Penetration of Bovine Serum Albumin into Dipalmitoylphosphatidylglycerol Monolayers: Direct Observation by Atomic Force Microscopy

Isao OHTSUKA and Shoko YOKOYAMA*

School of Pharmaceutical Sciences, Kyushu University of Health and Welfare; 1714–1 Yoshino-cho, Nobeoka, Miyazaki 882–8508, Japan. Received July 13, 2004; accepted October 19, 2004

The penetration of bovine serum albumin (BSA) into dipalmitoylphosphatidylglycerol (DPPG) monolayers was observed using atomic force microscopy (AFM) and surface pressure measurements. The effects of surface pressure, amount of BSA and the addition of ganglioside G_{M1} (GM1) were investigated. The surface pressure of the DPPG monolayer was increased by the penetration of BSA, and the increase in surface pressure was greater in the liquid-expanded film than that in the liquid-condensed film. The AFM images indicated that BSA penetrated into the DPPG monolayer. The amount of BSA that penetrated into the DPPG monolayer increased with time and with the amount of BSA added. On the contrary, the AFM image showed that BSA penetration into the mixed DPPG/GM1 (9 : 1) monolayer scarcely occurred. GM1 inhibited the penetration of BSA into the DPPG monolayer.

Key words bovine serum albumin; atomic force microscopy; dipalmitoylphosphatidylglycerol; penetration; ganglioside G_{M1}; surface pressure

Liposomal drug delivery systems have been widely researched for the purpose of reduction of drug toxicity and/or the targeting of drugs to specific cells.[1] Liposomes are, in some ways, ideal drug carriers in that they are biodegradable. There are, however, drawbacks to their use *in vivo*. Part of the liposomal bilayer membrane is destroyed through the interaction with blood components. It is important to clarify the stability of liposome products in the blood. The following findings have been made clear:[2,3] BSA adsorbs onto dipalmitoylphosphatidylglycerol (DPPG) liposomes by hydrophobic interactions; the adsorption of BSA brought about a phase separation in the liposomal bilayer membranes, thereby increasing the permeability of the liposomal bilayer membranes through the adsorption of BSA. Surface modified liposomes have been prepared: Klibanov *et al*.[4,5] and Maruyama *et al*.[6] reported that the conjugation of amphipathic poly(ethylene glycol) (PEG) with liposomes significantly increased the blood circulation half-life of the liposomes to a greater extent than those without PEG. We reported the membrane properties of mixed DPPG and ganglioside G_{M3} (GM3) liposomes:[7,8] GM3 incorporated into DPPG liposomes inhibited the adsorption of BSA on the liposomes, and the leakage of calcein as an aqueous-space marker from liposomes through adsorption of BSA decreased.

Atomic force microscopy (AFM) is a surface imaging technique capable of nanometer-scale lateral resolution, which operates by measuring the forces acting between the probe and the sample.[9] AFM images of membranes of phospholipids and gangliosides have been reported.[10—19] Furthermore, the distribution of drugs in the membrane has been shown by AFM.[20,21] However, AFM images of BSA adsorbed on the surfaces of phospholipid membranes have not yet been shown. In this study, the penetration of BSA into the DPPG monolayer was observed using AFM, varying the surface pressure of the membrane and the amount of BSA. Furthermore, the effect of ganglioside G_{M1} on the adsorption of BSA onto the DPPG membrane was also observed by AFM.

Experimental

Materials Sodium L-α-dipalmitoylphosphatidylglycerol (DPPG) and ganglioside G_{M1} (GM1), Galβ1→3Gal NAcβ1→4Galβ1(3←2αNANA)→4Glcβ1→1Cer, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. In this experiment, super pure water (Super-Q system) was used.

Measurement of Surface Pressure of the Monolayer Membrane DPPG was dissolved in chloroform, giving a 1 mM DPPG solution. After 150 μl of the DPPG solution was spread on water without surface disturbances using a microsyringe, the system was allowed to stand for 10 min. The surface pressure of the DPPG monolayer at the air/water interface was determined at 37°C by the Wilhelmy plate method using a surface pressure meter (HBM-A, Kyowa Interface Science Co., Ltd.) with a bar made of Teflon. The compression rate was 20 mm/min.

Observation of BSA Penetration into the DPPG Monolayer At 20 and 30 mN/m, 100—200 μl of 5 mg/ml BSA solution was injected into 1.6 l of the sublayer water without causing surface disturbances, and the time course of change in surface pressure of the DPPG monolayer was measured. Next, AFM images of BSA penetrated into the DPPG monolayer were observed at 15, 30 and 60 min after BSA was added to the sublayer water.

AFM Observation A model JSPM-5200 atomic force microscope (JEOL Ltd.) was used for AFM observations. The AFM probe used was a Micro Cantilever CSC38 (JEOL Ltd.) made of silicon and coated with Au, which had a spring constant of 0.08 N/m, a length of 250 μm, and a thickness of 1.0 μm. AFM observation was carried out with the contact mode in the air.

Results and Discussion

Surface Pressure–Area Isotherm The surface pressure (π) *versus* area (A) isotherm of the DPPG monolayer at 37°C is shown in Fig. 1.

The phase transition from the liquid-expanded film to the liquid-condensed film was observed at approximately 25 mN/m.

Penetration of BSA into the DPPG Monolayer Membrane The time course of change in surface pressure of the DPPG monolayer after adding BSA was measured, and the results are shown in Fig. 2.

The surface pressure of the DPPG monolayer increased with the addition of BSA to the sublayer water. This finding indicates penetration of BSA into DPPG monolayer membranes occurred. This result supports the previous

* To whom correspondence should be addressed. e-mail: s.yokoyama@phoenix.ac.jp

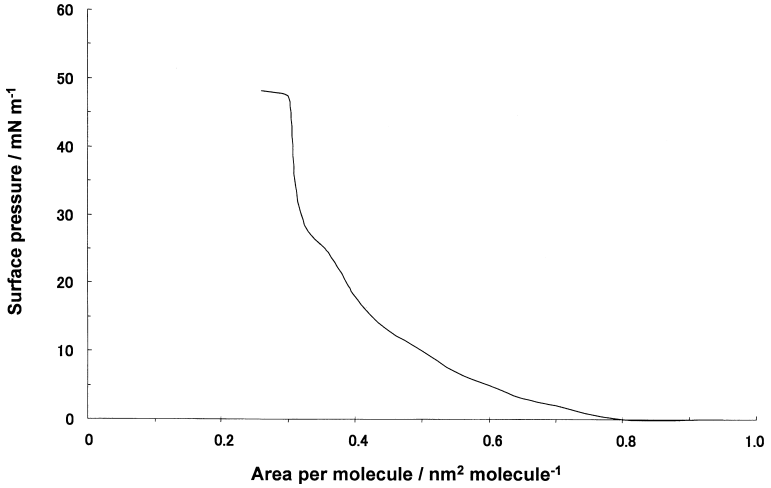

Fig. 1. Surface Pressure *vs*. Area per Molecule Isotherm for the DPPG Monolayer at 37°C

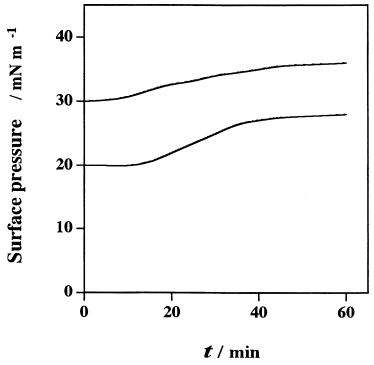

Fig. 2. Effect of BSA on the Surface Pressure of the DPPG Monolayer

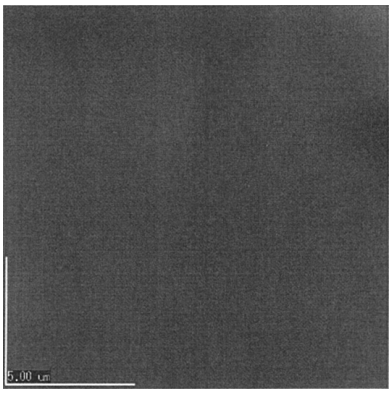

Fig. 3. AFM Image of the DPPG Monolayer Deposited at 30 mN/m

suggestion[2] that BSA adsorbed on DPPG liposomes penetrates into the liposomal bilayer membrane. At 20 and 30 mN/m, the DPPG monolayer is in the liquid-expanded and the liquid-condensed films, respectively. The increase in surface pressure of the DPPG monolayer in the liquid-expanded film was greater than that in the liquid-condensed film.

AFM Observation of BSA Penetrated into DPPG Monolayer First, the AFM image of the DPPG monolayer deposited at a surface pressure of 30 mN/m is shown in Fig. 3.

The DPPG monolayer had a uniform and flat appearance. Next, AFM images of the DPPG monolayers after the addition of 100 μl of 5 mg/ml BSA solution are shown in Fig. 4, where AFM images show the hydrophobic faces of the DPPG monolayers containing BSA.

BSA is visible as bright areas in the figures. The length of the lines drawn at the lower left corner in the figures is 5.0 μm. The three-dimentional images that BSA penetrated into the DPPG monolayer are shown in the right-hand figures. BSA is elliptical (41.6×140.9 Å) in shape.[22] The size of BSA observed as bright dots in Fig. 4c is nearly equal to the size of BSA molecule. It is considered that BSA penetrates lengthwise in the DPPG monolayers. The larger bright dots seem to be the dimmers and/or trimers[23] of BSA molecules or the molecular aggregates. BSA penetrates from the bulk aqueous phase to the DPPG monolayer, and then BSA interacts with DPPG by hydrophobic interaction. The three-domains model of BSA was presented by Brown and Shockley,[24] where the effective electric chages of the domains I, II and III are −10, −8 and 0, respectively. Each helix of BSA molecule has both hydrophilic and hydrophobic surfaces.[25] Furthermore, BSA has a configurational adaptability:[24] BSA has the hydrophilic surface in the bulk water phase and the hydrophobic surface in the hydrocarbon region of the DPPG monolayer. The larger bright areas shown in Figs. 4a and 4b may be related to the change in the conformation of BSA molecules. The amount of BSA adsorbed on the DPPG monolayer increased with time.

Figure 5 shows AFM images of BSA penetration into the DPPG monolayer in the liquid-expanded film state.

The amount of adsorption of BSA on the liquid-expanded film was slightly less compared with that on the liquid-condensed film. This is considered to be due to the fact that BSA is adsorbed onto the DPPG membrane by hydrophobic interactions[2,3] and that hydrophobic interactions between BSA and DPPG are greater in the liquid-condensed film. The amount of BSA that penetrated into the DPPG monolayer increased with time, and the height of the membrane also increased with time. BSA seems to easily penetrate into the liquid-expanded film compared with the liquid-condensed film because of the tightness of the latter film.

Next, the effect of the amount of BSA on the extent of penetration of BSA into DPPG monolayer was examined, and the results are shown in Fig. 6.

The penetration of BSA into the DPPG monolayer in-

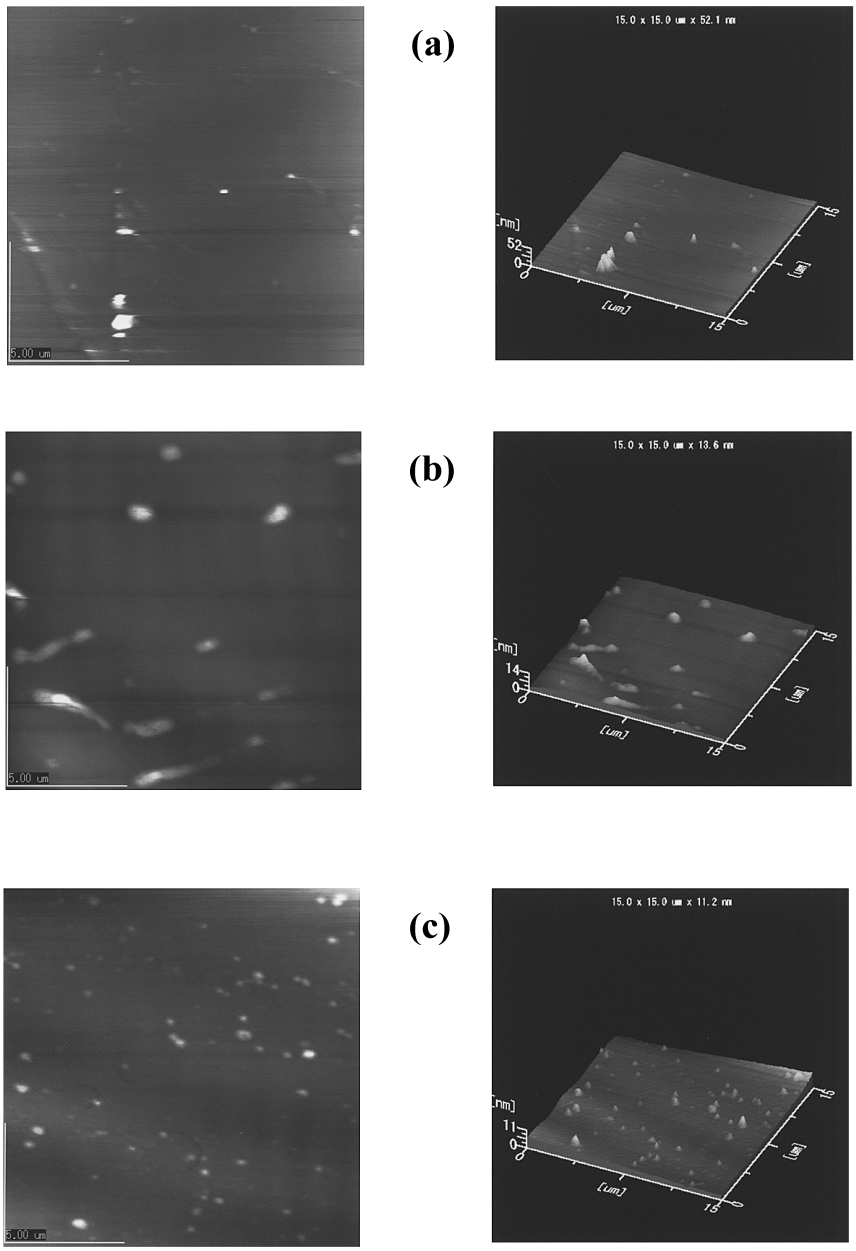

Fig. 4. AFM Images of BSA Penetration into DPPG Monolayers

Time after BSA was added: (a), 15 min; (b), 30 min; (c), 60 min. Surface pressure: 30 mN/m (liquid-condensed film). Amount of BSA added: 100 μl of 5 mg/ml BSA solution. Left-hand figures: two-dimensional figures (15 μm×15 μm). Right-hand figures: three-dimensional figures.

creased with the amount of BSA added. Especially, 30—60 min after BSA was added, a large amount of BSA was observed.

AFM Observation of Mixed DPPG/GM1 Monolayers and BSA In the previous paper,[7] it was found that the extent of adsorption of BSA on DPPG liposomes was decreased by the mixing of ganglioside into the DPPG liposomes. Last, the adsorption of BSA on the mixed DPPG/GM1 (9 : 1) monolayer was observed, and the result 60 min after BSA was added is shown in Fig. 7.

The adsorption of BSA on the mixed DPPG/GM1 (9 : 1) monolayer at 60 min was remarkably less than that on the DPPG monolayer at 60 min. Furthermore, the height of BSA molecules from the monolayer was extremely low, which seems to not be penetration and/or adsorption but rather a light touching of BSA to the DPPG/GM1 monolayer. GM1 inhibited the penetration of BSA into the DPPG monolayer. This is considered to be due to the increase in the hydrophilic property at the surface of the monolayer by adding GM1, thereby the hydrophobic interaction between BSA and DPPG was suppressed. The micrograph of the DPPG liposomes containing 0.15 mole fraction of ganglioside indicated the covered surfaces with the sugar chains.[7]

Measurement for mixed DPPG/GM1 monolayers with higher mole fractions of GM1 was not carried out, since mixed phospholipid/GM1 monolayers ($X_{GM1}>0.2$) bring about a change in the structure of the lipid membrane.[19] Nevertheless, a small amount of GM1 ($X_{GM1}=0.1$) effectively inhibited the adsorption and penetration of BSA. The fact[7] that a mixed DPPG/ganglioside liposome inhibited the adsorption of BSA was visually confirmed in this study by direct observation using AFM and DPPG/GM1 monolayers.

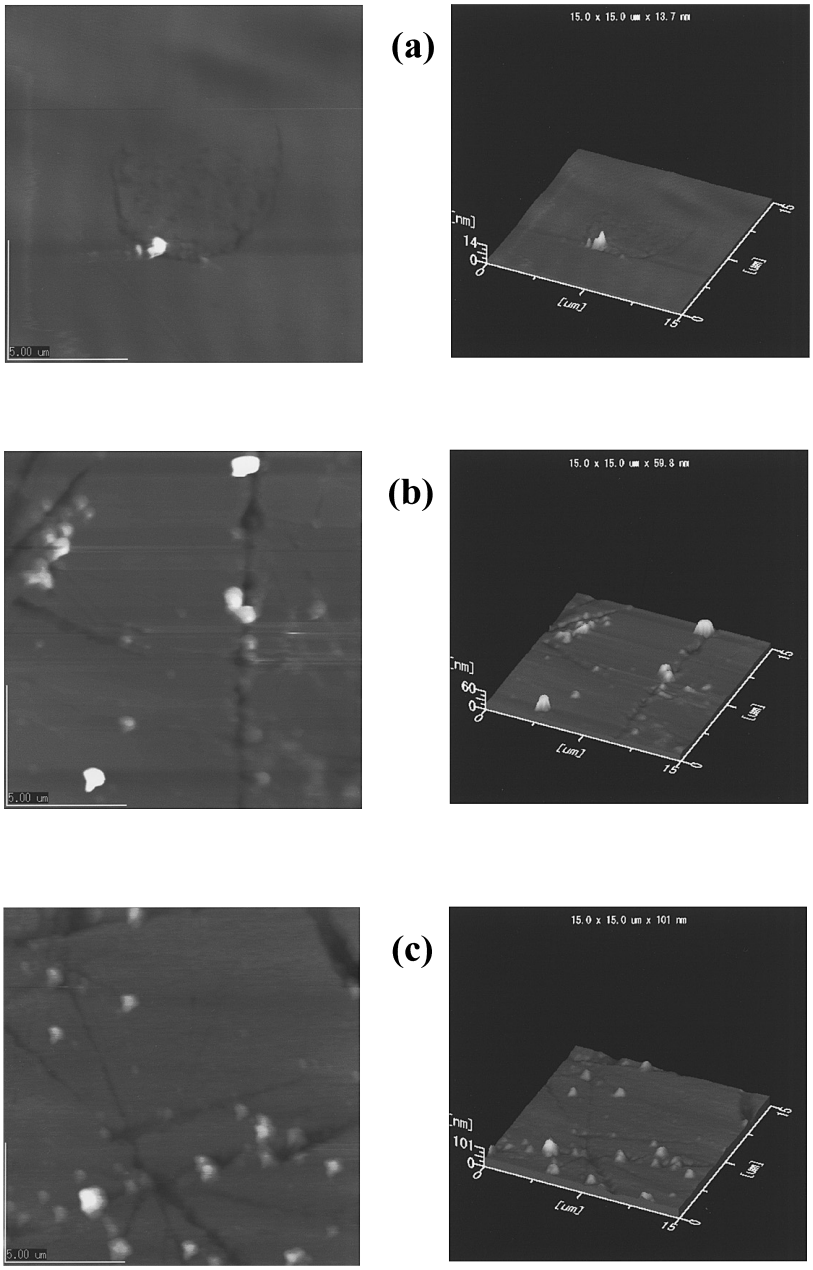

Fig. 5. AFM Images of BSA Penetration into DPPG Monolayers

Time after BSA was added: (a), 15 min; (b), 30 min; (c), 60 min. Surface pressure: 20 mN/m (liquid-expanded film). Amount of BSA added: 100 μl of 5 mg/ml BSA solution.

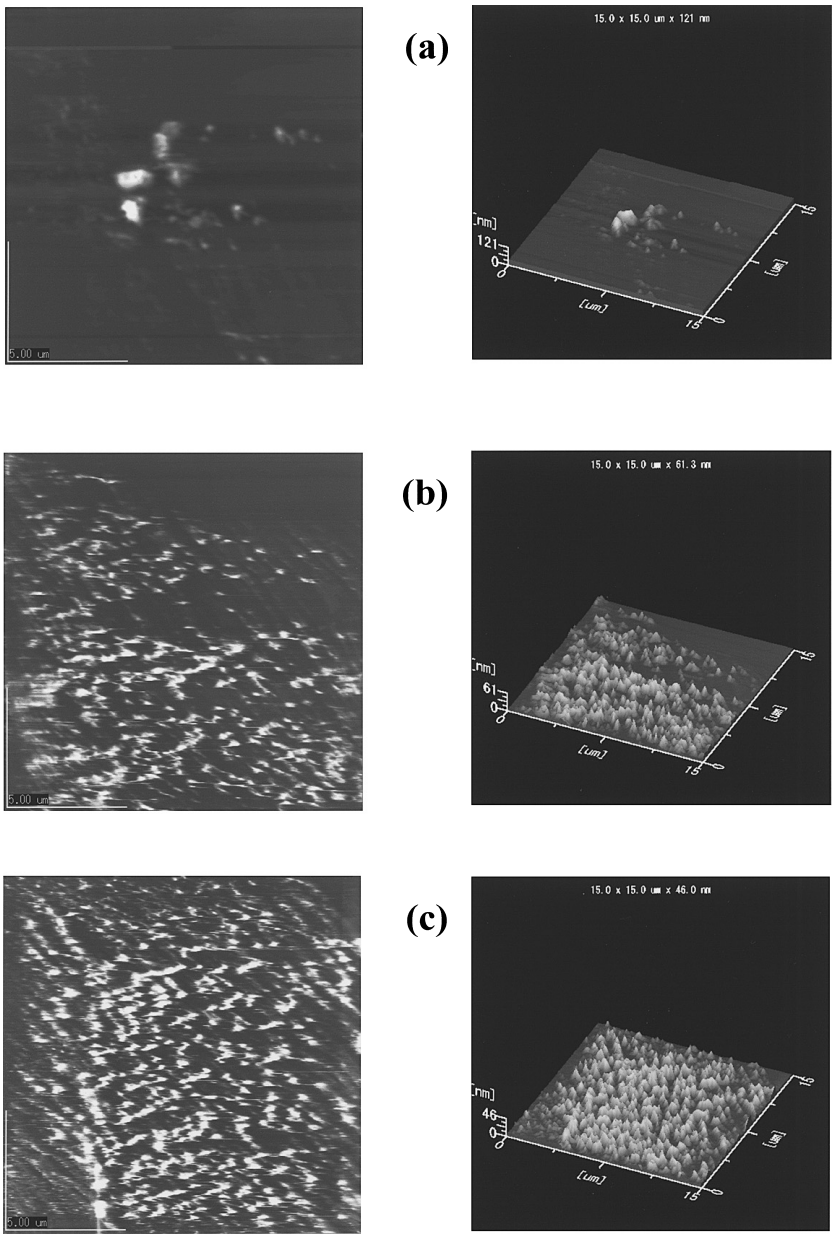

Fig. 6. AFM Images of BSA Penetration into DPPG Monolayers: Effect of the Amount of BSA

Time after BSA was added: (a), 15 min; (b), 30 min; (c), 60 min. Amount of BSA added: 200 ml of 5 mg/ml BSA solution. Surface pressure: 20 mN/m.

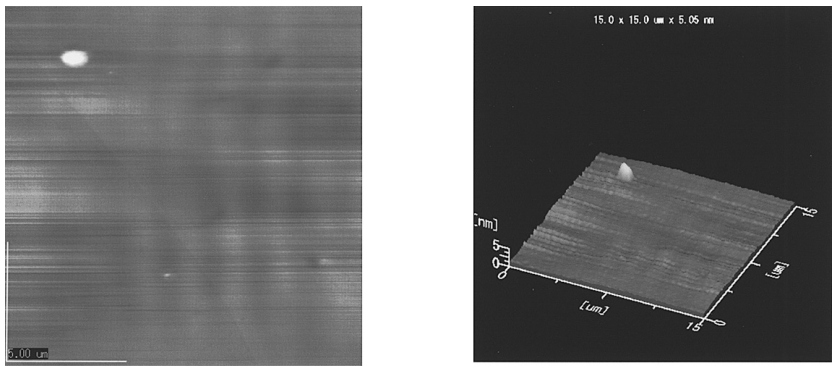

Fig. 7. AFM Images of BSA Penetration into the Mixed DPPG/GM1 (9 : 1) Monolayers

Time after BSA was added: 60 min. Surface pressure: 20 mN/m. Amount of BSA added: 100 μl of 5 mg/ml BSA solution.

Conclusion

BSA penetrated into DPPG monolayer. The amount of BSA that penetrated into the DPPG monolayer increased with the addition of BSA. A large amount of BSA penetrated into DPPG monolayer was observed 30—60 min after BSA was added. On the contrary, the penetration of BSA into the mixed DPPG/GM1 (9 : 1) monolayer scarcely occurred.

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