Effect of osmotic pressure on ganglioside-cholesterol-DOPC lipid mixture

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Abstract. By means of small-angle X-ray scattering (SAXS) method, we have studied the structure of the lipid mixtures of monosialoganglioside (GM1)-cholesterol-dioleoylphosphatidylcholine (DOPC) system as a model of lipid raft. The samples were small unilamellar vesicle (SUV) except for GM1 sample. The osmotic pressure was changed with varying the polyvinylpyrrolidone (PVP) concentration in the range from 0 to 25 % w/w. The increase of the PVP concentration is known to reduce the lamellar spacing due to the increase of the osmotic pressure. On the other hand the polar head region of GM1 was shown to be highly hydrophilic by the presence of oligosaccharide chain containing one sialic acid residue. In the cases of the GM1 micelle and GM1-cholesterol SUV the presence of PVP affects little on those aggregate structures. In the case of the SUVs of cholesterol-DOPC the stacking of the bilayers was induced with the increase of PVP concentration, especially at high cholesterol content. In the case of the SUVs of GM1-cholesterol-DOPC the multi-lamellar stacking was suppressed, but a minor change of the SUV structure was induced. The present results suggest that the coexistence of GM1 and cholesterol affords the lipid bilayer a resistance to the osmotic stress and avoids a multi-layered stacking.

1. Introduction

The formation of lipid microdomain in mammalian plasma membrane, so-called a lipid raft [1], has been assumed to be essentially important in signal transduction, cell adhesion and lipid/protein sorting [2-4]. A common feature of the raft is their peculiar lipid composition, being rich in glycosphingolipids (GSLs), sphingomyelin and cholesterol. Gangliosides treated in this report 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 result from the peculiar features of the ganglioside molecules both in their ceramide and oligosaccharide portions that act as hydrogen bond donor and acceptor [5].

By using elastic and quasi-elastic scattering methods (small-angle neutron scattering, SANS; small-angle X-ray scattering, SAXS; neutron spin-echo, NSE) techniques we have been studying the structure and dynamics of gangliosides and those aggregates with other lipids. We found several intrinsic structural characteristics of ganglioside micelles and ganglioside-containing vesicles depending on temperature, pH, salt concentration, lipid composition ratios, and so on. The main results are summarized briefly as follows. Gangliosides form a prolate ellipsoidal micelle in solutions, where the hydration of the polar head region sensitively changes depending on temperature which accompanying the conformational change of sugar chains [7-11] and the change of dissociation degree...
of sialics [12, 13]. For the ganglioside-cholesterol binary systems we found the presence of the maximum miscibility of cholesterols against gangliosides, the cholesterol-dependent micelle-to-vesicle transitions [14], and the Ca$^{2+}$-induced vesicle-to-lamellar transitions accompanying the formation of an interdigitated structure between the sugar heads of gangliosides in the opposing bilayers [15]. For the ganglioside-phospholipid binary systems gangliosides predominantly locate at outer-leaflet of the vesicle [16]. As similar as in the binary systems, the bilayer structures of the ganglioside-cholesterol-phospholipid ternary systems also show an asymmetric distribution of ganglioside-cholesterol rich regions at the outer leaflets of the vesicle bilayers [17]. On the dynamics of the lipid aggregates observed by the NSE experiments, the dehydration and bending of the ganglioside sugar heads suppress the undulation of the micellar structure of gangliosides [18]. In the case of ganglioside-cholesterol-phospholipid ternary SUV systems the bending modulus takes the smallest value at the lipid composition of [ganglioside]/[cholesterol]/[glycerophospholipid] ≈ 0.1/0.1/1 that is a similar composition as in intact neuronal cell membrane including rafts [19]. Another study shows that the coexistence of ganglioside and cholesterol enhances the permeability of water across the bilayer by K$^+$ ions [20]. These results suggest that the GSL microdomains afford the bilayer an appropriate fluidity and have a role to hold a homeostasis of the membrane environment for functional proteins. The above results strongly suggest that various physicochemical functions of GSLs would be deeply related to hydrodynamic properties of solutions.

On the other hand, so-called "osmotic stress" method by using high molecular weight neutral polymers has been used effectively to discuss about the hydration force between lipid bilayers [21-26]. This method is conventional and still powerful to change the osmotic pressure. As one of high molecular weight probes, the use of PVP is well established [24] as well as dextran [21]. PVP is also used for various industrial products such as medicines, cosmetics, and so on. With varying the PVP concentration from 0 to 25 % w/w we have studied the effect of the osmotic stress on the aggregate structures of G$_{M1}$/cholesterol/DOPC. In the present report we focus on the change of SUV or micelle structure depending on the osmotic pressure.

2. Material and methods

2.1. Materials

G$_{M1}$ was extracted and separated from bovine brain by using the method. The detail was described elsewhere [27-29]. Single band of the G$_{M1}$ on thin layer chromatography (TLC) plate (Kiesel gel 60; E. Merck, Darmstadt) was confirmed by a simple fluorometric method reported previously [30], and >90% purity for the G$_{M1}$ was determined by TLC-densitometry using G$_{M1}$ purchased from SIGMA Chemical Co. as a standard. Cholesterol, DOPC and PVP (Mt. 40,000) were purchased from SIGMA Chemical Co. and used without further purification. All other chemicals used were of analytical grade or better.

2.2. Preparation of SUV solutions

Required quantities of G$_{M1}$, cholesterol and DOPC were dissolved in a 2:1 (v/v) mixture of chloroform and methanol. The molar ratios of [G$_{M1}$]/[cholesterol]/[DOPC] prepared were 1/0/0, 1/1/0, 0.2/0.2/1, 0.1/0.1/1, 0.1/0/1, 0.2/0.2/1, 0/0.2/1, 0/0.1/1, and 0/0/1. The organic solvent was removed from the lipid mixture solutions under a stream of nitrogen gas, and the solutions were dried at 45 °C in vacuo for overnight. The dried mixtures were dissolved in 10 mM HEPES (N-(2-hydroxyethyl) piperazine-N’-(2-ethanesulfonic acid)) buffer (pH 7.0) to become 5 % w/v of total lipid concentration, and were vortexed for several minutes. For preparing SUV solutions the mixtures were sonicated for 10 minutes at ~10 °C by using a high-power probe-type ultrasonicator (Model UH-50 of SMT Co.) at 50 W. The PVP solutions with different PVP contents (10, 20, 30, 40, 50 % w/w) were also prepared using the same HEPES buffer. The above G$_{M1}$-cholesterol-DOPC SUV solutions and the PVP solutions were mixed by 1:1 in v/v, and we finally obtained the samples solutions used for the X-ray measurements. The lipid concentrations of the samples were 2.5 % w/v.
2.3. X-ray scattering measurements

SAXS measurements were performed by using the synchrotron radiation small-angle X-ray scattering spectrometer installed at BL10C line of the 2.5 GeV storage ring at the Photon Factory at the National Laboratory for High Energy Physics, Tsukuba, Japan. The X-ray wavelength used was 1.49 Å and the sample-to-detector distances were 80 cm (medium distance) and 190 cm (long distance). To check the correlation between the fatty acid tails, high-angle X-ray scattering measurements were also carried out by using the X-ray scattering spectrometer installed at BL40B2 of the 8 GeV synchrotron radiation source (SPring-8) of the Japan Synchrotron Radiation Research Institute (JASRI), Harima, Japan. The sample-to-detector distance and the X-ray wavelength used were 46 cm and 1.0 Å, respectively. The sample-cells were placed into a cell holder controlled at 25 °C. The exposure time for each measurement was 4 minutes for PF and 30 seconds for SPring-8.

![SAXS curves](image1)

**Fig. 1.** SAXS curves of GM1-cholesterol binary mixtures (in 10 mM HEPES buffer at pH 7.0 and at 25 °C) depending on PVP concentration, where (a) and (b) correspond to the molar ratio [GM1]/[cholesterol] = 1/0 and 1/1, respectively. The lipid concentrations are 2.5 % w/v.

![SAXS curves](image2)

**Fig. 2.** SAXS curves of cholesterol-DOPC binary mixtures (in 10 mM HEPES buffer at pH 7.0 and at 25 °C) depending on PVP concentration, where (a), (b) and (c) correspond to the molar ratio [cholesterol]/[DOPC] = 0/1, 0.1/1 and 0.2/1, respectively. The lipid concentrations are 2.5 % w/v. The change of PVP concentration from 5 to 25 % w/w corresponds to that of the osmotic pressures (ln $P$ (dyn/cm$^2$)) of 11.5 to 15.3.
3. Results and discussion

3.1. Effect of PVP on GM₁-cholesterol binary mixture

Fig. 1 shows the change of the SAXS curve $I(q)$ at the medium distance depending on PVP concentration, where (a) and (b) correspond to the samples with the molar ratios of $[\text{GM}_1]/[\text{cholesterol}] = 1/0$ and $1/1$, respectively. It has already shown that GM₁ forms a micelle and that the GM₁-cholesterol mixture forms a SUV at 1/1 [7, 11, 13, 15]. With increasing the PVP concentration the SAXS intensities below $\sim 0.05 \, \text{Å}^{-1}$ decreased gradually, whereas the broad humps around $q = \sim 0.1 \, \text{Å}^{-1}$ are mostly maintained. According to the radius of gyration $R_g$ estimation using the Guinier plot ($\ln I(q)$ vs. $q^2$) the $R_g$ value slightly changes from $56.5\pm1.1 \, \text{Å}$ to $53.8\pm1.4 \, \text{Å}$ for GM₁ and from $80.1\pm0.8 \, \text{Å}$ to $81.7\pm1.6 \, \text{Å}$ for 1/1, however these changes are relatively small. Thus the over-all structures of the aggregates of $[\text{GM}_1]/[\text{cholesterol}] = 1/0$ and 1/1 are mostly resistant to the addition of PVP up to 25 % w/w, namely the increase of osmotic pressure $\ln P$ (dyn/cm²) up to 15.3. The change of SAXS intensities below $\sim 0.05 \, \text{Å}^{-1}$ are attributable to the change of the average excess scattering density (so-called contrast) of the aggregate particles.

3.2. Effect of PVP on cholesterol-DOPC binary mixture

Fig. 2 shows the SAXS curve depending on both PVP and cholesterol concentrations, where (a), (b) and (c) correspond to the samples with the molar ratios of $[\text{cholesterol}]/[\text{DOPC}] = 0/1$, 0.1/1 and 0.2/1, respectively. In this binary system the increase of PVP concentration induces evidently the bilayer stacking of SUV, namely the transition from uni-lamellar to multi-lamellar structure, which appears as the development of the lamellar peak at $q = \sim 0.106 \, \text{Å}^{-1}$ that corresponds to the repeat distance of $\sim 59.3 \, \text{Å}$. The peak becomes to appear around 15 % w/w for 0/1 and 0.1/1 SUV samples and around 10 % w/w for 0.2/1 SUV sample. The growth rate of the peak becomes higher with increasing the cholesterol content. The broad hump or background above $\sim 0.05 \, \text{Å}^{-1}$ for each SAXS curve is attributable to the presence of the SUV components that do not form the lamellar stacking. Therefore the contribution of the appearance of the multilayered structure can be estimated as shown in Fig. 3. Fig. 3 clearly indicates that the increase of the cholesterol content makes the SUV membrane to be more sensitive to the elevation of the osmotic pressure.

![Fig. 3. Extracted diffraction peaks of the multi-layers obtained from the SAXS curves shown in Fig. 2, where (a), (b) and (c) correspond to those in Fig. 2. After normalizing the SAXS profiles at $q = 0.15$-0.16 Å⁻¹, the diffraction peaks of the multi-layers were separated from the globular scattering curves of the SUV components by subtracting the SAXS profile of PVP = 0 % w/w from those of PVP ≠ 0 % w/w.](image-url)
3.3. Effect of PVP on GM₁-cholesterol-DOPC ternary mixture

Fig. 4 shows the effect of the changes in both PVP concentration and lipid composition, where (a), (b) and (c) correspond to the molar ratios of [GM₁]/[cholesterol]/[DOPC] = 0.1/0/1, 0.1/0.1/1 and 0.2/0.2/1, respectively. As similar as in the case of the GM₁-cholesterol binary mixture in Fig. 1, despite of the increase of the PVP concentration the broad humps around \( q = \sim 0.1 \text{ Å}^{-1} \) are mostly maintained and do not induce an evident transition from vesicle to lamellar that occurs in the cholesterol-DOPC binary mixture. However, there exist some differences compared with the GM₁-cholesterol binary mixture. Thus, the SAXS intensities below \( \sim 0.05 \text{ Å}^{-1} \) significantly increase and those profiles also change to lose the deep grooves at \( q = \sim 0.035 \text{ Å}^{-1} \) with the rise of PVP concentration. These changes strongly suggest the deformation of the SUV structure, which is most clearly seen in the 0.1/0/1 SUV. In addition it can be recognized a germ of the lamellar stacking for the 0.1/0/1 SUV at 25 % w/w PVP.

![Fig. 4. SAXS curves of GM₁-cholesterol-DOPC ternary mixtures (in 10 mM HEPES buffer at pH 7.0 and at 25 °C) depending on PVP concentration, where (a), (b) and (c) correspond to the molar ratio [GM₁]/[cholesterol]/[DOPC] = 0.1/0/1, 0.1/0.1/1 and 0.2/0.2/1, respectively. The lipid concentrations are 2.5 % w/v.](image)

**4. Conclusion**

From the above results we can conclude as follows. The presence of cholesterol in the SUV reduces the fluidity of the bilayer and tends to flatten the bilayer, resulting in the assistance of the multi-layer stacking with the increase of osmotic pressure. On the other hand the presence of ganglioside essentially affords the SUV bilayer a resistance to the osmotic stress, which is attributable to the intrinsic characteristics of ganglioside molecules, namely, the huge hydrophilic sugar head with sialic acids. Both Coulomb and hydration repulsions would hinder the approach between the bilayers under the osmotic pressure to some extent.

Noticeably, the present results clearly show that the coexistence of ganglioside and cholesterol within the lipid bilayer enhances significantly the resistance to the osmotic stress. This is reasonably interpreted as the result of the preferential clustering of ganglioside and cholesterol at the outer leaflet of the SUV bilayer [16, 17] since the presence of the ganglioside-cholesterol rich region at the outer leaflet would interfere in the multi-layered structure formation through those steric and electrostatic hindrances. Alternatively, the formation of ganglioside-cholesterol microdomains has a role to hold a homeostasis of membrane structure against the change of osmotic stress, which would be essentially important to hold various physiological functions and reactions occurring on membrane surfaces.

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