the film.

packing density of the upper part of the phospholipid acyl chains. The thermodynamically favorable state of the mixed cholesterol-DPPC film depends on the density of the molecular packing and must satisfy Kitaigorodskii's close-packing principle [21]. Because of mismatches in the cross-sectional area between the bulky hydrophobic body and the small hydroxyl group of cholesterol and in the molecular length of DPPC and cholesterol, incorporation of the sterol molecules into the phospholipid matrix may lead to voids in the film. At low χ_{CHOL}, the higher vertical position of cholesterol satisfies the hydrophobic contacts in the upper part of the film whereas voids created on the head group level are compensated by the corresponding tilt of DPPC phosphocholine groups [Fig. 2(b)]. For elevated χ_{CHOL}, the potential density of voids at the head group level becomes higher than could be compensated by the tilted phosphocholine groups. As a result, cholesterol molecules occupy a lower vertical position and the inevitable voids in the hydrophobic upper part of the film are compensated by the tilt of DPPC acyl chains [Fig. 2(b)].

Importantly, in contrast to conclusions of Ratajczak *et al.* for DChol-SM mixed monolayers [8], our XR data strongly suggest that in the mixtures with elevated χ_{CHOL} all cholesterol molecules descend into the head group region of DPPC, not only uncomplexed cholesterol. The critical χ_{CHOL} for cholesterol-DPPC mixed monolayers is approximately 0.4, as estimated from the XR and surface pressure-mean molecular area isotherm data (see supplementary material [17]). We propose that the downward shift of cholesterol may be responsible for a number of phenomena observed in model membranes, e.g., a sharp jump in the rate of cholesterol release to β cyclodextrin from cholesterol-phospholipid mixtures at χ_{CHOL} around 0.4 [1] (see supplementary material [17]).

Biological membranes are hypothesized to contain a heterogeneous collection of cholesterol-enriched domains with distinct lipid and protein composition known as lipid rafts [2,3]. The lipids in rafts have been widely accepted to be in a less fluid state than the surrounding membrane. Our GIXD results demonstrate that cholesterol cannot promote long-range ordering of lipids in membrane domains at the entire range of concentrations. Even in a binary mixture with DPPC, a lipid rafts resident with two saturated acyl chains, cholesterol forms ordered domains of only a few nm in diameter, whereas the bulk of the phospholipids in membrane domains contains at least one monounsaturated acyl chain and is less inclined to ordering [22]. This finding does not support the theory that membrane domains exist as independent ordered lipid-cholesterol entities ready to accommodate protein molecules. Indeed, it has been recently identified that at least the lipids of the influenza viral envelope are in a disordered liquid state at physiological temperatures [23].

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