Physicochemical Studies on the Phospholipid Bilayer Incorporated Sialoglycolipid

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With the intention of obtaining a novel liposome as a drug carrier able to avoid clearance by the reticuloendothelial system (RES), the authors synthesized two sialoglycolipids, *i.e.*, 2-*O*-(8-hexadecanoylamino-3,6-dioxaoctyl)-β-*N*-acetylneuraminic acid sodium salt (Sia-t-pa) and 2-*O*-[8-(2-hexadecyloctadecanoylamino)-3,6-dioxaoctyl]-β-*N*-acetylneuraminic acid sodium salt (Sia-t-psa) and modified the surface of their liposomal membrane.

Sia-t-pa was found to be eluted from the liposomal membrane by serum albumin, whereas Sia-t-psa was not, using the gel filtration method. In order to clarify the reason why there was such a difference between Sia-t-pa and Sia-t-psa, we investigated the properties of the phospholipid bilayer-incorporated sialoglycolipids physicochemically. The effect of the anchor structure of the sialoglycolipids, *i.e.*, single acyl chain (Sia-t-pa) or double acyl chain (Sia-t-psa), on the fluidity and calorimetric properties was noticeably different. It was inferred that the effect was attributable to a difference in the position of the sialoglycolipid incorporated in the bilayer membrane.

Key words sialoglycolipid; phospholipid bilayer; liposome; reticuloendothelial system avoidance; ganglioside G_{M1}; drug delivery system

Liposomes have a high potential as drug carriers[2]; however, liposomes administered i.v. are rapidly taken up by phagocytic cells of the reticuloendothelial system (RES).[3] The rapid clearance of circulating liposomes from the bloodstream has been an obstacle in targeting tumors with anticancer drugs.[3] In order to avoid or delay such uptake by the RES, efforts have been made to find liposomes that remain in the blood circulation for a relatively long period of time. It has been found that liposomes containing ganglioside G_{M1},[4-7] hydrogenated phosphatidylinositol[5,8] or *N*-(polyethyleneglycol)phosphatidylethanolamine[9-11] have the ability to avoid clearance from the RES. Particularly, it is worth applying ganglioside G_{M1}, having an *N*-acetylneuraminic acid at the terminal position of its glycolipid moiety, in the form of liposomes as a drug carrier, since glycolipid is present on the biological cell surface. However, ganglioside G_{M1} is unlikely to be used for drug delivery studies because its production is complicated and relatively costly. Therefore, Higashi *et al.*[12,13] synthesized sialoglycolipids to mimic ganglioside G_{M1} with the intention of giving the ability to avoid the RES to liposomes consisting of modified sialoglycolipids as well as to those containing ganglioside G_{M1}.

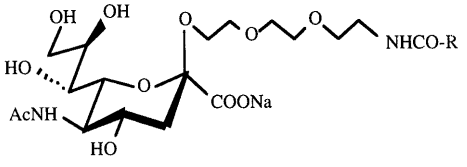
In the present study, we investigated the physicochemical properties of the liposomal membrane-incorporated synthesized sialoglycolipids by fluorescence polarization and differential scanning calorimetry to obtain a novel liposome having the ability to avoid the RES.

Experimental

Materials Two sialoglycolipids, 2-*O*-(8-hexadecanoylamino-3,6-dioxaoctyl)-β-*N*-acetylneuraminic acid sodium salt (Sia-t-pa, single acyl chain amphiphile) and 2-*O*-[8-(2-hexadecyloctadecanoylamino)-3,6-dioxaoctyl]-β-*N*-acetylneuraminic acid sodium salt (Sia-t-psa, double acyl chain amphiphile) were synthesized. The structures of the sialoglycolipids are shown in Fig. 1 and the synthetic route of the ligands is outlined in Chart 1. β-Sialoglycolipid was prepared according to the method of Higashi *et al.*[12] The β configuration of the glycosidic bond was established by ^{1}H-NMR spectroscopy. The spacer and anchor moieties were introduced using the method of Sasaki *et al.*[13]

L-α-Phosphatidylcholine dipalmitoyl (DPPC), cholesterol (CHOL), dicetyl phosphate (DCP) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *N*-Dansylhexadecylamine (DSHA) was prepared according to the method described in the literature.[14] Phosphate buffered saline (PBS) was obtained from Nissui Pharmceutical Co., Ltd. (Tokyo, Japan). All other reagents and solvents were of analytical reagent grade.

Preparation of Liposomes Liposomes containing DPH or DSHA were prepared as described below. Multilamellar vesicles (MLVs) were prepared according to the method of Bangham *et al.*[15] with some modification as follows: DPPC, CHOL, and sialoglycolipid were dissolved in an appropriate amount of chloroform–methanol (1 : 1 v/v), and the fluorescent probe, DPH dissolved in tetrahydrofuran or DSHA dissolved in methanol was added to the lipid solution. The molar ratios of the fluorescent probes to phospholipid were maintained at 1 : 130 based on the literature[14,16-19] where there was no interference by light scattering with fluorescence anisotropy measurements. The solvent was removed by distillation in a stream of nitrogen, followed by drying under reduced pressure overnight. The residues were hydrated with 0.1 M aqueous NaCl. The resultant suspensions were vortexed mechanically and sonicated for 2 min with a bath-type sonicator (Branson B1200; Emerson-Japan, Tokyo, Japan). The liposomes were extruded through polycarbonate membranes (Nuclepore Co., MA, U.S.A.) with successive pore sizes of 0.2 and 0.1 μm. These procedures were carried out at a temperature above the gel-liquid crystalline phase transition temperature (T_c) of the phospholipid. The resulting liposome suspensions were gel-filtered at room temperature (20—25 °C) by passing them through a Sephadex G-50 column (15 mm i.d. × 16 cm) pre-equilibrated with 0.1 M aqueous NaCl to give multilamellar liposome suspensions. The size of the liposomes was determined using a submicron particle analyzer



I: Sia-t-pa R= n-$C_{15}H_{31}$
II: Sia-t-psa R= CH(n-$C_{16}H_{33}$)$_2$

Fig. 1. Structure of Synthesized Sialoglycolipids

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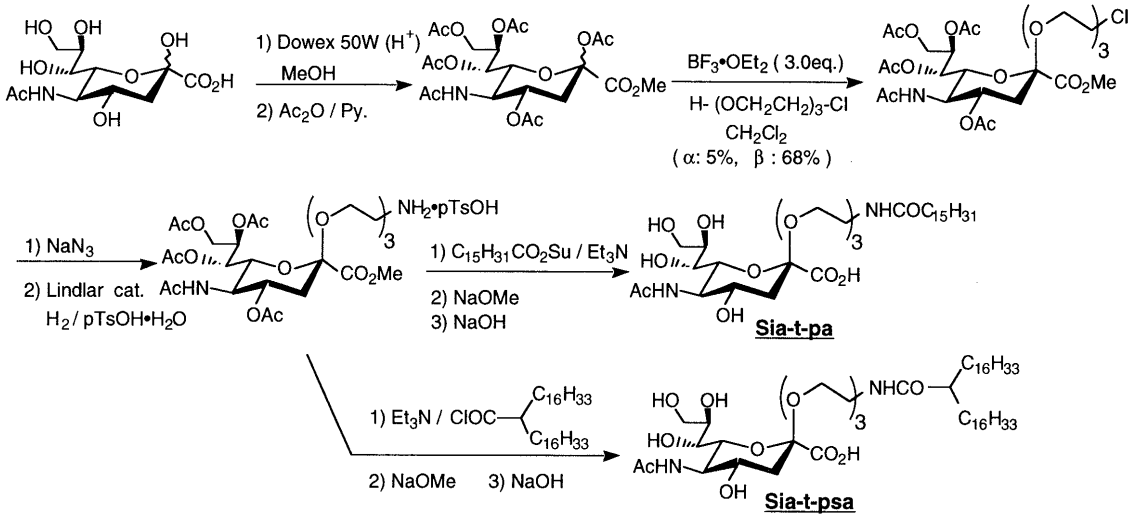

Chart 1. Synthesis of Sialoglycolipids

(NICOMP 370, Pacific Scientific, MD, U.S.A.). The mean diameter of the liposomes ranged from 100—170 nm, except for pure DPPC liposomes or DPPC : CHOL liposomes without electric repulsion on the membrane surface and DPPC liposomes containing large amounts of Sia-t-pa. The mean diameter of the series of DPPC or DPPC : CHOL liposomes ranged from 500—740 nm by the aggregation between liposome particles and that of DPPC : Sia-t-pa liposomes ranged from 12—90 nm by the surfactant-like effect of Sia-t-pa. Liposomes for evaluating the elution patterns of Sia-t-pa and Sia-t-psa *i.e.*, DPPC : CHOL : DCP : Sia-t-pa (molar ratio, 10 : 10 : 1 : 2) and DPPC : CHOL : DCP : Sia-t-psa (molar ratio, 10 : 10 : 1 : 2) were prepared according to the method for the preparation of liposomes containing fluorescent probe.

Fluorescence Measurements For steady-state fluorescence anisotropy measurements, the concentration of probe entrapped in liposome suspensions was kept constant at 3.85×10^{-7} M for DPH and 1.93×10^{-6} M for DSHA, respectively, and the lipid concentration was kept constant at 5×10^{-5} M for DPH and 2.5×10^{-4} M for DSHA, respectively. Fluorescence anisotropy measurements were based on the intensity of the fluorescence emission of DPH (excitation, 357 nm; emission, 428 nm) or DSHA (excitation, 337 nm; emission 533 nm), respectively, and determined using a Jasco fluorescence spectrofluorometer F-777 (JASCO Corporation, Tokyo, Japan) with a fluorescence anisotropy dipolarization meter ADP-301 coupled to a Jasco temperature controller.

The steady-state fluorescence anisotropy of the fluorescent probe, r_s, can be obtained from Eq. 1,

$$r_s = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

where I is the fluorescence intensity and subscripts V and H indicate the vertical and horizontal orientations of the excitation (first term) and analyzer(second term) polarizers, respectively. G ($= I_{HV}/I_{HH}$) is a grating correction factor. The temperature scanned was between 20 and 60 °C.

Differential Scanning Calorimetry All calorimetric scans were performed with a SEIKO DSC 120 (Seiko Instruments Inc., Tokyo. Japan). On the basis of these experiments, the systems remained closed to equilibrium throughtout their phase transition. The calorimeter was designed for measuring heat capacities and heat effects accompanying thermally induced transition of phospholipid molecules in 0.1 M aqueous NaCl. The temperature range for the instrument is 20—60 °C and the total volume of the sample compartment (Ag pan) is approximately 60 μl. The concentration of phospholipid was 10 mM. The thermal and enthalpic correction of the instrument was performed by indium and DPPC. All the experiments in this study were carried out at a scanning rate of 0.5 °C/min.

Determination of Sialoglycolipid by HPLC According to the method described in the previous paper,[20] Sia-t-pa and Sia-t-psa were determined by HPLC. The mobile phase was $CH_3OH : H_2O : HClO_4 = 800 : 200 : 1$ for Sia-t-pa, and $= 950 : 50 : 1$ for Sia-t-psa. Under these conditions, the retention times of Sia-t-pa and Sia-t-psa were 7.9 min and 14.4 min, respectively.

Results and Discussion

Evaluation of Siaglycolipid-Modified Liposomes by Gel Filtration Figure 2 shows the elution patterns of Sia-t-pa and Sia-t-psa by gel filtration using Sephacryl S-400 pre-equilibrated with PBS. About 50% of Sia-t-pa was eluted into the serum albumin fraction after the Sia-t-pa-modified liposomes were incubated with 50% rat serum albumin-containing PBS for 30 min at 37 °C. This confirmed that Sia-t-pa binds to serum albumin, and is released from liposomes. However, no Sia-t-psa eluted in the case of Sia-t-psa-modified liposomes incubated with 50% rat serum albumin-containing PBS. Gel filtration after incubation with PBS resulted in the elution of Sia-t-pa or Sia-t-psa intercalated in the liposomal bilayers into the void, confirming the incorporation of Sia-t-pa and Sia-t-psa into the liposome membranes.

Fluorescence Polarization Measurement Fluorescence polarization is a potent and convenient technique for providing information about fluidity in the lipid bilayer. In this study, DPH and DSHA were used as a tool to monitor the motion of molecules in the hydrophobic and hydrophilic domains of the lipid bilayer, respectively.[14,18,19,21-23] As seen in Fig. 3, the steady-state fluorescence anisotropy, r_s, of DPH intercalated in the liposomal bilayer, whose lipid composition was DPPC : Sia-t-pa (a) or DPPC : Sia-t-psa (b), was measured as a function of temperature. For pure DPPC liposomes, an abrupt change in r_s was observed at around the T_c (*ca.* 41 °C) of DPPC. For DPPC : Sia-t-pa liposomes (a), with increasing Sia-t-pa, r_s values in the gel state ($< T_c$) were smaller, whereas those in the liquid crystalline state ($> T_c$) were greater compared with those of pure DPPC liposomes. This means that the single straight acyl chain of Sia-t-pa tends to fluidize the liposomal bilayer even in the gel state and stabilize the liposomal bilayer in the liquid crystalline state. In the gel state, the addition of Sia-t-pa disturbed the packing of the DPPC molecules, and these DPPC molecules may increase the conformational freedom. In the liquid crystalline state, the swinging motion of the fatty acid chains will be suppressed by the hydrophobic moiety of Sia-t-pa. This dual character of Sia-t-pa , which fluidizes lipids in the gel state while it immobilizes them in the

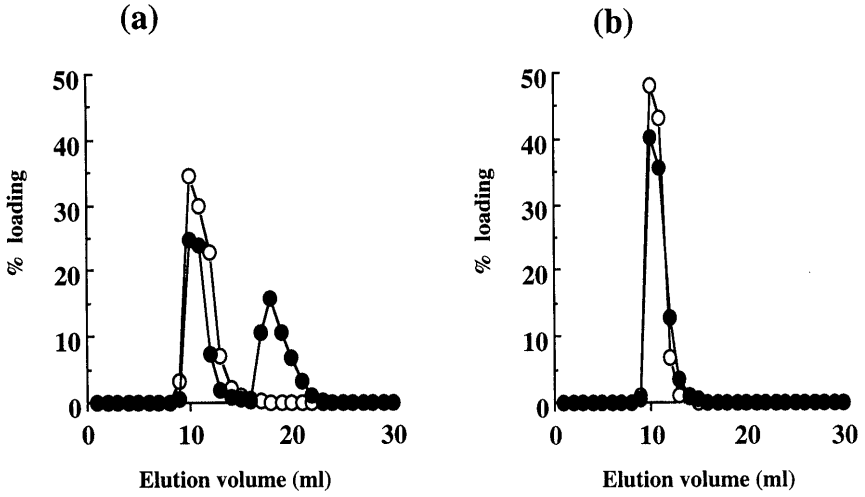

Fig. 2. Elution Profiles of Two Sialoglycolipids on Liposomes after Incubation with PBS or Rat Serum Albumin-Containing PBS

Incubated solution (1 ml) was loaded onto a Sephacryl S-400 column (12 mm i.d. × 15 cm) equilibrated with PBS (pH 7.4). (a), DPPC : CHOL : DCP : Sia-t-pa (molar ratio, 10 : 10 : 1 : 2); (b), DPPC : CHOL : DCP : Sia-t-psa (molar ratio, 10 : 10 : 1 : 2); ○, incubated with PBS; ●, incubated with 50% rat serum albumin-containing PBS.

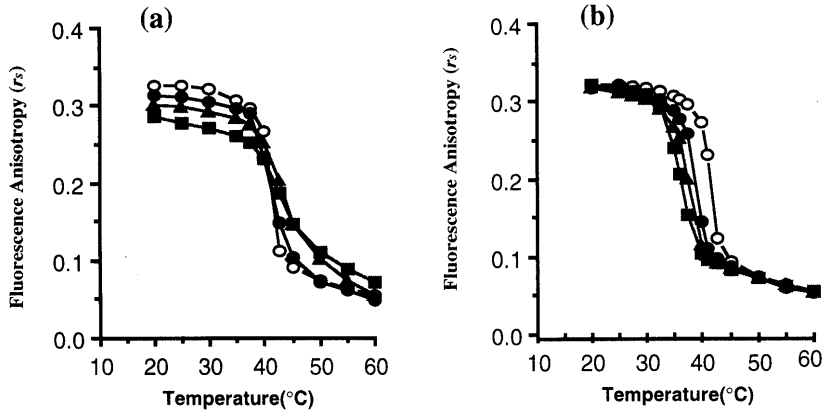

Fig. 3. Steady-State Fluorescence Anisotropy (r_s) of 1,6-Diphenyl-1,3,5-hexatriene in (a) DPPC : Sia-t-pa Liposomal Membranes and (b) DPPC : Sia-t-psa Liposomal Membranes as a Function of Incubation Temperature

Molar ratio of DPPC : sialoglycolipid: ○, 10 : 0; ●, 10 : 2.5; ▲, 10 : 5.0; ■, 10 : 7.5.

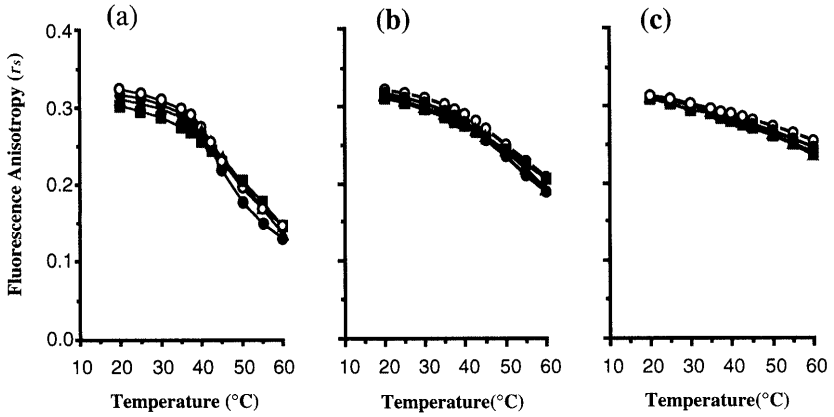

Fig. 4. Steady-State Fluorescence Anisotropy (r_s) of 1,6-Diphenyl-1,3,5-hexatriene in (a) DPPC : CHOL : Sia-t-pa (Molar Ratio, 10 : 2.5 : x, x = 0—7.5) Liposomal Membranes, (b) DPPC : CHOL : Sia-t-pa (Molar Ratio, 10 : 5.0 : x, x = 0—7.5) Liposomal Membranes, (c) DPPC : CHOL : Sia-t-pa (Molar Ratio, 10 : 10 : x, x = 0—7.5) Liposomal Membranes as a Function of Incubation Temperature

○, x = 0; ●, x = 2.5; ▲, x = 5.0; ■, x = 7.5.

liquid crystalline state, closely resembles the phenomena observed when cholesterol is added to pure DPPC liposomal bilayers,[24] except that T_c remains almost unchanged. For DPPC : Sia-t-psa liposomes (b), with increasing Sia-t-psa, r_s values both in the gel state and liquid crystalline state were similar to those of pure DPPC liposomes, whereas T_c was reduced.

As seen in Fig. 4, with increasing the molar ratio of

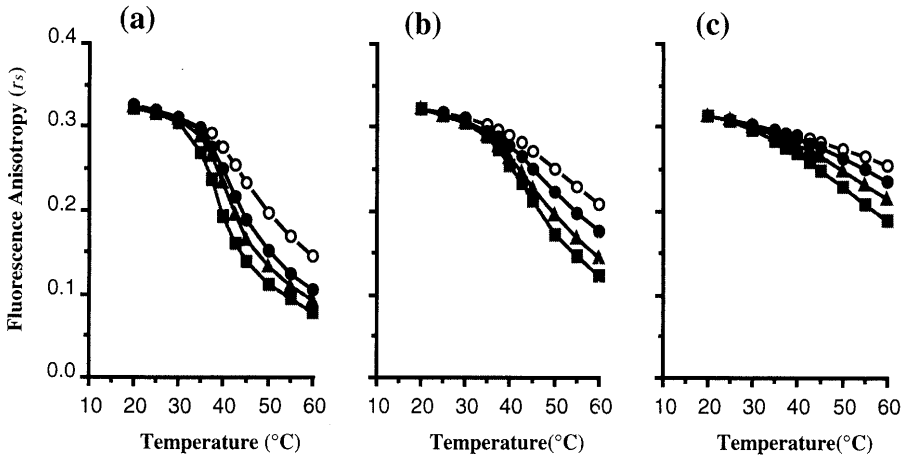

Fig. 5. Steady-State Fluorescence Anisotropy (r_s) of 1,6-Diphenyl-1,3,5-hexatriene in (a) DPPC : CHOL : Sia-t-psa (Molar Ratio, 10 : 2.5 : x, x=0—7.5) Liposomal Membranes, (b) DPPC : CHOL : Sia-t-psa (Molar Ratio, 10 : 5.0 : x, x=0—7.5) Liposomal Membranes, (c) DPPC : CHOL : Sia-t-psa (Molar Ratio, 10 : 10 : x, x=0—7.5) Liposomal Membranes as a Function of Incubation Temperature

○, x=0; ●, x=2.5; ▲, x=5.0; ■, x=7.5.

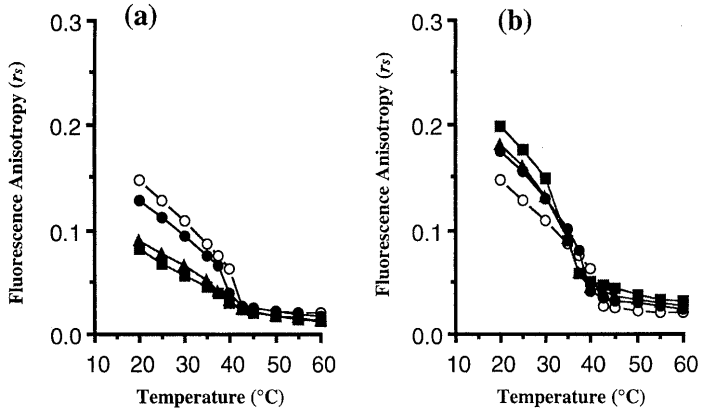

Fig. 6. Steady-State Fluorescence Anisotropy (r_s) of *N*-Dansylhexadecylamine in (a) DPPC : Sia-t-pa Liposomal Membranes and (b) DPPC : Sia-t-psa Liposomal Membranes as a Function of Incubation Temperature

Molar ratio of DPPC : sialoglycolipid: ○, 10 : 0; ●, 10 : 2.5; ▲, 10 : 5.0; ■, 10 : 7.5.

cholesterol, for DPPC : CHOL liposomes, a change in r_s gradually became smaller, and for DPPC : CHOL : Sia-t-pa liposomes, the effect of Sia-t-pa on decreasing r_s disappeared; in other words, the effect of Sia-t-pa on the hydrophobic region of DPPC molecules was not stronger than that of cholesterol when the molar ratio of cholesterol was larger.

For DPPC : CHOL : Sia-t-psa liposomes (Fig. 5), the effect of Sia-t-psa on decreasing r_s value was observed, and the effect was smaller with increasing cholesterol content, in the higher temperature region; no effect in the lower temperature region was observed, even in the presence of cholesterol. It is known that DPH, which is a fluorescent probe for monitoring the motion of molecules around it, is located in the hydrophobic domain of the lipid bilayer consisting of the acyl chains of phospholipids.[21-23] From the results on the temperature dependence of r_s and the fact that the influence on T_c noticeably differed between Sia-t-pa and Sia-t-psa, it is inferred that Sia-t-psa was located deeper than Sia-t-pa in the hydrophobic region of the liposomal bilayers up to the position at which Sia-t-psa influenced T_c.

As seen in Fig. 6, the r_s of DSHA intercalated in the liposomal bilayers was measured as a function of temperature, where it is considered that there was no interaction between the sialoglycolipids and the DSHA probe because the motion of the DSHA probe was sensitive to the additional amount of sialoglycolipids, although the molar ratio of DSHA/Sia-t-pa was below 1/30. For pure DPPC liposomes, an obvious change in r_s was also observed at around T_c, although it was smaller than that observed for DPH. For DPPC : Sia-t-pa liposomes(a), the change in r_s observed at around T_c gradually became more indistinct and the r_s values in the gel state were smaller compared with that of pure DPPC liposomes with increasing Sia-t-pa over a range of incubation temperatures. From this result, it is considered that the interaction based on hydrogen bonding between the hydrophilic regions of DPPC molecules was weakened by intercalation of Sia-t-pa molecules. For DPPC : Sia-t-psa liposomes (b), the r_s values in both gel-state and liquid crystalline state were greater compared with pure DPPC liposomes, which might be caused by the network based on hydrogen bonding between DPPC molecules and Sia-t-psa formed by amide bonds in Sia-t-psa in a region close to the surface of the lipid bilayer membranes,[25] accompanying the reduction

in T_c with increasing Sia-t-psa.

In the case of the series using the DSHA probe, a change in r_s gradually smaller with increasing the molar ratio of cholesterol, as well as using the DPH probe, and r_s values were smaller in the lower temperature region with increasing Sia-t-pa than with pure DPPC liposomes. On the other hand, r_s values were greater compared with those of pure DPPC liposomes over a range of incubation temperatures with increasing Sia-t-psa. With the DSHA probe, the effects of the sialoglycolipids on the r_s values were also very different for Sia-t-pa and Sia-t-psa and this was also the case for the DPH probe.

It is known that DSHA is located in the hydrophilic region (in the vicinity of the surface) which consists of the headgroups of DPPC molecules.[14] It is assumed that the hydrophilic moiety of Sia-t-psa located in the hydrophilic region of the DPPC molecule and the interaction between neighboring headgroups of DPPC molecules and Sia-t-psa may be stronger than that between the headgroups of pure DPPC molecules, whereas Sia-t-pa may weaken the interaction between neighboring headgroups of DPPC molecules although Sia-t-pa has the amide bond like Sia-t-psa. Then, since cholesterol is located in the hydrophobic region of liposomal membranes,[18] the effect of cholesterol on the fluidity of the hydrophilic region may be slight and cholesterol can not prevent the reduced fluidity of the hydrophilic region following the addition of Sia-t-pa.

From these observations, we can offer a schematic representation of the location of Sia-t-pa and Sia-t-psa in the phospholipid bilayer at lower temperatures ($< T_c$) as presented in Fig. 7. This scheme shows that Sia-t-psa is intercalated deeper in the hydrophobic region of liposomal bilayers than Sia-t-pa and the amide bonds of Sia-t-psa are located in the hydrophilic region and reinforce the interaction between the hydrophilic moieties of DPPC molecules and Sia-t-psa. On the other hand, since the amide bonds of Sia-t-pa are not located in the hydrophilic region, although Sia-t-pa has an amide bond like Sia-t-psa, the interaction between the hydrophilic moieties of DPPC molecules and Sia-t-pa is considered to be weak.

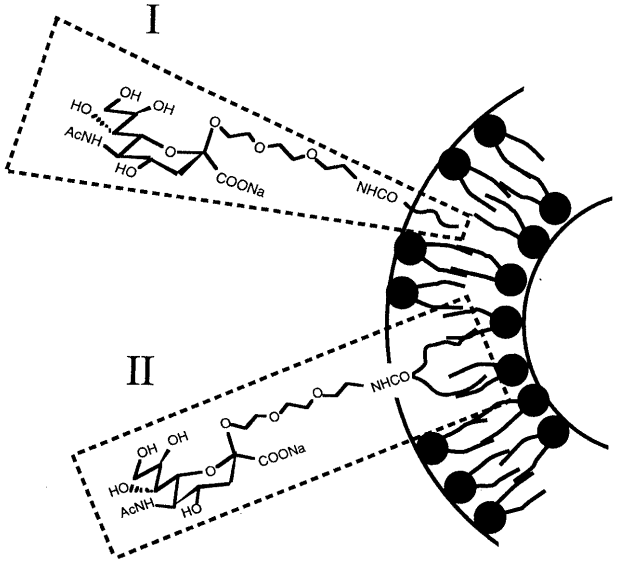

Fig. 7. Schematic Representation of Location of Sia-t-pa (I) and Sia-t-psa (II) in the Phospholipid Bilayer

Differential Scanning Calorimetry (DSC) High sensitivity differential scanning calorimetry has been used to study the thermotropic behavior of dispersed liposomes, the lipid composition of which is pure DPPC, DPPC : Sia-t-pa or DPPC : Sia-t-psa over the temperature range 20—60 °C. The reproducible calorimetric scan data were obtained for pure DPPC liposomes and a variety of DPPC : Sia-t-pa and DPPC : Sia-t-psa liposomes. Representative scans are shown in Fig. 8. The scan for pure DPPC was characteristic of multilamellar dispersions of phospholipid with a main phase transition at about 39 °C. The enthalpy of the main phase transition was 44 ± 9 mJ/mg. As the DSC data were obtained under experimental conditions where the DPPC concentration was very low (7.3 mg/ml), the pre-phase transition disappeared and the main phase transition temperature was seen to be smaller than that in the literature.[26] When Sia-t-pa was added in increasing amounts to pure DPPC, the main phase transition temperature remained almost constant in good agreement with the data from the fluorescence polarization experiment, whereas the enthalpy of the main phase transition became smaller with increasing Sia-t-pa. For DPPC : Sia-t-pa (10 : 7.5) liposomes, as the enthalpy became almost zero, it would seem that the liposomes

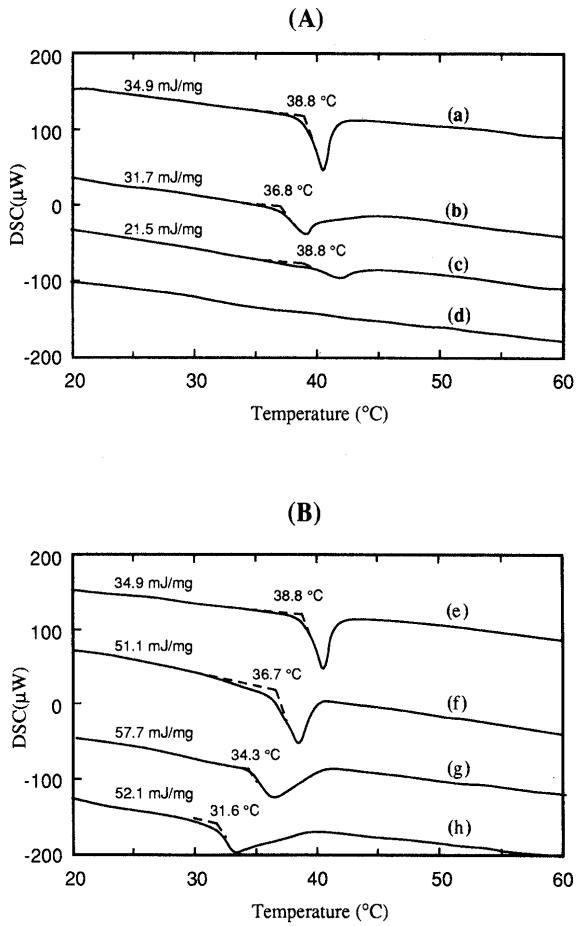

Fig. 8. Representative Calorimetric Heating Scans of Mixtures of DPPC with Sia-t-pa (A) and Sia-t-psa (B)

The main DPPC gel–liquid crystal transition temperature and enthalpy with the main transition are shown in the Figures. Molar ratio of DPPC : Sia-t-pa: (a) 10 : 0, (b) 10 : 2.5, (c) 10 : 5.0, (d) 10 : 7.5. Molar ratio of DPPC : Sia-t-psa: (e) 10 : 0, (f) 10 : 2.5, (g) 10 : 5.0, (h) 10 : 7.5.

changed their conformation from a DPPC bilayer to mixed micelles formed by DPPC and Sia-t-pa. However, fluorescent anisotropy was observed for DPPC : Sia-t-pa (10 : 7.5) liposomes, showing that the gel state, which was not detected by DSC, existed to some extent in DPPC : Sia-t-pa micelles. These experimental results can be explained well by interpreting the results as indicating that the acyl chain of Sia-t-pa is not located in the hydrophobic region of the DPPC bilayer and, hence, can disrupt the structure of the hydrophobic domain to some extent. On the other hand, when Sia-t-psa was added in increasing amounts to pure DPPC, the main phase transition temperature shifted toward the lower temperature, and the enthalpy of the main phase transition gradually increased. This inferred that the acyl chain of Sia-t-psa was intercalated into the hydrophobic region of the DPPC bilayer and the hydrophobic region became packed. The experimental results suggest that the location model in Fig. 7 is a suitable one.

The present data support the interpretation that the single acyl chain amphiphile, Sia-t-pa, can be incorporated into the DPPC bilayer. However, at lower temperatures ($< T_c$), no strong interaction in the hydrophilic region was produced and Sia-t-pa can be extracted from the surface of the liposomal membrane by plasma components, whereas the double acyl chain amphiphile, Sia-t-psa, remains in the lipid membrane as this amphiphile is intercalated deeper in the DPPC molecules than Sia-t-pa and produces a strong interaction between Sia-t-psa and DPPC molecules in the hydrophilic and hydrophobic regions and hydrogen bonding between DPPC molecules; the amide bond of Sia-t-psa also contributes to this stability. In our opinion the difference in stability of intercalation in the lipid bilayer between Sia-t-pa and Sia-t-psa is associated with the difference in their intercalating position in the lipid bilayer.

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