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Mutual recognition between polymerized liposomes: enzyme and enzyme inhibitor system

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In order to examine the usefulness of polymerized liposomes as a model for cell membranes, a mutual recognition phenomenon between different liposomes on which complementary ligands were attached was examined. We used trypsin- and soybean trypsin inhibitor (STI)-carrying polymerized liposomes to attain high sensitivities. The STI which was immobilized on the polymerized mono-dienoylphosphatidylcholine liposome showed a definite inhibitory effect on the catalytic activity of the trypsin which was immobilized on another polymerized liposome, whereas the inhibitory effect of the STI which was immobilized on the di-dienoylphosphatidylcholine liposome was much smaller than that of the mono-dienoylphosphatidylcholine system because of the larger rigity of the di-dienoylphosphatidylcholine liposome. These results suggest that the mutual recognition between complementary ligands can be realized by using polymerized liposomes with a physical stability and moderate deformability as their carriers.

Introduction

Mutual recognition of cells in vivo is an essential factor in organization of tissues and organs, and also in immunological protection system. In a cellular system main sites for a recognition of other cells exist on the surface of membranes. Cell membranes consist of lipid bilayers stabilized by polypeptides and polysaccharides basically, and many membrane proteins are floating (extrinsic protein) or dipping (intrinsic protein) in these lipid bilayers [1]. Some kinds of membrane proteins such as histocompatibility antigen ($H\text{-}2K^k$ [2–3], for example) do play an essential role in the cell-cell recognition system.

Recently polymerized liposomes, which are spherical bilayers stabilized by polymer chains, have been paid keen attention [4–10], because they are quite suitable analogues of biomembranes. Catalytic behaviors [11,12], usabilities as carriers of proteins [13–15], heme [16,17] and sugars [18], of polymerized liposomes have been extensively studied.

Many kinds of proteins such as bacteriorhodopsin [13], rhodopsin [14] and ATPase [15] have been examined to immobilize onto polymerized liposomes. These proteins are, however, typical intrinsic membrane proteins which are soluble in water only in the presence of surfactants. Only a few reports have been published about the immobilization of non-membrane proteins onto the polymerized liposomes [19]. Since the polymerized

Abbreviations: MDPE, monodienoylphosphatidylethanolamine; DDPC, di-dienoylphosphatidylcholine; MDPC, monodienoylphosphatidylcholine; TLME, *N*-tosyl-L-lysine methyl ester hydrochloride; STI, soybean trypsin inhibitor; DMS, 1,6-dimethylsuberimidate dihydrochloride; DSS, 1,6-dihydroxysuccinimidylsuberate.

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liposomes are useful as carriers of many biological substances such as drugs [5,20], it is worthwhile to examine polymerized liposomes as carriers of non-membrane proteins [21].

In this report, we studied the usefulness of polymerized liposomes as a model for cell membranes by examining a mutual recognition between polymerized liposomes modified with complementary ligands. We used phosphatidylcholine and phosphatidylethanolamine with one or two dienoyl aliphatic acids as liposome-forming monomer lipids and copolymerized these lipids by ultraviolet irradiation or radical copolymerization. Onto these liposomes we immobilized trypsin and soybean trypsin inhibitor (STI) as the ligands, because these proteins associate with each other very strongly and specifically ($K_i \doteqdot 10^{-10}$ M). The degree of recognition can be estimated from the catalytic and inhibitory properties of the proteins immobilized.

This is one of the investigations in a new research field, so called 'chemo-mimetic biology', because the proteins used here are only 'tools' of recognition, and biological meanings of these proteins are ignored. Such an idea would not be made by 'biologists' but by 'chemists' who are not largely obsessed by the 'raison d'être' of proteins in nature.

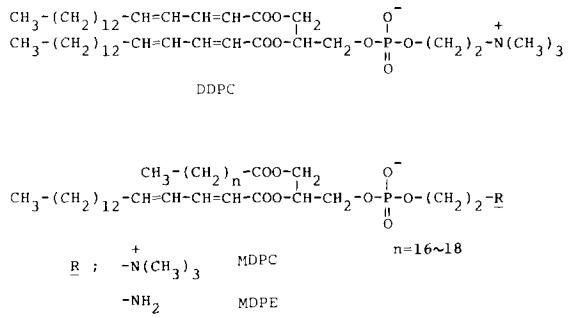

Fig. 1. Chemical structure of polymerizable lipids.

Experimental

Materials

Trypsin (EC 3.4.21.4) (bovine pancreas, T-8253, 11 000 units/mg, Sigma) and soybean trypsin inhibitor (12 000 BAEE units/mg, Miles 36-572) were used without further purification. Milli-Q grade water was used for preparation of solutions.

Polymerized liposomes

Phosphatidylethanolamine and phosphatidylcholine with a dienoyl aliphatic acid (MDPE, MDPC), and phosphatidylcholine with two dienoyl aliphatic acids (DDPC) (Fig. 1) were kindly donated by Dr. Y. Nagata, Tosoh Corporation, Tokyo. The purities of these lipids were confirmed by both NMR and thin-layer chromatography. These lipids (45 mg MDPC or DDPC and 5 mg MDPE) were dissolved in 2 ml of dichloromethane in a round-bottomed flask and the solvent was evaporated using a vacuum pump. Water (10 ml) was added and the suspension was mixed using a vortex-mixer for 5 min at 40 °C while N_2 gas was continuously passed into the flask. The turbid suspension was ultrasonicated under a flow of N_2 gas for 30 min at 40 °C using a Kontes cell-disruptor. Afterwards, the liposome suspension in a quartz cell was irradiated using an Ushio high-pressure mercury lamp (250 W) for several hours. A polymerization process of these lipids was followed by the decrease in absorbance of dienoyl groups at 258 nm.

In the DDPC-MDPE system, we examined radical copolymerization. At first 5 mol% of AIBN (azobisisobutyronitrile) was added to the liposome suspension to polymerize dienoyl groups in the hydrophobic region, and the suspension mixture was incubated at 60 °C. After 22 hours (conversion of polymerization; 68%) 5 mol% of V-50 (2-azobis(2-methylpropamidinium) dichloride, Wako Pure Chemicals, Osaka) was added to polymerize residual dienoyl groups in the hydrophilic region of the liposome [22], and the suspension was further incubated for 20 h at 60 °C (conversion of polymerization; 87%). To complete the polymerization, the suspension was finally ultraviolet irradiated for 10 hours.

The presence of polymer was confirmed by the gel permeation chromatography (mobile phase; 50% chloroform-methanol) using Cosmosil columns (5GPC-100 (4.6 × 250 mm), 5GPC-300 (4.6 × 250 mm)) (Nakarai Chemicals, Kyoto) connected to a Waters HPLC system.

Diameters of the polymerized liposomes were estimated using a dynamic light scattering ap-

paratus (BI 2230, Brookhaven, New York) with a He-Ne laser (6328 Å, NEO-15MS, Japan Science Engineering, Osaka) [23].

Immobilization of trypsin. The polymerized liposome suspension (4 ml) was mixed with 1,6-dihydroxysuccinimidylsuberate (2.5 mg) (DSS) or 1,6-dimethylsuberimidate dihydrochloride (3.0 mg) (DMS) at pH 8 or 10, respectively. The pH of the suspension was adjusted by using 0.1 M NaOH or HCl solution. The amounts of bifunctional reagents added were 3–4-times in excess of MDPE residues in the liposome to avoid cross-linking between liposomes. After 5 min the suspension mixture was quickly carried on a Sepharose 6B column (10×2 cm) at 4° C and at pH 8 (DSS) or 10 (DMS), respectively.

The liposome fraction was collected using a Pharmacia fraction collector (KM-100) connected to a mini UV monitor (Type II, Atto, Tokyo). The activated liposome obtained was immediately coupled with trypsin (1.5 mg) or STI (2.0 mg) at room temperature and pH 8.0–8.5 for 30 min and, afterwards, the suspension was continuously stirred at 4° C for 18 hours. The suspension mixture was carried onto a Sepharose 6B column (23×2 cm) again to separate the protein-carrying liposome from the unreacted protein at 4° C and pH 8.0.

The amount of protein bound onto the liposome was determined from the absorption of the filtrate. By the microscopic observation (Axiomat, Carl-Zeiss) and dynamic light scattering technique, we could confirm the absence of self aggregation of cross-linking of the polymerized liposomes during the modification process.

Kinetic measurements. The catalytic activity of the immobilized trypsin was followed using a pH stat titrator (RTS 622, Radiometer, Copenhagen). The reaction vessel (5 ml) was thermostated at 25 ± 0.05° C by a Neslab waterbath RTE-8. The catalytic reaction was started by the addition of small amount of immobilized trypsin suspension to the solution containing 0.016 M $CaCl_2$ and various amount of substrate (*N*-tosyl-L-lysine methyl ester hydrochloride, TLME). In the case of inhibition reactions, the suspension mixture containing trypsin and STI was incubated for 30 min at 25° C, and, afterwards, the substrate solution was added.

Association processes of the protein-carrying liposomes were observed by the turbidity change at 300 nm using a high-sensitivity spectrophotometer (SM-401, Union Giken, Osaka, Japan).

Fluorescence anisotropy depolarization. The lipid structural order of the polymerized liposome was measured using a fluorescence anisotropy depolarization method. We used a single-photon counting system consisting of a photomultiplier (PRA model 1550), a time-to-amplitude converter (ORTEC model 457), a discriminator (ORTEC model 436), a constant fraction discriminator (ORTEC model 583), and a multichannel analyzer (Hitachi 505). As the exciting light apparatus, a hydrogen gas-filled flash lamp (PRA model 510B) was used (pulse width about 2 ns). The exciting light was polarized by means of a polarizing filter (Polaroid HNP'B), and the fluorescence intensity was measured through the same kind of filter [24]. As a fluorophore we used 1,6-diphenylhexatriene.

DSC measurements. The phase transition point of liposomes was determined by a differential scanning calorimetry using a SSC580 (Daini-Seikosha, Tokyo, Japan). The concentration of lipid suspensions was 25 $mg \cdot ml^{-1}$, and the sample volume was 50 μl. The raising rate of temperature was 0.5 $C° \cdot min^{-1}$.

Results and Discussion

A. Trypsin-carrying polymerized liposome

Using a dynamic light scattering technique the average diameters of liposomes before and after polymerization were estimated to be 2000 Å and 1200 Å (MDPC-MDPE, ultraviolet irradiation), 1500 Å, 1400 Å (DDPC-MDPE, ultraviolet irradiation) and 1100 Å (DDPC-MDPE, radical initiation followed by ultraviolet irradiation), respectively.

The degree of polymerization for the MDPC-MDPE was evaluated to be 4 by the GPC. As for the DDPC-MDPE, we could not estimate the degree of polymerization because of the extremely low solubility of the polymerized DDPC-MDPE due to the two-dimensional networks [25].

The temperatures of the midpoint of phase transition (T_m) of the MDPC liposome were 31° C and 34° C before and after the polymerization, respectively. The T_m value of the DDPC was

about 25 °C before polymerization, but we could not detect the phase transition after polymerization.

The amount of proteins bound to liposomes were estimated to be 0.3–0.4 mg/10 mg lipid from the absorption of filtrate. The surface concentration of trypsin immobilized onto polymerized liposomes was estimated to be one trypsin molecule per 1430 lipid molecules (210 MDPE molecules) for the MDPC-MDPE liposome, and 1990 lipid molecules (360 MDPE molecules) for the DDPC-MDPE liposome (ultraviolet irradiation) and 590 lipid molecules (90 MDPE molecules) for DDPC-MDPE liposome (radical initiators and ultraviolet irradiation), respectively. Trypsin molecules were considered to be homogeneously distributed on the liposome surface, because we could not find a phase separation of lipid molecules by the DSC measurements.

Assuming that the liposomes consist of five bilayers and the occupied area per one lipid molecule is 58 $Å^2$ in the liquid-crystal phase [26], the number of trypsin bound to one liposome was roughly estimated to be about 460 (MDPC-MDPE), 600 (DDPC-MDPE, ultraviolet irradiation) and 730 (DDPC-MDPE, radical initiators and ultraviolet irradiation), respectively.

The trypsin immobilized onto the polymerized liposome had a satisfactorily high catalytic activity. The percent of immobilization (ratio of immobilized and supplied trypsins) was estimated to be 3.7% (MDPC-MDPE), 5.7% (DDPC-MDPE, ultraviolet irradiation) and 10.2% (DDPC-MDPE, radical initiators + ultraviolet irradiation), respectively.

The relative activity (ratio of activity of free and immobilized trypsins) were 33% (MDPC-MDPE), 9.2% (DDPC-MDPE, ultraviolet irradiation) and 7.5% (DDPC-MDPE, radical initiators + ultraviolet irradiation), respectively. The percent of immobilization was lower than those of literature values (onto glutaraldehyde-activated albumin microspheres 47% [27], onto azide group-containing latex particles; 50% [28]), because we added a large amount of trypsin to the reacting suspension to avoid cross-linking of liposomes mediated by trypsin molecules.

Fig. 2 shows the pH dependence of the catalysis by the immobilized and free trypsins. By the

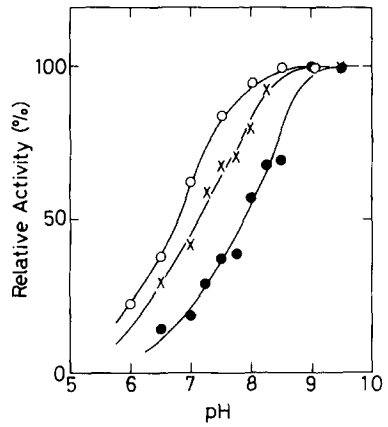

Fig. 2. Effect of pH on the catalytic activity of trypsins at 25 °C. [TLME] = 400 μM, [$CaCl_2$] = 16 mM. ○, free, ×, trypsin immobilized by the DMS method. ●, trypsin immobilized by the DSS method.

immobilization of trypsin onto polymerized liposomes using DSS, the pH profile of the catalytic activity shifted to alkaline region by 1.0 pH unit, probably because of the residual phosphate (anionic) group of the phosphatidylethanolamine and the carboxyl group of the suberic acid bound to the MDPE residue. In the case of the liposome modified by using DMS, the pH profile of the immobilized trypsin was between that of free trypsin and that of trypsin immobilized by using DSS, probably because of the compensation between the introduction of the cationic cross-linking reagent and the consumption of amino groups. A similar pH shift induced by the ionic groups on carriers [29] has been observed very often in the catalysis of enzymes immobilized onto albumin-glutaraldehyde microspheres [27] and carboxyl group-containing polymer latex particles [30–32].

The Michaelis constant (K_m) and activation energy (E_a) of free and immobilized trypsins are shown in Table I. The values of K_m and E_a for free and immobilized trypsins are similar with each other, which suggests that the immobilization onto polymerized liposome does not strongly influence the catalytic behavior of trypsin.

The storage stability of trypsin immobilized to the polymerized MDPC-MDPE liposome was also examined at 10 °C. The catalytic activity of the immobilized trypsin gradually decreased with time to 50% of the initial activity after two weeks. As

TABLE I

KINETIC PARAMETERS OF THE TRYPSIN CATALYZED REACTION AT pH 8.0

	Free	Immobilized
K_m (μM)	71 $\pm$10 [a]	60 $\pm$10 (DMS [b])
		49 $\pm$ 8 (DSS [b])
E_a (kcal·mol^{-1})	11.4$\pm$ 0.2	12.6$\pm$ 0.6 (DSS [b])

[a] Literature value: 74$\pm$10 μM [31], 101$\pm$7 μM [33], 41.8$\pm$4.3 μM [34].
[b] Reagent used for immobilization.

for free trypsin, only 3% of the initial catalytic activity remained after the storage for two weeks at 10 °C.

B. Interaction of trypsin with STI on polymerized liposome

Next we examined the inhibitory effect of STI on the catalytic activity of trypsin immobilized on the polymerized liposome (MDPC-MDPE). Fig. 3 shows the inhibitory effect of the immobilized STI on the immobilized trypsin. By the curve-fitting method at the low concentration range of STI the inhibition constant (K_i) for various trypsin-STI systems were estimated roughly (Table II).

By the immobilization the apparent inhibitory effect became lower but still definite. The unexpectedly small decrease in inhibition effect of STI by the immobilization is due to the curve fitting at the low STI concentration region. If we calculate the K_i value at the high STI concentration region,

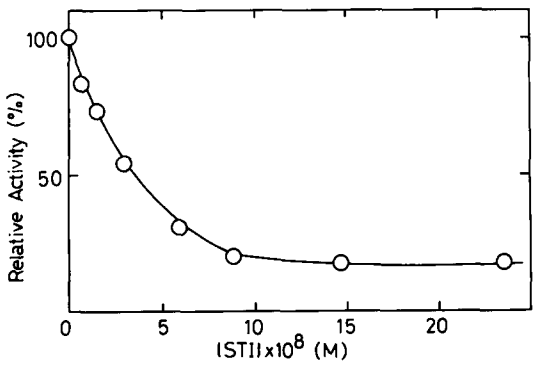

Fig. 3. Inhibitory effect of the STI-liposome on the hydrolysis of TLME catalyzed by the trypsin-liposome. MDPC-MDPE system. 25 °C. pH 8.0. [trypsin] = 7.2 nM, [TLME] = 400 μM, [$CaCl_2$] = 16 mM.

TABLE II

K_i VALUES OF FREE AND IMMOBILIZED PROTEIN SYSTEMS [a]

Trypsin	STI	K_i (M)
Free	Free	$1\cdot10^{-10}$
DSS [b]	Free	$2\cdot10^{-9}$
DMS [b]	Free	$5\cdot10^{-9}$
Free	DSS [b]	$3\cdot10^{-8}$
DSS [b]	DSS [b]	$2\cdot10^{-8}$
DMS [b]	DSS [b]	$3\cdot10^{-8}$

[a] pH 8.0, 25 °C. [$CaCl_2$] = 0.016 M.
[b] Reagent used for immobilization.

the K_i value would become much larger in the immobilized protein system as easily understood from Fig. 3. The inhibitory effect of low-molecular mass inhibitor, thionine, on the immobilized trypsin was similar to that on free trypsin (K_i for free trypsin; 31 $\pm$ 6 μM, that for the immobilized trypsin; 27 $\pm$ 7 μM at pH 8.0, [$CaCl_2$] = 16 mM).

The degree of inhibition in the liposome system can be considered to reflect the degree of surface-surface contact between different liposomes directly. As shown in Fig. 3, the immobilized STI largely inhibited the catalytic activity of the immobilized trypsin, which means that the trypsin-carrying liposome contacts with the STI-carrying liposome at wide areas. About 20% of the initial catalytic activity remained even by the addition of excess amount of STI-liposome, which might be due to the 'dead space' between associated liposomes.

When both proteins were immobilized covalently onto polymerized DDPC-MDPE liposome, the inhibitory effect was smaller than that of MDPC-MDPE system, and even by the addition of excess amount of STI-liposome, there still remained a large catalytic activity of the immobilized trypsin (Fig. 4). This is because the deformability of the liposome was lowered by the polymerization of two alkyl chains in the DDPC lipid, and the dead space between associated liposomes became much larger than that of the MDPC system.

Fig. 5 shows the effect of temperature on the inhibition constant (K_i) of the STI-liposome–trypsin-liposome system. In the free-trypsin–free STI system, the van't Hoff plot was linear and the

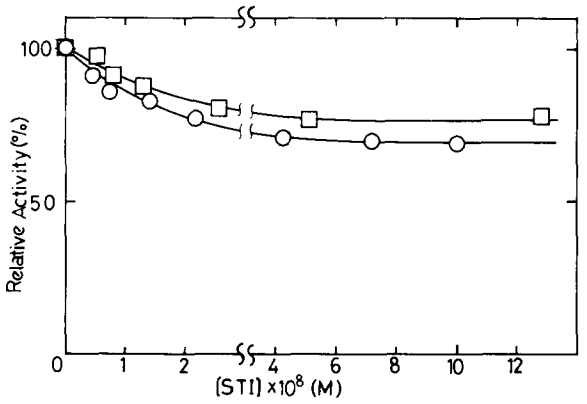

Fig. 4. Inhibitory effect of the STI-liposome on the hydrolysis of TLME catalyzed by the trypsin liposome. DDPC-MDPE system. 25 °C. pH 8.0. [TLME] = 400 μM, [$CaCl_2$] = 16 mM. ○, polymerized by ultraviolet irradiation ([trypsin] = 4.4 nM, $K_i = 7 \cdot 10^{-8}$). □, polymerized by radical initiators and ultraviolet irradiation ([trypsin] = 4.7 nM. $K_i = 1 \cdot 10^{-7}$ M).

thermodynamic parameters were reported to be -12.3 kcal · mol^{-1} (ΔG), 8.6 kcal · mol^{-1} (ΔH) and -69.8 eu (ΔS), respectively [35]. In the immobilized STI-immobilized trypsin system (lipid; MDPC), the van't Hoff plots were not linear and seemed to have a bending point at about 25 °C. The bending point might correspond to the initial point of the phase transition (T_c) of the polymerized MDPC. This result supports that the inhibition effect is affected strongly by the deformability of the carrier liposome.

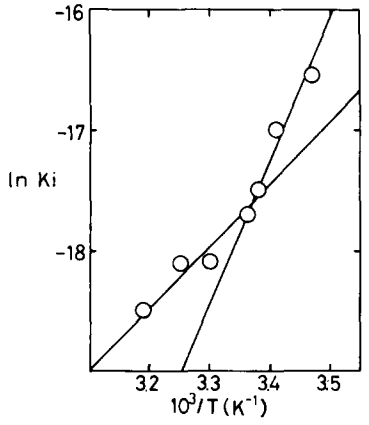

Fig. 5. Temperature dependence of K_i, pH 8.0. [TLME] = 400 μM, [$CaCl_2$] = 16 mM. $\Delta H = 22.5$ kcal · mol^{-1} ($T > 25$ °C), $\Delta H = 10.3$ kcal · mol^{-1} ($T < 25$ °C).

To examine a correlation of the lipid structural order with the degree of contact between protein-conjugated liposomes, we carried out the fluorescence anisotropy depolarization measurements. The effect of polymerization on the lipid structural order, however, could not be clearly observed because of the localized distribution of fluorescent probe, diphenylhexatriene [36], in the clefts formed between blocks of polymerized lipids on the liposome surface [37,38].

In the latex system which we examined previously, we could not clearly detect the inhibitory effect of immobilized STI on the catalysis by the immobilized trypsin, though the association of the STI-latex with the trypsin-latex could be observed by the spectrophotometric measurements [31]. This is because a steric hindrance of the rigid latex particle is extremely large. Using a microscopic technique, we could directly determine rate constants of the association processes between latex particles [39–42]. The degree of recognition and surface-contact between different latex particles, however, could not be quantitatively estimated.

In contrast to polymer latex systems, we could detect the surface-recognition quite easily in the polymerized liposome system as described above. These results could be interpreted by the deformability of the polymerized liposomes to permit the interaction between proteins immobilized onto them to a large extent. It should be mentioned here that Sackmann et al. observed an increase in the bending elasticity of the dimyristoylphosphatidylcholine (DMPC) liposome by the addition of 20 mol% of the methacryloyl group-containing polymerized phospholipid [43].

Association process of protein-carrying liposomes

In order to clarify the dynamic association process between the trypsin-liposome and the STI-liposome, we examined the spectrophotometric measurements of liposome suspension. Fig. 6 shows the effect of the STI-liposome on the initial absorption change of the suspension at 300 nm after mixing the STI-liposome with the trypsin-liposome. The figure shows that, by the addition of the STI-liposome, the absorption change of the suspension was increased. By the denaturation of STI molecules bound onto the liposome, the association phenomena disappeared,

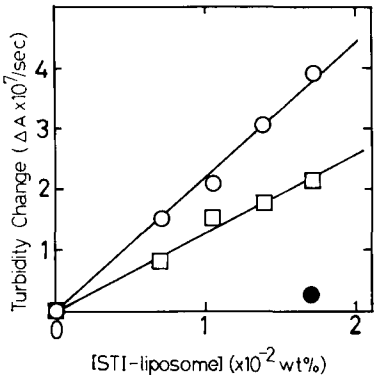

Fig. 6. Turbidity measurements of the trypsin-liposome–STI-liposome system. pH 8.0. [trypsin-liposome] = $2.7 \cdot 10^{-2}$ wt%. ○, 30 °C, □, 25 °C. ●, with the denatured STI-liposome at 30 °C.

which shows that the association could be solely attributed to the specific interaction between trypsin and STI molecules immobilized onto the polymerized liposome.

To further analyze the dynamic aspect of liposome-liposome association processes directly, we labelled the proteins by fluorescein isothiocyanate (FITC) and rhodamine isothiocyanate (RITC) separately, and immobilized these proteins onto the polymerized liposomes. We could observe the association of two kinds of liposomes on a slide glass. Because of a small size of the liposomes (≒ 1000 Å), however, it was impossible to make a dynamic analysis of the association processes. We are now trying to modify large unilamellar polymerized liposomes with fluorescent trypsins and STI's separately, and will report about the kinetic data obtained directly by the fluorescence microscope in the near future.

In conclusion, a liposome-liposome recognition phenomena could be realized by the introduction of trypsin and soybean trypsin inhibitor onto the polymerized liposomes. The degree of recognition was strongly dependent upon the deformability of carrier liposomes, and the deformability was easily controlled by the monomers used.

Acknowledgments

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