Estimation of Surface State of Poly(ethylene glycol)-Coated Liposomes Using an Aqueous Two-Phase Partitioning Technique

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Poly(ethylene glycol)-coated liposomes (PEG-liposomes) were prepared from distearoylphosphatidylcholine (DSPC)/cholesterol (Ch) (1:1, molar ratio) with various amounts of distearoyl-*N*-(monomethoxy poly(ethylene glycol)succinyl)phosphatidylethanolamine (DSPE-PEG). Surface potentials of PEG-liposomes showed negative values, however, the zeta potentials were almost neutral under physiological conditions (150 mM NaCl). Taking these electrical surface properties into consideration, a non-charge-sensitive phase system consisting of 5% PEG8000 and 5% dextran T-500, 0.01 M sodium phosphate, 0.15 M sodium chloride (pH 7.0) was used to estimate the alteration of surface state of PEG-liposomes after interaction with plasma *in vitro* and *in vivo*. PEG-liposomes showed increased partitioning to the PEG phase with increasing amount of DSPE-PEG. One hundred percent partitioning to the PEG phase was obtained when 2 or 1 mol% of DSPE-PEG1K or 2K was incorporated into the liposomes, respectively. This PEG/lipid ratio (mol/mol) thus afforded complete protection of the liposomal surface by the PEG moiety. When these PEG-liposomes were incubated with plasma protein (*in vitro*) or were recovered from liposome-injected mice (*in vivo*), they showed decreased partitioning to the PEG phase. However, when the *in vivo*-treated PEG-liposomes were purified by column chromatography and ultracentrifugation, their partitioning to the PEG phase was restored to that of PEG-liposomes incubated in phosphate-buffered saline. Thus although PEG acts as a steric barrier against the attachment of plasma protein to the liposome surface and slows down liposome clearance from the circulation *in vivo*, a weak interaction remains between PEG-liposome and plasma protein when the incorporated amount of DSPE-PEG is low.

Key words liposome; polyethylene glycol; phase partition system; dextran; drug delivery system

The main disadvantage of liposomes as a drug carrier is their marked accumulation in the reticulo-endothelial system (RES) after intravenous administration. However, this has been largely overcome by surface modification of liposomes, *i.e.*, by incorporating amphipathic poly(ethylene glycol) (PEG) derivatives into the liposome membrane (PEG-liposomes).[1-3] PEG-liposomes have a long lifetime in the bloodstream as well as reduced accumulation in the RES compared to conventional liposomes. It had been proposed that the marked steric hindrance and/or hydrophilic barrier due to the PEG moiety could prevent liposomes from being opsonized by serum proteins.[4] Torchilin *et al.* have presented a hypothetical model of the molecular mechanisms of protective action of PEG on liposomes *in vivo*.[5,6] The protective layer of PEG on the liposome surface is thought to exist as a statistical "cloud" of possible conformations of the PEG moiety in the solvent. Computer simulation demonstrated that a hydrophilic and flexible polymer molecule can create a dense conformational cloud over the liposome surface, preventing opsonizing protein molecules from contacting the liposome. Thus, the nature of the surface of PEG-liposomes should be taken into consideration in evaluating the stability and long-circulating character *in vivo*.

Aqueous two-phase partitioning in PEG–dextran or Ficol–dextran is a simple, readily accessible method to investigate the surface properties of liposomes.[7-11] The kinetics of the partitioning of liposomes in aqueous two-phase partition systems is dependent upon the vesicle size,[9] net charge,[10] hydrophobicity[11] and the presence of other components. Thus, since partitioning behavior provides an index of surface properties, this method can be used analytically. If two liposome preparations show different partitioning behavior in the same phase system, their surface properties must differ. By changing the composition of the two-phase system with respect to the concentration and molecular weight of polymer, and/or concentration and type of buffer salts, partitioning of liposomes can be made sensitive to their charge or to non-charged surface components. Senior *et al.* demonstrated that the retention of PEG on the surface of liposomes and the association between plasma proteins and the liposomal surface, when PEG-liposomes (tresylated PEG derivative with an average molecular weight of 5000 incorporated at 10 mol% of total lipids, sized to 144 ± 28 nm) were incubated with plasma, were readily detected by partition in a two-phase system of PEG 8000 and dextran T-500.[12]

In this study, the alteration of surface state of PEG-liposomes after interaction with plasma *in vitro* and *in vivo* was estimated using a PEG–dextran two-phase partition system. For the *in vivo* study, the influence of PEG moieties at the liposomal surface on the interaction of liposomes with plasma protein and on the retention time of liposomes in the bloodstream was investigated using liposome samples collected from mice after injection of PEG-liposomes.

Experimental

Materials Distearoylphosphatidylcholine (DSPC), distearoylphosphatidylethanolamine (DSPE), dipalmitoylphosphatidylethanolamine (DPPE), dimiristoylphosphatidylcholine (DMPC), and monomethoxy poly(ethylene glycol)succinimidyl succinate (PEG-OSu) with an average molecular weight of 1000 (1K), 2000 (2K) or 5000 (5K) daltons were generously donated by NOF (Tokyo, Japan). Distearoyl-*N*-(monomethoxy poly(ethylene glycol)succinyl)phosphatidylethanolamine (DSPE-PEG) and dipalmitoyl-*N*-(monomethoxy poly(ethylene glycol)succinyl)phosphatidyl-ethanolamine (DPPE-PEG) were synthesized

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as described previously.[3] Cholesterol (Ch) was purchased from Wako Pure Chemical (Osaka, Japan), and PEG with an average molecular weight of 8000 and dextran T-500 were from Sigma (MO, U.S.A.) and Pharmacia Biotech (Tokyo, Japan), respectively. ^{3}H-Cholesterol was obtained from New England Nuclear Japan (Tokyo). 6-(*p*-Toluidino)-2-naphthalenesulfonic acid (TNS) purchased from Aldrich Chem. (WI, U.S.A.) was used for surface potential measurement. All other reagents were commercial products of analytical grade.

Liposome Preparation PEG-liposomes were composed of DSPC and Ch (1 : 1, m/m) with several mol% of DSPE-PEG. ^{3}H-Cholesterol was incorporated as a marker. Small unilamellar liposomes (SUV, mean size of 120 nm) were prepared by the reverse-phase evaporation (REV) method followed by extrusion (Lipex Biomembranes, Canada) through a polycarbonate membrane filter (Nuclepore Co., CA, U.S.A.) with a pore size of 0.2 or 0.1 μm. Normal saline was used as the liposomal aqueous phase. In this preparation method, PEG chains are present on both sides of the liposomal membrane. The average size of these liposomes was measured by a dynamic light scattering method (Coulter Model N4 SD sub-micron particle analyzer, FL, U.S.A.). Phospholipid content of the liposome solution was determined by phosphorus assay.[13]

Surface Potential Measurement Surface potential (Ψ) was determined by TNS fluorescence as described.[14,15] Briefly, TNS was dissolved in distilled water and added to liposome samples at a concentration of 1 μM TNS at four lipid concentrations, 1 : 50, 1 : 100, 1 : 150 and 1 : 200, by dilution. In this experiment, we used liposomes prepared with DMPC instead of DSPC as the phospholipid, because the gel-to-liquid crystalline phase transition temperature (*Tc*) of DSPC is very high (*Tc* = 55 °C). The transition temperature influenced the membrane fluidity and, as a result, the binding of fluorescent probe on the liposomal membrane. The fluorescence intensity of TNS was measured at 25 °C with a Shimadzu RF 5000 spectrofluorometer (Kyoto, Japan). The excitation and emission wavelength were 321 and 445 nm, respectively. Surface potential was calculated using the relation:

$$f(-)/f = \exp[F\Psi/(RT)]$$

where F is the Faraday constant, R is the gas constant, T is the absolute temperature (25 °C), and $f(-)$ and f are fluorescence intensity of DMPC/Ch liposomes and PEG-liposomes (DMPC/Ch/DSPE-PEG2K, 1 : 1 : 0.13, mol/mol), respectively. The results are expressed as the average at each lipid concentration ($n = 4$).

Zeta Potential Measurement Zeta potential was determined with an ELS 800 (Otsuka Electronics Co., Tokyo). All measurements were done at 25 °C and the liposomes were moved at an electric field of below 38 V/cm.[14,16] Zeta potential (z) was calculated by means of the following Helmholtz–Smolukowski equation from the electrophoretic mobility (μ):

$$z = \mu\eta/\varepsilon$$

where η and ε are the viscosity and the dielectric constant of the solution, respectively. We used the values of $\eta = 8.93 \times 10^{-4}$ Ns/m^2 (= 0.893 cP), and $\varepsilon = 7.86 \times 10^{-10}$ F/m (= 78.6 CGSesu) at 25 °C.

Preparation of Partitioning Phase System A two-phase system of 5% PEG 8000 and 5% dextran T-500 in 1 : 1 volume ratio was prepared in 0.11 M sodium phosphate (pH 7.0) (charge-sensitive system) and 0.01 M sodium phosphate (pH 7.0), containing 0.15 M sodium chloride (non-charge-sensitive system) by mixing the appropriate weights of the following stock solutions: 22% dextran T-500, 30% PEG 8000, 0.44 M sodium phosphate (pH 7.0), 0.6 M sodium chloride and distilled water.[17] The two phases were equilibrated at 25 °C, separated and stored at 4 °C until use.

Phase Partitioning of PEG-Liposomes One hundred μl of ^{3}H-Ch-labeled liposomes was added to a mixture of PEG 8000 (top phase) and dextran T-500 (bottom phase) (0.75 ml of each) equilibrated at 25 °C in an 80 × 10 mm glass tube. The phases were mixed for 1 min by repeated inversion, then a 50 μl aliquot was taken to count total radioactivity. The mixture was left at 25 °C for an additional 30 min, then 25 μl of each phase (PEG 8000/top phase, dextran T-500/bottom phase) was sampled for counting and the distribution of liposomes between the phases was determined. The amount of liposomes at the interface was calculated by subtracting the sum of the radioactivity in the PEG 8000 and dextran T-500 phases from total radioactivity.

Phase Partitioning of Liposomes Treated with Plasma *in Vitro* and *in Vivo* For liposomal samples *in vitro*, 80 μl of ^{3}H-Ch-labeled liposomes was incubated with 320 μl of ddY mouse fresh plasma at 37 °C. At intervals, 100 μl aliquots of the incubated samples were collected and applied to the two-phase partition system. As controls, liposomes incubated with PBS at 37 °C for 3 h and those not incubated with mouse plasma (mixed liposomes and plasma) were used.

Partitioning of liposomes treated *in vivo* was also determined using the following two different liposomal samples. Liposomes labeled with ^{3}H-Ch (150 μl) were injected i.v. into male ddY mice *via* the tail vein. Plasma containing liposomes was obtained by centrifugation at 2500 × g for 20 min from blood drawn at 30 min post-injection, and applied to the two-phase partition system. Another liposome sample was obtained by gel-filtration (Bio-Gel A15m column) followed by ultracentrifugation (200000 × g for 30 min) of the above-mentioned plasma containing liposomes.

Partitioning of plasma protein of ddY mouse was done similarly, and the protein concentration was measured by Lowry's protein assay.

Results and Discussion

Surface Properties of PEG-Liposomes Aqueous two-phase partitioning is a simple method to investigate the surface properties of liposomal membranes. In order to study the membrane surface state of PEG-liposomes *in vitro* and *in vivo*, we first employed the above method to investigate the electrochemical surface properties of PEG-liposomes. In the PEG-liposomal formulation, DSPE-PEG was used as an amphipathic PEG derivative, with a negative charge. The surface potential of PEG-liposomes coated with DSPE-PEG in which the PEG has an average molecular weight of 1000 or 5000 is shown in Fig. 1. Their surface potential decreased with increasing amount of PEG, with no significant difference between the two specimens. In contrast, the zeta potential of PEG-liposomes increased with increasing salt concentration and molecular weight of PEG (Fig. 2). However, zeta potential was neutral at the physiological condition (150 mM NaCl). These results indicated that the surface potential of PEG-liposomes did not influence their zeta potential under this experimental condition.

By altering the sodium phosphate concentration, a charge-sensitive or a non-charge-sensitive two-phase system can be made. Three kinds of two-phase partitioning system were prepared to investigate the difference of partitioning behavior of PEG-liposomes. Two-phase systems of 5% PEG 8000 and 5% dextran T-500 were prepared in 0.11 M sodium phosphate (pH 7.0) (charge-sensitive phase systems), 0.068 M sodium phosphate/

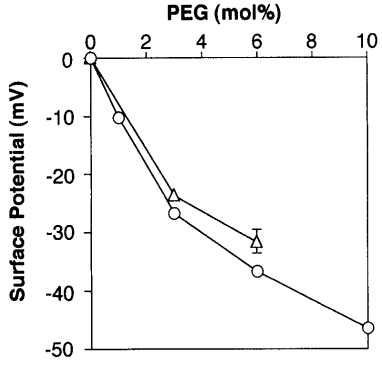

Fig. 1. Dependence of Surface Potential of DMPC/Ch Liposomes on Mole Percent of DPPE-PEG1K or 5K

○, DMPC/Ch/DPPE-PEG1K liposomes; △, DMPC/Ch/DPPE-PEG5K liposomes. Values represent the mean ±S.D. of triplicate measurements.

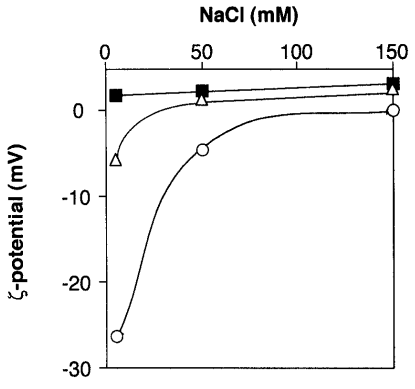

Fig. 2. Effect of NaCl Concentration on the Zeta Potential of Bare Liposomes and PEG-Liposomes

Liposomes were composed of ■, DSPC/Ch (1 : 1, mol/mol); ○, DSPC/Ch/DSPE-PEG1K (1 : 1 : 0.13, mol/mol); or △, DSPC/Ch/DSPE-PEG5K (1 : 1 : 0.13, mol/mol).

Each data point represents the mean ±S.D. of triplicate measurements.

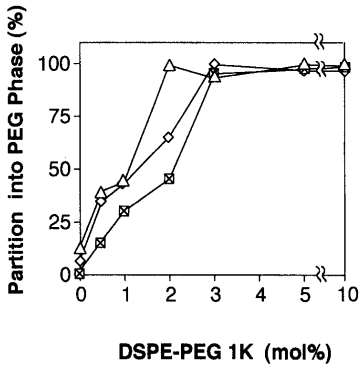

Fig. 3. Effect of Sodium Phosphate Concentration on the Partitioning Behavior of PEG-Liposomes (Labeled with ^{3}H-Cholesterol) Containing Various Amounts of DSPE-PEG1K in a Two-Phase Partition System of PEG 8000 and Dextran T-500

Sodium phosphate concentration was △, 0.01 M; ◇, 0.068 M; ⊠, 0.11 M. $n=3$, ±S.D. < 5%.

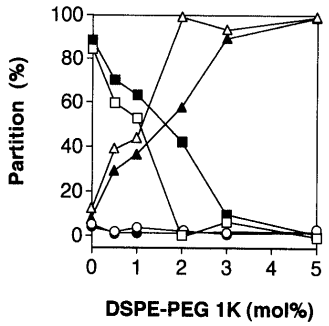

Fig. 4. Effect of Liposomal Size (120 and 200 nm Mean Diameter) on the Partitioning Behavior of PEG-Liposomes Composed of DSPC/Ch/DSPE-PEG1K (1 : 1 : 0.13, mol/mol) in the Non-charge-Sensitive Phase System Consisting of 5% PEG 8000 and 5% Dextran T-500, 0.01 M Sodium Phosphate, 0.15 M Sodium Chloride (pH 7.0)

Partitioning of PEG-liposomes 120 nm in mean diameter (open symbols) into the PEG phase (△, ▲), interface (□, ■) and dextran phase (○, ●) and that of PEG-liposomes 200 nm in mean diameter (closed symbols) are shown as percentage of the total. $n=3$, ±S.D. <5%.

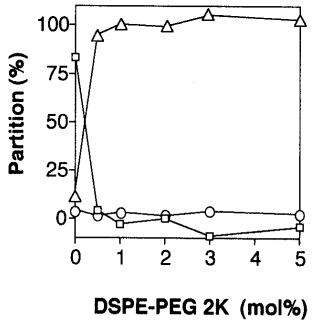

Fig. 5. Partitioning Behavior of PEG-Liposomes Containing Various Amounts of DSPE-PEG2K in the Non-charge-Sensitive Phase System

Liposomes were labeled with ^{3}H-cholesterol. Partitioning into the PEG phase (△), interface (□) and dextran phase (○) is shown as percentage of the total. $n=3$, ±S.D. <5%.

0.075 M sodium chloride (pH 7.0), and 0.01 M sodium phosphate/0.15 M sodium chloride (pH 7.0) (non-charge-sensitive phase systems), which have relatively high, medium and low electrostatic potential differences between the phases ($\Delta\Psi$), respectively.[17]

Figure 3 shows the partitioning behavior of PEG-liposomes prepared with DSPC/Ch (1 : 1, mol/mol) containing various amounts of DSPE-PEG1K in the three kinds of two-phase systems. Partitioning behavior of PEG-liposome was different from the two-phase systems and by increasing the incorporated amount of DSPE-PEG1K into DSPE/Ch liposomes (120 nm mean diameter), the higher partitioning into the PEG phase was observed in the non-charge-sensitive system as compared with the charge-sensitive system. PEG-liposomes not partitioned into the PEG phase were partitioned to the interface between PEG and dextran phase (data not shown). So, in these partitioning systems, the non-charge-sensitive phase system can sensitively distinguish between the presence and absence of the PEG moiety on the liposomal surface, which may correspond to the neutral zeta potential at the physiological condition. Therefore, in the following experiment, the non-charge-sensitive phase system consisting of 5% PEG 8000 and 5% dextran T-500, 0.01 M sodium phosphate, and 0.15 M sodium chloride (pH 7.0) was used to estimate the alteration of the surface state of PEG-liposomes.

Partitioning behavior of liposomes is also influenced by particle size, lipid composition, and solvent. The effect of particle size (120 nm *versus* 200 nm in average diameter) on the partitioning of PEG-liposomes (DSPC/Ch/DSPE-PEG1K) is shown in Fig. 4. Partitioning into the PEG phase of PEG-liposomes after standing for 30 min was affected by both vesicle size and PEG incorporation rate. One hundred percent partitioning into the PEG phase of small liposomes was obtained at 2 mol% incorporation of DSPE-PEG1K. In contrast, 3—5 mol% incorporation of DSPE-PEG1K was needed for the relatively large liposomes. Thus, at low incorporation of DSPE-PEG, there is a size dependency to the partitioning of PEG-liposomes in the non-charge-sensitive phase system.

Figure 5 shows the partitioning of PEG-liposomes (DSPC/Ch = 1 : 1, mol/mol, 120 nm in average diameter) incorporating various amounts of DSPE-PEG2K in the non-charge-sensitive phase system. Partitioning of PEG-liposomes into the PEG phase was increased with increasing incorporated amount of DSPE-PEG. One hundred percent partitioning into the PEG phase was obtained at 1 mol% incorporation of DSPE-PEG2K into liposomes. Bare liposomes (without DSPE-PEG) in this system were distributed 83.5% and 4.0% into the inter-

Table 1. Partitioning of Plasma Proteins

Sodium phosphate concentration (M)	Phase	Partition (%)
0.11	PEG phase	17.3
	Interface	21.6
	Dextran phase	61.1
0.068	PEG phase	11.0
	Interface	32.4
	Dextran phase	56.7
0.01	PEG phase	8.8
	Interface	36.5
	Dextran phase	54.7

$n=3$, $\pm$S.D. $<5\%$.

face and the dextran phase, respectively. In the case of DSPE-PEG5K-containing liposomes, only 0.5 mol% incorporation of DSPE-PEG5K resulted in 100% PEG phase partitioning (data not shown).

Torchilin and Papisov have reported that the PEG/lipid ratio (mol/mol) required for complete protection of the liposomal surface by the PEG moiety was 1/24 or 1/48 for PEG1K or 2K, respectively.[5] As indicated in Figs. 4 and 5, the PEG/lipid ratio of PEG-liposomes which showed 100% PEG phase partitioning was 1/25 or 1/50 for PEG 1K or 2K, respectively. These results are in reasonable accord with the values theoretically calculated by Torchilin and Papizov. Thus, the non-charge-sensitive phase system can be used to detect analytically not only the presence of PEG on the surface of liposomes, but also the state of surface protection by PEG.

Partitioning of PEG-Liposomes *in Vitro* and *in Vivo* Before investigating the partitioning behavior of PEG-liposomes *in vitro* and *in vivo*, we have to consider the possibilities of cleavage of the PEG moiety from the lipid anchor and removal of the entire DSPE-PEG molecule from liposome membranes. Parr *et al.* have reported that the succinyl linkage, the same as that in DSPE-PEG used in this experiment, was more labile than that in other PEG derivatives, such as amide and carbamate, and direct-linked PEG derivatives.[18] Their study involved incubation of micellar PE-PEG in serum. Under the conditions of our experiment, we could not detect cleavage up to 24 h (data not shown). Therefore, under our *in vitro* experimental conditions, cleavage of the PEG moiety from the lipid anchor seems to be negligible. They also examined the removal of DSPE-PEG using ^{3}H-labeled DSPE-PEG2K, and observed no removal of DSPE-PEG. Thus, we consider that cleavage of PEG and loss of DSPE-PEG do not occur during incubation of liposomes with plasma under our experimental conditions.

The alteration of the surface properties of PEG-liposomes composed of DSPC/Ch/DSPE-PEG1K (1 : 1 : 0.13, mol/mol, 120 nm in mean diameter) after interaction with plasma *in vitro* and *in vivo* was estimated using the non-charge-sensitive phase system consisting of 5% PEG 8000 and 5% dextran T-500, 0.01 M sodium phosphate, and 0.15 M sodium chloride (pH 7.0). The partitioning behavior of the plasma proteins themselves was also measured and the results are summarized in Table 1. Plasma proteins tended to partition to the dextran phase (54.7%) in the non-charge-sensitive phase system.

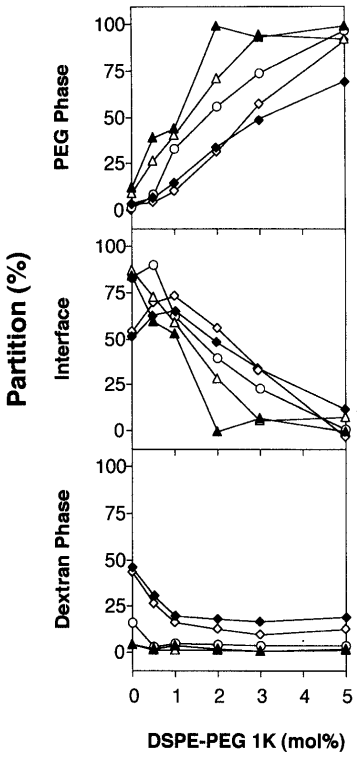

Fig. 6. Partitioning Behavior of PEG-Liposomes (Labeled with ^{3}H-Cholesterol) Containing Various Amounts of DSPE-PEG1K, Which Were Treated with PBS or Mouse Plasma *in vitro*, in the Non-charge-Sensitive Phase System Consisting of 5% PEG8000 and 5% Dextran T-500, 0.01 M Sodium Phosphate, 0.15 M Sodium Chloride (pH 7.0)

▲, intact PEG-liposomes; △, PEG-liposomes incubated with PBS for 3 h; ○, PEG-liposomes not incubated with ddY mouse plasma (liposome and plasma mixed before applying); ◇, and ◆, PEG-liposomes incubated with mouse plasma for 0.5 and 3 h, respectively. $n=3$, $\pm$S.D. $<8\%$.

Figure 6 shows the partitioning of PEG-liposomes which had been incubated with ddY mouse plasma *in vitro*. As controls for intact PEG-liposomes, liposomes incubated with PBS and liposomes not incubated with mouse plasma (*i.e.*, mixed just before testing) were used. Intact PEG-liposomes and PEG-liposomes incubated with PBS showed similar partitioning behavior except at 2 mol% incorporation of DSPE-PEG1K, whereas liposomes not incubated with mouse plasma showed decreased partition to the PEG phase and increased distribution to the interface. These results indicate that weak interaction between liposomes and plasma proteins occurred during the partitioning procedure.

After incubation with plasma, bare liposomes and PEG-liposomes with low DSPE-PEG content (<2 mol%) showed apparent partitioning to the dextran phase, reflecting their association with plasma protein. Compared with non-incubated samples, both samples showed decreased partitioning to the PEG phase. In PEG-liposomes with a large amount of DSPE-PEG (5 mol%), decreased PEG phase partitioning was observed following incubation for 3 h.

Partitioning behavior of plasma samples containing bare liposomes and PEG-liposomes, obtained at 0.5 h after i.v. injection in ddY mice (*in vivo* samples), are shown in Fig.

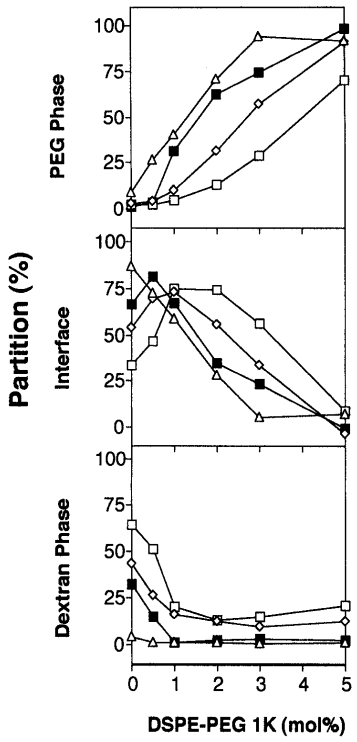

Fig. 7. Partitioning Behavior of PEG-Liposomes (Labeled with ^{3}H-Cholesterol) with Various Amounts of DSPE-PEG1K, Obtained from ddY Mice at 0.5 h after i.v. Injection

△, PEG-liposomes incubated with PBS for 3 h; ◇, PEG-liposomes incubated with mouse plasma for 0.5 h; □, PEG-liposomes obtained from ddY mice at 0.5 h after i.v. injection, ■, PEG-liposomes obtained from ddY mice at 0.5 h after i.v. injection and purified by column separation and ultracentrifugation. $n=3$, ±S.D. <8%.

7. Partitioning behavior of bare liposomes was similar to that of plasma itself (Table 1). Compared with *in vitro* samples (incubated with plasma), the partitioning to the PEG phase of *in vivo* PEG-liposomes was considerably decreased. However, partitioning of purified samples, which were obtained from plasma samples by column separation and ultracentrifugation, was similar to that of PEG-liposomes incubated with PBS. These results strongly suggested that plasma proteins associate with the surface of bare liposomes and that the presence of PEG moieties prevents this interaction.[12] Thus, the incorporated amount of PEG might be an important factor for sterical stabilization of liposomes.

Blood residence of PEG-liposomes composed of DSPC/Ch/DSPE-PEG1K (1 : 1 : 0.13, mol/mol, 100—120 nm in mean diameter) was about 70—80% of the dose at 0.5 h and 40—50% of the dose at 3 h after i.v. injection in mice.[19] It is known that the amount of PEG influences the blood residence time of PEG-liposomes[3] and theoretically, the liposome surface is completely covered with the PEG moiety at more than 2 mol% of DSPE-PEG1K.[5] However, the partitioning behavior of liposomes with less than 5 mol% of DSPE-PEG1K (Fig. 7) suggests that there is an interaction between PEG-liposomes and plasma protein, though the results with *in vivo*-treated PEG-liposome purified by column separation and centrifugation indicate that this interaction is weak. Although PEG-liposomes with 10 mol% DSPE-PEG1K also showed 100% partition to the PEG phase,[12] we should take the possibility of micelle formation of DSPE-PEG into consideration.

In conclusion, the non-charge-sensitive phase system consisting of 5% PEG 8000 and 5% dextran T-500, 0.01 M sodium phosphate, 0.15 M sodium chloride (pH 7.0) is suitable for estimation of the surface properties of liposomes. This system is able to detect the association of plasma protein with liposomes *in vitro* and *in vivo*.

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