In the BE guidelines published in Japan, testing is essentially required to be performed under fasting conditions, not fed conditions, except for drugs with markedly poor bioavailability or with a high frequency of serious adverse events under fasting conditions. Thus, there is no published information about the effects of food on the bioavailability of the generic products tested in this study. The pharmacokinetics of the innovator and Product D were previously compared in humans both after a meal and before a meal.[27] In that study, the innovator and Product D exhibited the same behavior, and both the *AUC* and C_{max} before the meal were 1.2-fold higher than after the meal, in line with the data reported in the interview form of Neoral. Thus, the other 3 generic products might exhibit the same behavior as Product D under fed conditions because their physical appearance and particle size in FeSSIF were quite similar. On the other hand, Kees *et al.* reported that the *AUC* and C_{max} of Cicloral, which is approved in Europe, are significantly increased after a meal and, under fasted conditions, the C_{max} and *AUC* of Cicloral were lower than those of the innovator.[15] Therefore, in the case of products with specific formulation characteristics like microemulsions, further bioequivalence studies under fed conditions may be required in the future.

Because we investigated the pharmacokinetics of CsA in rats treated with capsule contents dissolved in water before oral administration, we did not evaluate the disintegration behavior of the capsules themselves, either *in vitro* or *in vivo*, despite the fact this disintegration behavior can affect the pharmacokinetics of CsA. In addition, long-term storage might affect the physicochemical properties of the capsule membrane, as well as the capsule contents (microemulsion/emulsion pre-concentrate), leading to a change of pharmacokinetics of CsA and its metabolites. Therefore, capsule disintegration and the effect of long-term storage on capsule membrane/content will also have to be evaluated both *in vitro* and *in vivo* in the future.

Acknowledgment This research was supported by Health and Labor Sciences Research Grants (Research on Regulatory Science of Pharmaceuticals and Medical Devices).

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Polyethylene Glycol Prevents *in Vitro* Aggregation of Slightly Negatively-Charged Liposomes Induced by Heparin in the Presence of Bivalent Ions

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Liposomes are of great interest as drug delivery vehicles, and studies have focused on understanding how the physical and chemical characteristics of liposomes can be modified to improve their *in vivo* behavior. In a previous study, we found that the slightly negatively-charged liposomes aggregate only in the culture medium of human umbilical vein endothelial cells, whereas the liposomes modified with polyethylene glycol (PEG) (PEGylated) did not aggregate. In the present study, we investigated the underlying mechanism of this phenomenon. Firstly, it was found that heparin in the culture medium is one of the factors that cause aggregation of the non-PEGylated liposomes. Since the addition of ethylenediaminetetraacetic acid (EDTA) prevented the aggregation, metal ions, such as Ca^{2+} and Mg^{2+}, in the culture medium could also be important in driving the aggregation. In the presence of heparin, higher concentrations of Ca^{2+} or Mg^{2+} increased the particle size of the non-PEGylated liposomes, although no change in the particle size of PEGylated liposomes was observed. Under conditions in which aggregation occurred, we measured the binding and uptake of liposomes by macrophages *in vitro*. The binding and uptake of non-PEGylated liposomes were significantly increased with increasing Ca^{2+} concentrations, whereas those of PEGylated liposomes were unchanged. While the formation of aggregations of cationic or anionic liposomes has been reported previously, there are few reports addressing the aggregation of slightly negatively-charged or neutral liposomes. Thus, our data provide useful insights on the effect of PEGylation on liposomal aggregation and *in vivo* behavior.

Key words liposome; heparin; aggregation; polyethylene glycol; Ca^{2+}

Various liposomal products have been developed and applied to clinical treatment. Since methods to control the size of liposome and to improve the *in vitro* and *in vivo* stability of liposome had been developed, liposomes have attracted more attention. Incorporation of polyethylene glycol (PEG)-conjugated lipids (PEGylated) into liposomes is known to improve the circulation time of liposomes and prevent their uptake by the reticuloendothelial system (RES).[1–4] It was reported that PEGylation inhibits the adsorption of serum proteins *in vitro*,[5,6] and decreases the uptake of liposomes by cells such as macrophages.[7] From these reports, the extension of the circulation time by PEGylation is widely considered to be caused by the formation of a hydration layer, and steric hindrance can also prevent protein (such as opsonin) adsorption following recognition by cells of the RES, such as macrophages. However, the mechanisms producing these effects of PEGylation remain controversial. In particular, as regards protein adsorption, one report suggested that PEGylation can reduce protein adsorption by liposomes,[8] whereas other reports indicated that the total protein adsorption from plasma was not changed or increased by PEG.[9,10] In another report, it was indicated that PEG-modification of negatively charged liposomes can inhibit the binding of fibrinogen to liposomes, but there were no effects of PEG on nearly neutral liposomes (PC : PG : PE : Chol = 69 : 4 : 4 : 23).[11] Negatively charged liposomes have often been used to show the inhibition of protein adsorption by PEG as described above, but the effect of PEG on nearly neutral or slightly negatively-charged liposomes remains unclear.

In our previous and ongoing studies, we have addressed basic questions about drug release from liposomes under various conditions, in order to develop an *in vitro* method for testing liposomal drug release. While *in vitro* drug release tests will be very useful for the development of liposomal drug formulations and the evaluation of their lot-to-lot uniformity, there are currently no official or proven methods available for this purpose.[12] Because the purpose of formulation testing is not only the control of the manufacturing process and the quality, but also ultimately the assurance of the clinical efficacy and safety of the product, the *in vivo* environment, in which the drug or formulation is ultimately employed, should be taken into account for establishing the *in vitro* testing conditions as much as possible. Therefore, we assessed drug release from liposomes in various solutions, such as serum/plasma and cell culture media to mimic the *in vivo* environment,[13] as well as aqueous solutions of salt, sugar, and buffer.[14] In a previous study, we observed the formation of aggregates in the test medium of slightly negatively-charged liposomes only in the culture medium of human umbilical vein endothelial cells (HUVECs), whereas no aggregates were formed when the PEGylated liposomes were used. As described above, the mechanisms by which PEGylation affects nearly neutral or slightly negatively-charged liposomes have not been fully clarified. If the aggregate we observed in the culture medium is also formed *in vivo*, the prevention of that aggregation by PEGylation could be one of the factors that could extend the circulation time of such liposomes. It is well known that the circulation time of small or relatively neutral liposomes is longer than that of large or negatively charged liposomes,[15,16] thus the aggregates can be easily eliminated from blood circulation. In this study, we attempted to elucidate why the non-PEGylated liposomes, which had a very weak negative surface charge, aggregated in the HUVEC medium whereas the PEGylated liposomes did not.

The authors declare no conflict of interest.

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MATERIALS AND METHODS

Materials The phospholipids, hydrogenated soybean phosphatidylcholine (HSPC) and (*N*-(carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE-PEG2000), were purchased from NOF Corporation (Tokyo, Japan). Cholesterol (Chol) was of analytical grade (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Adriacin® injection 10 (Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan), a doxorubicin hydrochloride (DXR) injection, was purchased from a general sales agency for drugs. The PD-10 desalting columns were purchased from GE Healthcare Japan (Tokyo, Japan). Centrifugal filter units, Amicon Ultra (10k MWCO), were purchased from MILLIPORE (Tokyo, Japan). HUVEC culture medium, HuMedia-EG2, which consisted of maintenance medium HuMedia-EB2, 2% Fetal Bovine Serum (FBS), 10 μg/L human epidermal growth factor, 5 mg/L human basic fibroblast growth factor, 10 mg/L heparin, 1 mg/L hydrocortisone, and antibiotics, was purchased from KURABO (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) with high glucose and antibiotic cocktail were purchased from Invitrogen (Tokyo, Japan). Human serum (Biopredic International, Rennes, France) was obtained from KAC Co., Ltd. (Kyoto, Japan). Heparin sodium and carboxyfluorescein (CF) were reagent special grade (Wako Pure Chemical Industries, Ltd.).

Liposome Preparation Liposomes, liposome-encapsulated DXR, and liposome-encapsulated CF were prepared by the modified ethanol injection method.[17] DXR was encapsulated into liposomes by remote loading using an ammonium sulfate gradient.[18] Briefly, all lipids (200 μmol) were dissolved in about 5 mL of ethanol in different compositions: PEG-modified liposomes (sterically stabilized liposomes, SL), HSPC/Chol/DSPE-PEG2000 (55/40/5 mol/mol); normal liposomes (L), HSPC/Chol (6/4 mol/mol). The ethanol was removed with a rotary evaporator leaving behind about 1 mL of the ethanol solution. Next, 8 mL of 300 mM ammonium sulfate (for DXR-SL and DXR-L) or 100 mM CF dissolved in 10 mM Tris–HCl (pH 8.0) (for CF-SL and CF-L) was added to the ethanol solution. Liposomes formed spontaneously after further evaporation of the residual ethanol. Liposomes were then extruded through a series of polycarbonate filters (Nucleopore, Pleasanton, CA, U.S.A.) with pore sizes ranging from 0.4 to 0.1 μm. The mean diameter of extruded liposomes was in the range of 100–150 nm. Following extrusion, liposomes were ultracentrifuged at 80000 rpm for 45 min at 4°C, and suspended in normal saline or 10 mM Tris–HCl (pH 8.0) for liposome-entrapped DXR or CF respectively. Phospholipid concentration was determined by a colorimetric assay using Phospholipids C Test from Wako (Wako Pure Chemical Industries, Ltd.). For encapsulation of DXR, DXR was added to the ammonium sulfate-containing liposomes at a DXR/liposome ratio of 0.2 : 1 (w/w), and the liposomes were incubated for 1 h at 55°C. The liposome-encapsulated DXR and liposome-encapsulated CF were exchanged by eluting through a PD-10 desalting column equilibrated with normal saline.

Incubation of Liposome Liposomes were diluted with each test solution to a final lipid concentration of 0.2 mM in glass test tubes, and incubated at 37°C for 30 min in a water bath, without agitation. We observed that the aggregation of non-PEGylated liposomes with heparin occurred immediately. Thus, the intervals and temperature values were chosen to keep the experimental conditions constant. Only for photography, liposomes were incubated at 37°C for 6 h to observe significant precipitations. The final concentrations of ethylenediaminetetraacetic acid (EDTA) and heparin were 20 mM and 10 μg/mL respectively, unless otherwise indicated. The following solutions, 150 mM NaCl (saline), 150 mM KCl, 100 mM $CaCl_2$, 150 mM $MgSO_4$, 300 mM Glucose, and phosphate buffered saline (PBS) (pH 7.5) were prepared. Human serum was filtered by an ultrafiltration membrane before mixing with liposomes.

Zeta Potential Analysis Zeta potential was measured using an ELSZ-1000 (Otsuka Electronics Co., Ltd., Osaka, Japan), which is based on laser Doppler velocimetry in an electric field. CF-L, CF-SL, and negatively charged liposome (COATSOME EL-01-A, NOF Corporation) were diluted with saline to a final lipid concentration of 0.2–0.5 mM.

Particle Size Analysis The particle size distribution and mean diameter of each liposomal preparation after incubation was measured using a dynamic light scattering (DLS) photometer DLS-7000 (Otsuka Electronics Co., Ltd.) equipped with a He-Ne laser source (wavelength, 632.8 nm). All DLS measurements were made at a scattering angle of 90°. Data were gathered using a counting period of 100 s. Histogram analysis was performed to calculate the average particle size and standard deviation.

Binding and Uptake of Liposomes To assess the uptake or binding of liposomes by mononuclear phagocytes, we used a mouse macrophage cell line, RAW 264.7, which was kindly given by Dr. Tsunoda, Laboratory of Biopharmaceutical Research, National Institute of Biomedical Innovation. Cells were maintained in DMEM supplemented with 10% FBS and 1% antibiotic cocktail. Cells were seeded into 12-well plates (1×10^6 cells/well) and incubated at 37°C for 24 h. After incubation, aliquots of medium were removed, and cells were treated with CF-liposomes (0.2 mM) at 37°C for 1 h. The CF-liposomes were suspended in FBS- and antibiotic-free DMEM with the indicated concentration of $CaCl_2$ and 10 μg/mL heparin. After incubation, the cells were washed twice with ice-cold PBS and lysed by adding 500 μL of 0.5 M NaOH. Each lysate was diluted 4-fold with distilled water, and fluorescence of the CF in the lysate was measured at 490/520 nm (emission/excitation) using a spectrofluorometer (JASCO, Tokyo, Japan).

RESULTS AND DISCUSSION

The HUVEC culture medium, namely HuMedia EG-2, in which the aggregation of non-PEGylated liposomes had previously been observed in our study, is optimized for the maintenance and proliferation of normal cells. The HuMedia EG-2, which is composed of base medium HuMedia EB-2 and additives to enhance HUVEC proliferation, is a specialized culture medium. Among the additives in the medium (fetal bovine serum, antibiotics, heparin, hydrocortisone, human epidermal growth factor, human fibroblast growth factor), heparin was likely to interact with liposomes. Thus, DXR-L or DXR-SL was dispersed in base medium HuMedia EB-2, HuMedia EB-2 with heparin, and HuMedia EG-2, and we monitored the aggregation properties of these solutions. In the solution of DXR-L dispersed in base HuMedia EB-2, the aggregate was not observed, while significant aggregation occurred in

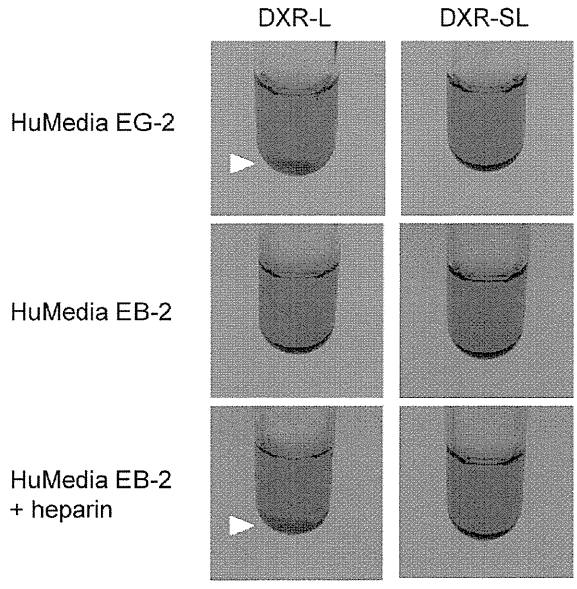

Fig. 1. Photographs of Liposomes (DXR-L and DXR-SL) Dispersed in HuMedia EG-2, HuMedia EB-2, or HuMedia EB-2 with Heparin

These photographs were taken after 6h incubation at 37°C. An arrow indicates the precipitate of aggregates.

the solution of DXR-L dispersed in HuMedia EB-2 with heparin or HuMedia EG-2 (Fig. 1). There were no such changes in any of the solutions of the PEGylated liposome, DXR-SL. From these data, we concluded that the interaction of heparin with liposomes is responsible for the aggregation of DXR-L in HuMedia EG-2.

Next, to confirm whether heparin is the only cause of aggregation, DXR-L was dispersed in Eagle's minimum essential medium (MEM), which is commonly used for cell culture. There were no aggregates (data not shown). Heparin is a highly sulfated polymer that consists of a repeating disaccharide unit, including uronic acid and glucosamine, and is strongly negatively charged.[19] On the other hand, the non-PEGylated liposome DXR-L, mainly composed of phosphatidylcholine with a slightly negative charge, was quite unlikely to interact with heparin by itself to form aggregates. While the detailed composition of HuMedia EB-2 is proprietary, HuMedia EB-2 includes microelements (such as Zn, Cu, and Fe ions), which are not contained in common culture media, in addition to Ca^{2+} and Mg^{2+}. Thus, HuMedia EB-2 seems to be more similar to body fluids than other common culture media. Because of these factors, we hypothesized that bivalent ions are most likely to be involved in the interaction of non-PEGylated liposomes and heparin. We tested this hypothesis by adding the cation-chelating agent EDTA to the culture

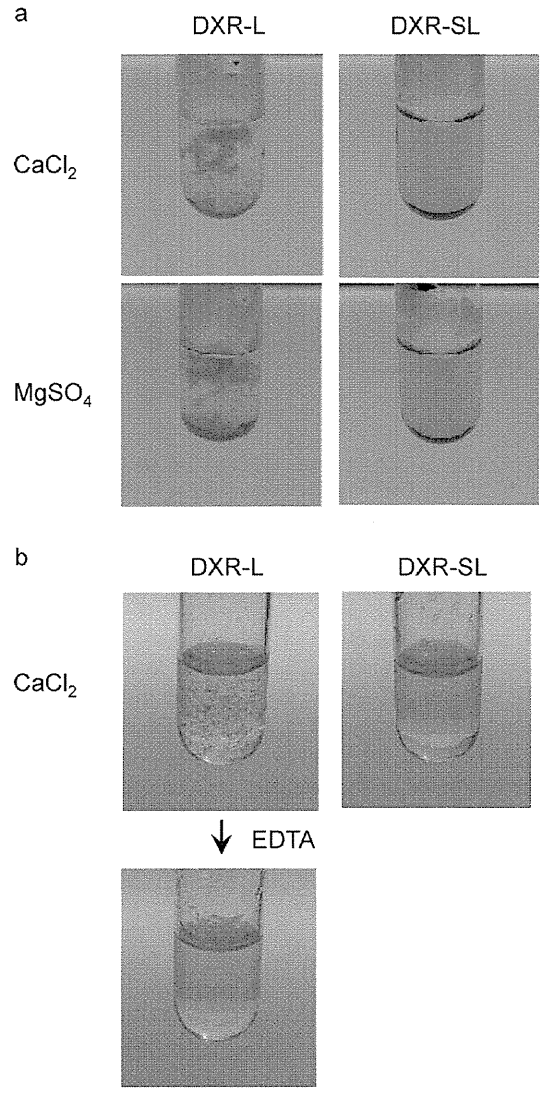

Fig. 2. Photographs of Liposomes (DXR-L and DXR-SL) Dispersed in $CaCl_2$ (100mM) or $MgSO_4$ (150mM) in the Presence of Heparin (10μg/mL) (a)

Photograph of DXR-L dispersed in $CaCl_2$ (100mM) in the presence of heparin (10μg/mL) before and after the addition of EDTA, and that of DXR-SL as control (b).

medium, and then measured the particle size of DXR-L. We found that there were no changes in the particle size of DXR-L dispersed in the HuMedia EB-2 with heparin and EDTA, whereas the particle size of DXR-L in HuMedia EB-2 with heparin alone was increased to about 1000nm (Table 1). These data suggest that bivalent ions are needed to form aggregates of liposomes with heparin.

Table 1. Effect of Ion for the Formation of Aggregation of DXR-L with Heparin in HUVEC Medium

	Particle size (nm)			
	HuMedia EG2	HuMedia EB2	HuMedia EB2 with heparin	HuMedia EB2 with heparin, EDTA
DXR-L	1266.7±49.6	142.8±0.6	942.4±90.2	144.3±1.3
DXR-SL	137.4±5.3	137.0±12.2	139.5±4.5	132.3±6.5

Each value represents the mean±S.D. (n=3). DXR-L and DXR-SL represent DXR encapsulated conventional liposome and PEGylated liposome, respectively.

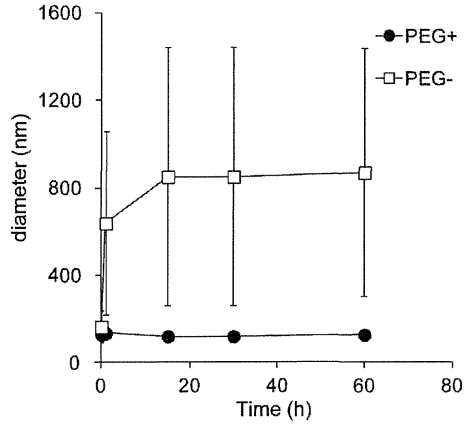

Fig. 3. The Change of the Particle Size of Liposomes (PEG +: CF-SL, PEG −: CF-L) with Time in the Presence of $CaCl_2$ (5 mM) and Heparin (10 μg/mL) at 37°C

Data represent the average particle size±S.D. (n=2) calculated by histogram analysis.

Next, to clarify the effect of bivalent ions, liposomes were dispersed in a solution of $CaCl_2$ or $MgSO_4$ as a source of Ca^{2+} or Mg^{2+}, which are the major bivalent ions in blood. Whereas the solution of DXR-SL in which the liposome was uniformly dispersed was red, the solution of DXR-L in which the liposome aggregated and precipitated was nearly clear (Fig. 2a). When EDTA was added after aggregation of DXR-L with heparin and Ca^{2+}, the formation of aggregation was reversed and the solution became clear (Fig. 2b). These results revealed that non-PEGylated liposome interacted with heparin and formed aggregates in the presence of bivalent ions, and the aggregations were reversible and did not involve the fusion of lipid membrane. Additionally, when the particle size of liposomes in other solutions (NaCl, KCl, glucose, and PBS) with heparin was measured, there were no aggregates of liposomes in these solutions (Table 2). This result emphasizes the importance of bivalent ions in driving the formation of liposomal aggregates with heparin.

In the above experiments, we used only one concentration of $CaCl_2$ (100 mM) or $MgSO_4$ (150 mM) solution. Thus, we assessed the dependence of the aggregation of liposome with heparin on Ca^{2+} and Mg^{2+} concentrations. To enable easy handling, we used carboxyfluorescein (CF)-encapsulated liposomes, namely CF-L or CF-SL, in this experiment. Firstly we investigated the time course of the formation of aggregation at 37°C. When CF-L was dispersed in the saline with heparin, its particle size was immediately increased (Fig. 3). Thus the samples were incubated for 30 min to keep the experimental conditions constant. The particle size of CF-L was significantly increased with increasing concentrations of Ca^{2+} and Mg^{2+} (Fig. 4). The increase of particle size was observed in

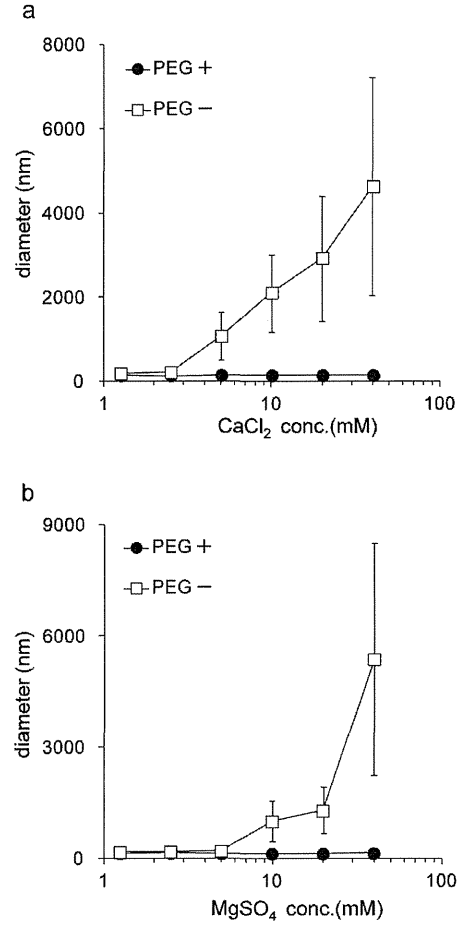

Fig. 4. The Effect of the $CaCl_2$ (a) or $MgSO_4$ (b) Concentration on the Particle Size of Liposomes (PEG +: CF-SL, PEG −: CF-L) in the Presence of Heparin (10 μg/mL)

Data represent the average particle size±S.D. (n=3) calculated by histogram analysis.

the presence of 2.5–5.0 mM Ca^{2+} or 5–10 mM Mg^{2+}. Because Ca^{2+} has a high affinity for heparin, Ca^{2+} could induce aggregation even at lower concentrations. Next, the effect of varying heparin concentrations on aggregation was assessed under a constant concentration (10 mM) of Ca^{2+} and Mg^{2+}. The particle size of CF-L was increased even at a low concentration of heparin (about 0.15 μg/mL) (Fig. 5). In the case of the PEGylated liposome CF-SL, no changes in the particle size were observed. It was possible that the surface charge prevent the aggregation of liposomes, because PEG-conjugated lipid (DSPE-PEG 2000) has negative charge. Thus, the surface charge of each liposome was measured (Table 3). As a result, non-PEGylated liposome CF-L and PEGylated liposome CF-SL exhibited a slightly negative surface charge, and there

Table 2. Particle Size of Liposome in Each Solution with Heparin

	Particle size (nm)					
	NaCl	KCl	$CaCl_2$	$MgSO_4$	Glucose	PBS
DXR-L	139.4±2.3	138.9±7.3	2830.0±137.8	4089.1±227.3	136.8±1.7	137.4±0.5
DXR-SL	128.3±8.8	132.3±0.4	129.8±0.5	141.3±1.8	137.9±3.0	131.9±8.3

Each value represents the mean±S.D. (n=3).

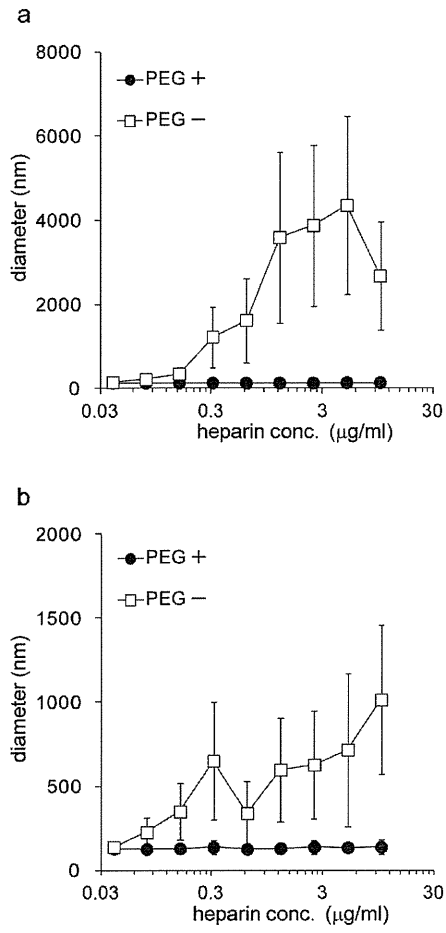

Fig. 5. The Effect of Varying Heparin Concentrations on the Particle Size of Liposomes (PEG +: CF-SL, PEG −: CF-L) in the Presence of $CaCl_2$ (10 mM) (a) or $MgSO_4$ (10 mM) (b)

Data represent the average particle size±S.D. (n=3) calculated by histogram analysis.

were no significant differences between CF-L and CF-SL in their zeta potential. Therefore, the prevention of aggregation could be due to a steric hindrance by PEG, which inhibits the interaction of the lipid membrane and heparin.

The aggregation induced by Ca^{2+} has been studied extensively in liposomes composed of negatively charged lipids.[20,21] It was also reported that, in liposomes composed of phosphatidylserine, Ca^{2+} induced aggregation, followed by membrane fusion.[22,23] The formation of aggregates observed in our study would not be accompanied by membrane fusion because the aggregate was reversible by the addition of EDTA. In the case of positively charged liposomes, formations of complexes with DNA and anionic polyions were also reported.[24,25] However, the aggregation of nearly neutral or slightly negatively-charged liposomes is poorly studied. Therefore, our results provide useful findings on the aggregation of such liposomes.

Table 3. Zeta Potential of Liposomes in Saline

Liposomes	Zeta-potential (mV)
CF-L	−3.46±0.72
CF-SL	−2.98±0.33
Anionic liposome	−44.29±1.29

Each value represents the mean±S.D. (n=2).

The normal levels of calcium and magnesium in serum are about 2.5 mM and 1.0 mM, respectively. In addition to calcium and magnesium, blood contains other positive ions, such as Fe, and Cu ions. The predicted lipid concentration after the administration of the liposomal product DOXIL® to human will be around 0.1 μmol/mL, which is close to the lipid concentration used in this study. Additionally the aggregation of non-PEGylated liposomes began at a concentration of Ca^{2+} between 2.5 and 5.0 mM in the above experiment. Therefore the possibility that the same phenomenon can occur *in vivo* cannot be eliminated. Next, to estimate the change in particle size of liposomes in blood circulation, the particle size of liposomes dispersed in ultrafiltered serum, which does not contain high molecular weight proteins, was measured. The particle size of DXR-L was significantly increased in the ultrafiltered serum with heparin, and the addition of EDTA inhibited the increase in particle size (Table 4). The particle size of the PEGylated liposome DXR-SL was slightly increased, and this was assumed to be caused by the interaction of DXR-SL with low molecular weight proteins or polypeptides in a multivalent ion-related fashion. This result suggested that slightly negatively-charged liposomes could possibly interact or aggregate with heparin in the blood circulation. However, when the same experiment was conducted using a different lot of serum, significant aggregation of DXR-L was not observed. More detailed examination is needed to demonstrate the aggregation of slightly negatively-charged liposomes in the blood circulation. In biological and human body conditions, in addition to heparin, there are other glycosaminoglycans, such as heparin sulfate and chondroitin sulfate. Therefore, the interaction of slightly negatively-charged liposomes with these anionic polymers also needs to be investigated in the future.

It has been assumed that the prolonged life-time of PEGylated liposomes is brought about by escape from the RES, whereas non-PEGylated liposomes are rapidly eliminated from blood circulation by phagocytes such as macrophages in the RES.[26] That is, whether the liposomes get trapped or evade the RES is an important feature that impinges on the *in vivo* behavior of liposomes. Therefore, we then assessed the effect of aggregate formation on the uptake of liposomes by macrophages. In this experiment, we used CF-L and CF-SL,

Table 4. Particle Size of Liposomes in Ultrafiltrated-Serum

	Particle size (nm)			
	None	With EDTA	With heparin	With heparin, EDTA
DXR-L	401.0±17.8	127.6±3.9	2201.1±56.8	136.1±2.2
DXR-SL	174.5±10.2	131.3±1.5	165.0±9.6	142.3±1.4

Each value represents the mean±S.D. (n=3).

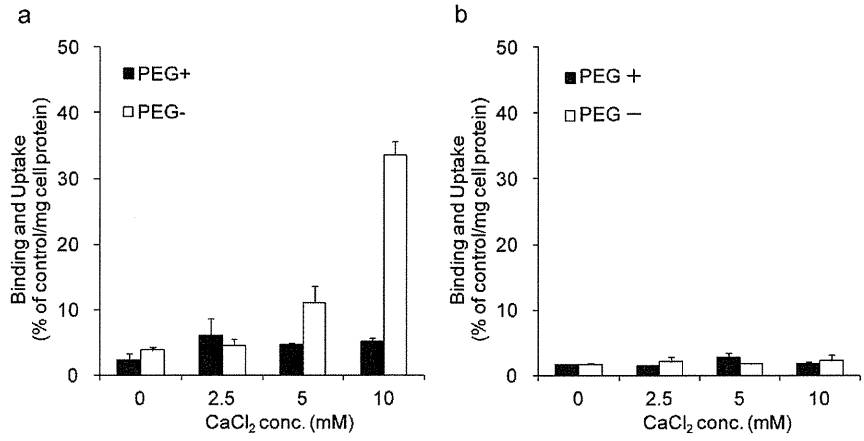

Fig. 6. Uptake and Binding Rate of Liposomes (PEG +: CF-SL, PEG −: CF-L) by Macrophages in the Presence of Ca^{2+} and Heparin (a) or in the Absent of Heparin (b)

Liposomes, dispersed in DMEM with $CaCl_2$ at the indicated concentration in the presence or the absent of heparin (10 μg/mL), were incubated for 30 m at 37°C, and added to cells. After incubating for 1 h at 37°C, the cells were washed, lysed with 0.5 M NaOH, and the concentration of the CF in cells was calculated. Each value represents the mean±S.D. of 3 replicates.

and murine RAW 264.7 cells as the test macrophage cell line. We measured the binding and uptake of these liposomes by RAW 264.7 cells at the indicated concentration of Ca^{2+} in the presence of 10 μg/mL heparin. The binding and uptake rates of CF-L were increased with increasing concentrations of Ca^{2+}, whereas in the case of the PEG-liposome CF-SL, the binding and uptake rates were not increased (Fig. 6). Therefore, these data revealed that the binding and uptake rates of CF-L were increased upon increasing the aggregation of CF-L with heparin/Ca^{2+}. This increased binding and uptake is likely caused by the tendency of phagocytes, such as macrophages, to take up larger particles more readily.[27] Additionally, scavenger receptors, whose ligands are anionic macromolecules as well as degenerated-LDL, are expressed on the cell surface of macrophages, and are involved in the phagocytosis of foreign substances and waste products.[28] Therefore, the increase in uptake rate with increasing particle size could be affected by the strong negative charge of the heparin contained in the aggregate. On the other hand, the uptake of CF-SL was not changed, because CF-SL did not interact with heparin and its particle size remained small even at a high concentration of Ca^{2+}.

Our data indicate that slightly negatively-charged liposomes aggregate with heparin in the presence of bivalent ions such as Ca^{2+} and Mg^{2+}. Our data also indicate that the interaction of such liposomes with heparin can be prevented by modification with PEG. The measurement of particle size by DLS revealed the quantitative relationship between Ca^{2+}/Mg^{2+} (or heparin) and the formation of aggregates. Additionally, we measured the binding and uptake of liposomes by macrophages, and found that the heparin-mediated aggregation of liposomes can enhance the binding and uptake of liposomes.

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ポストインサーション法によって調製したPEG修飾リポソームの表面物性に関する研究

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Study of Surface Properties of Poly(Ethylene Glycol)–Modified Liposomes in Post–Insertion Method

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Post–insertion method is now widely used to modify liposomes with poly(ethylene glycol) (PEG) lipids. In this study, we examined the physicochemical properties of surface layers of distearoylphosphatidylethanolamine-PEG2000–modified liposomes composed of distearoylphosphatidylcoline, distearoylphosphatidylglycerol and cholesterol prepared in post–insertion method. Zeta potential measurements showed that the estimated aqueous layer thickness of PEG liposomes increases with increasing concentrations of PEG lipids added. Size exclusion chromatography demonstrated that the incorporation efficiency of PEG lipids into liposomes gradually decreases with increasing addition amount of PEG lipids. Quenching experiments of fluorescence–labeled PEG lipids confirmed that almost all PEG lipids distribute at the outermost layer of liposomes in the post–insertion preparation whereas PEG lipids distribute equally at inner and outer layers of liposomes in the conventional pre-mixed method. By correcting the incorporation efficiency and the outermost distribution of PEG lipids in liposomes, we found that the dependency of increases in aqueous layer thickness of PEG liposomes upon the outermost content of PEG lipids is similar in the both post–insertion and pre–mixed methods. Fluorescence resonance energy transfer measurements suggested that the increasing behavior of aqueous layer thickness is closely correlated with the conformational transition of PEG chains at the liposome surface. These results provide comparative information of the surface properties of PEG–modified liposomes prepared by the post–insertion and pre–mixed methods.

Key words : liposome／poly(ethylene glycol)／post–insertion／surface property／fixed aqueous layer thickness

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1. 緒　　言

リポソームは，毒性や抗原性が低く，脂溶性薬物

や水溶性薬物，タンパク質など種々の物質を封入できることから，有用な薬物キャリアーとして期待されている．ところが，血中投与されたリポソームは異物として認識され，肝臓などの細網内皮系（RES）に捕獲されてしまい，その血中滞留性の向上が課題とされてきた[1]．この課題を解決する方法として，親水性高分子による膜修飾が知られている．膜修飾を行う親水性高分子の修飾剤としては，一般的に，ポリエチレングリコール（PEG）にリン脂質またはコレステロールなどの脂質を結合したPEG誘導体が用いられている[2]．リポソーム表面をPEG脂質で修飾したPEGリポソームの高い血中滞留性は，リポソーム表面のPEG鎖が形成する水和層やPEG鎖自身の立体障害により，オプソニンなどの血清タンパク質の結合や単核食細胞系などとの相互作用，血漿中でのリポソーム粒子間での凝集などが抑制されるためと考えられている[3～5]．高い血中滞留性が得られることにより，腫瘍組織や炎症部位などの血管透過性が亢進した組織への受動的な集積（EPR効果）が可能となり[6]，ドキソルビシン製剤であるDoxil®を初めとしていくつかの製剤が既に実用化されている[7]．

リポソームのPEG修飾法としては，リポソーム調製時にあらかじめPEGリン脂質を加える方法（pre-mixed法）が従来行われてきたが，近年，リポソーム調製後にPEGリン脂質を加えるポストインサーション（post-insertion）法が広く利用されている[8～10]．前者の手法で調製されたPEGリポソームにおいては，リポソーム膜内外層の双方ともにPEG脂質が修飾されているのに対し，後者ではリポソーム外膜のみをPEG脂質で修飾することが可能である．したがって，ポストインサーション法ではより効率的にリポソーム外膜をPEG修飾できることに加えて，リポソーム調製後にPEG修飾量の調節や抗体などによるPEG鎖への機能付加が可能などの利点もある．しかし，ポストインサーション法で調製されたPEG修飾リポソームの物性や生物活性について，従来のpre-mixed法との比較を行った研究例は少ない[11,12]．そこで本研究では，両法で作製したPEG修飾リポソームについて，ゼータ電位測定によるリポソーム表面固定水和層の厚さ（fixed aqueous layer thickness, FALT）を求めるとともに[13]，蛍光標識PEGリン脂質を用いたリポソーム膜内外層間のPEGリン脂質の分布やリポソーム膜表面でのPEG鎖コンフォメーション変化などに関する蛍光分光学的評価を行った．これらの結果から，ポストインサーション法の特性を明らかにするとともに，PEG修飾リポソームの表面物性指標としてのFALTの有用性を検証した．

2. 実　験

2.1 試　薬

リポソーム構成脂質としては，distearoylphosphatidylcholine（DSPC）とdistearoylphosphatidylglycerol（DSPG）は日油株式会社から，cholesterolはSigma–Aldrich社から購入したものを用いた．PEGリン脂質としては，分子量2000のPEG鎖が付加したリン脂質誘導体であるDSPE–PEG2000（日油株式会社）を用い，PEG鎖末端がアミンで活性化されたDSPE–PEG2000–NH_2は日油株式会社から供与を受けた．PEG脂質の蛍光標識にはNHS–Fluorescein（Thermo SCIENTIFIC社）とNBD succinimidyl ester（Invitrogen）を用いた．また，蛍光標識リン脂質であるNBD–PEとrhodamine–PEはInvitrogenから購入した．

2.2 リポソームの調製

DSPC/DSPG/cholesterol（5/3/5モル比）をメタノール：クロロホルム（1：2 v/v）に溶解後，ロータリーエバポレーターを用いて薄膜を形成させ，一晩減圧乾燥により溶媒を除去した．薄膜に10 mM Tris-HCl緩衝液（pH 7.4）を加え，約60 ℃でボルテクスすることにより脂質を分散させた．これを60 ℃付近に保ったまま，Mini–Extruder（Avanti Polar Lipid社）を用いて200 nmのポリカーボネートフィルターを十数回通過させてリポソームを調製した．Pre–mixed法では，PEGリン脂質はメタノール：クロロホルム（1：2 v/v）に脂質と共に混和させ，上述と同様の方法でPEGリポソームを作製した．Post–insertion法においては，予め調製したリポソーム懸濁液に10 mM Tris–HCl緩衝液（pH 7.4）に溶解させたPEGリン脂質を加え，60 ℃に加熱しながら60分間ボルテクスを行うことでPEGリポソームを作製した．Post–insertion法とpre–mixed法で作製したリポソームの粒子径はいずれも約200 nm（post–insertion法：194 ± 40 nm，pre–mixed法：197 ± 32 nm）であり，挿入法による粒子径の違いは認められなかった．リン脂質の定量はBartlett法[14]により行った．

2.3 リポソームの粒子径およびゼータ電位の測定

調製したリポソームの粒子径とゼータ電位はNICOMP社製NICOMP 380 ZLSを用いて測定した．測定には光路長1 cmのプラスチックセルを用い，脂質濃度は0.5 mMで行った．リポソームのゼータ電位の絶対値は，共存する塩濃度（イオン強度）の増大によって減少する．これは，グイ–チャップマン理論

による表面電位ψ_xと粒子表面からの距離との関係式

$$\psi_x \approx \phi_0 \exp(-\kappa x) \tag{1}$$

から説明される[15]．ここで，ϕ_0は$x=0$のときの電位を表し，κはデバイパラメータである．κはイオン強度Iと

$$\kappa = \sqrt{\frac{2000 N_A e^2 I}{\varepsilon_r \varepsilon_0 kT}} \tag{2}$$

の関係にあり，NaClのような1：1電解質の場合，25℃では

$$\kappa = \sqrt{[\mathrm{NaCl}]}/0.304 \tag{3}$$

となる．したがって，NaCl濃度を変えてゼータ電位ζ（$\approx\psi_x$）を測定することで

$$\ln\zeta = \ln\phi_0 - \kappa x \tag{4}$$

の関係から，粒子表面からすべり面までの距離x，すなわち固定水和層の厚さ（FALT）を求めることができる[16]．なお，(3)式のκの算出には，Trisのp*K*a（8.08）から解離型として存在するTrisの割合を求めることで緩衝液（pH 7.4）中の10 mM Trisの寄与を考慮した．

2.4 FluoresceinおよびNBD標識PEGリン脂質の調製

PEG鎖末端がアミンで活性化されたDSPE-PEG2000-NH_2を50 mMホウ酸緩衝液（pH 8.5）に溶解し，DSPE-PEG2000-NH_2の10倍量（モル比）のNHS-FluoresceinあるいはNBD succinimidyl esterのdimethylsulfoxide溶液を加え，一晩攪拌して反応を進行させた．未反応のNHS-FluoresceinあるいはNBD succinimidyl esterはSephadex G-25カラムを用いたゲルろ過と透析により除去した．

2.5 ゲルろ過クロマトグラフィー

Fluorescein標識PEGリン脂質を1%の割合で混合したDSPE-PEG2000を用いてリポソームのPEG修飾を行った後，Sepharose CL-6Bカラム（直径1 cm×長さ40 cm）を用いたゲルろ過によりリポソーム膜に挿入されたPEGリン脂質と未挿入のPEGリン脂質の分布を求めた．ゲルろ過は，流速1 mL/min，フラクション体積0.75 mL，溶離液は10 mM Tris-HCl bufferを用いて行った．各フラクションのfluorescein蛍光は励起波長490 nm，蛍光波長535 nmで測定した．

2.6 NBD消光実験

亜ジチオン酸ナトリウム（$Na_2S_2O_4$）によってNBD基が不可逆的に還元され蛍光消光が起こる現象[17]を利用し，DSPE-PEG2000-NBDの蛍光強度の減少率からリポソーム膜外層側に存在するPEGリン脂質の割合を求めた．PEGリン脂質に対して5 mol%の割合でDSPE-PEG2000-NBDを加えたDSPE-PEG2000を用いてリポソームを調製し，外水相に15 mMの$Na_2S_2O_4$を添加した後のNBD蛍光の消光挙動を励起波長460 nm，蛍光波長540 nmで観察した．

2.7 蛍光共鳴エネルギー移動（FRET）測定

アクセプター分子であるrhodamine-PEを全脂質に対して0.05 mol%を含むリポソーム膜に，ドナー分子であるDSPE-PEG2000-NBDを5 mol%含むDSPE-PEG2000をpost-insertion法により挿入し，未挿入のPEGリン脂質はSepharose CL-6Bカラムにより除去した．このNBD/rhodamine二重標識PEG修飾リポソームについて，NBDの励起波長である460 nmで励起したときのNBDの蛍光強度の減少とrhodamineの蛍光強度の増加からFRETを評価した．

ドナー分子単独での蛍光強度をF_D，アクセプター分子存在下におけるドナー分子の蛍光強度をF_{DA}とすると，励起エネルギー移動効率Eは次式で表される．

$$E = 1 - (F_{DA} / F_D) \tag{5}$$

また，ドナー-アクセプター分子間距離をR，励起エネルギー移動効率が50%となるドナー-アクセプター分子間距離をR_0とすると，Eは次式のように表される．

$$E = R_0^6 / (R_0^6 + R^6) \tag{6}$$

ここで，ドナー分子とアクセプター分子がそれぞれNBDとrhodamineの場合のR_0値として3.2 nmを用いた[18]．

3. 結果および考察

3.1 ゼータ電位測定によるPEG修飾リポソームの固定水和層長（FALT）の評価

Post-insertion法によって調製したPEG修飾リポソームのゼータ電位変化の挙動をFig. 1Aに示す．PEGリン脂質の添加量に依存してゼータ電位の絶対値は

Table 1 FALT for PEG-modified liposomes in post-insertion method

PEG lipid added /total lipid (mol%)	0.9	1.8	3.6	5.4	7.2	9.0
FALT (nm)	2.4 ± 0.2	3.2 ± 0.1	3.8 ± 0.1	4.0 ± 0.1	4.2 ± 0.1	4.3 ± 0.1

Table 2 Insertion efficiency of PEG lipids into liposomes in post-insertion method at 60 ℃

PEG lipid added /total lipid (mol%)	0.9	1.8	3.6	5.4	7.2	9.0
Insertion efficiency of PEG lipid (%)	90 ± 1	88 ± 1	86 ± 1	83 ± 2	82 ± 1	77 ± 2

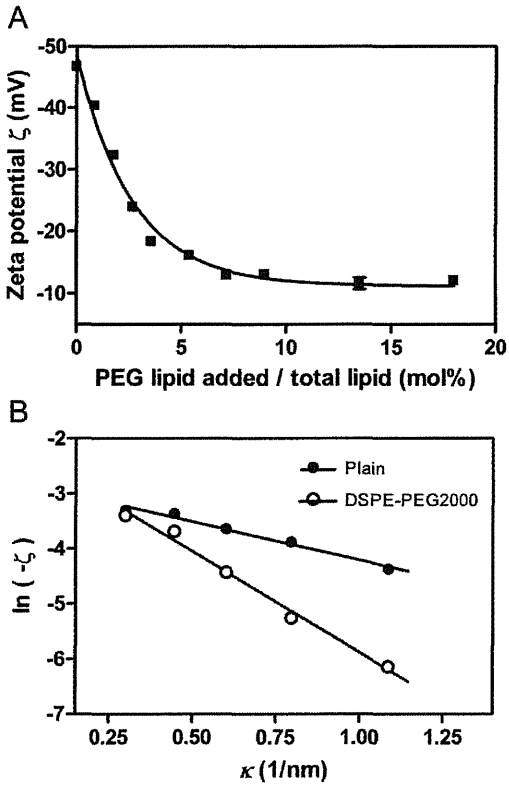

Fig. 1 (A) Change in zeta potential with addition of DSPE-PEG2000 to DSPC/DSPG/cholesterol (5:3:5) liposomes prepared post-insertion in Tris-HCl buffer (0 mM NaCl). (B) Linear relationship of ln (-ζ) with Debye parameter (κ). Liposomes with (○) or without (plain) (●) modification of 3.6 mol% of DSPE-PEG2000 in the post-insertion method.

減少し，リポソーム表面PEG鎖による固定水和層の形成が示唆された．そこで，グイ-チャップマン理論を適用してPEG修飾リポソームのFALTを求めた[16]．Fig. 1Bに未修飾（plain）及びDSPE-PEG2000を3.6

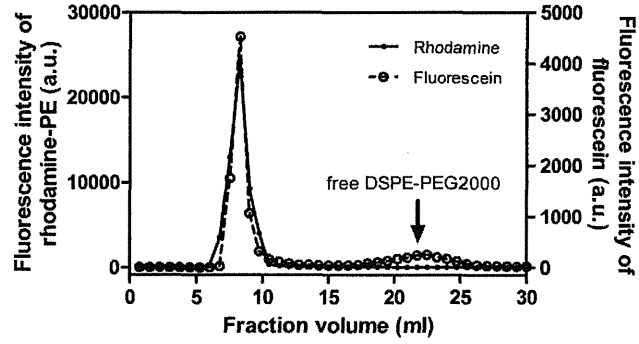

Fig. 2 Size exclusion chromatograph of DSPE-PEG2000-modified liposomes on a Sepharose CL-6B column. Liposomes were doubly labeled with rhodamine-PE (●) and DSPE-PEG2000-fluorescein (○). Liposomes were modified with DSPE-PEG2000 (3.6 mol% to total lipids) in the post-insertion method.

mol%添加したリポソームのゼータ電位とデバイパラメータκとの関係を示す．この直線の傾きから，PEG修飾によってFALTが1.4 nmから3.8 nmに増加していることが示された．なお，Fig. 1Bの切片より求められる表面電位はPEG被覆によって変化しているが，これはDSPE-PEG2000が有するリン酸基部分の負電荷の影響と考えられる．Table 1にはpost-insertion法におけるPEGリン脂質の添加量とFALTとの関係を示した．PEGリン脂質添加量に依存してFALTが増加すること，また添加量として7 mol%程度でFALTが一定に達することが示された．

3.2 リポソーム膜最外層におけるPEGリン脂質被覆率とFALTとの相関

DSPE-PEG2000のリポソーム膜に対する挿入効率を求めるために，リポソーム膜をrhodamine-PEで

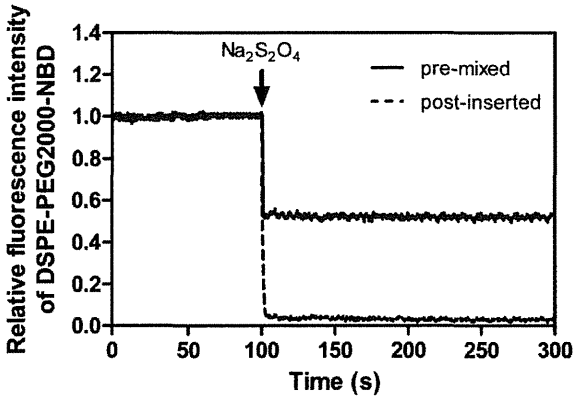

Fig. 3　Fluorescence quenching of NBD for estimating the fraction of NBD-labeled PEG lipids distributed in the outermost layer of PEG-modified liposomes. 15 mM of $Na_2S_2O_4$ was added to PEG liposomes labeled with DSPE-PEG2000-NBD (3.6 mol% to total lipids).

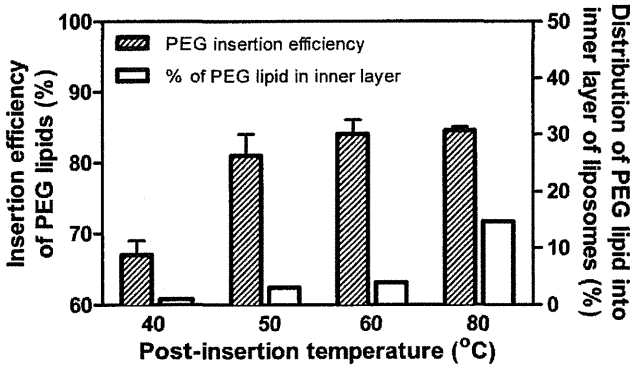

Fig. 4　Effects of post-insertion temperature on insertion and distribution of PEG lipids into liposome membranes. Liposomes were modified with DSPE-PEG2000 (3.6 mol% to total lipids) in the post-insertion method. PEG insertion efficiency was determined by size exclusion chromatography (Fig. 2). Distribution of PEG lipid into the inner layer of liposome membranes was estimated by NBD quenching experiments (Fig. 3).

DSPE-PEG2000をfluoresceinでそれぞれ標識し，それらの蛍光を指標にSepharose CL-6Bゲルろ過による分離分析を行った（Fig. 2）．Rhodamineとfluoresceinの蛍光が重なっているピークはリポソームに挿入されたPEGリン脂質を，低分子量側のfluorescein単独のピークはリポソームに挿入されていないPEGリン脂質をそれぞれ表している．この2つのピークの曲線下面積からpost-insertion法におけるリポソーム膜へのPEGリン脂質の挿入効率を求めた．Table 2にはpost-insertion法におけるPEGリン脂質の添加量と

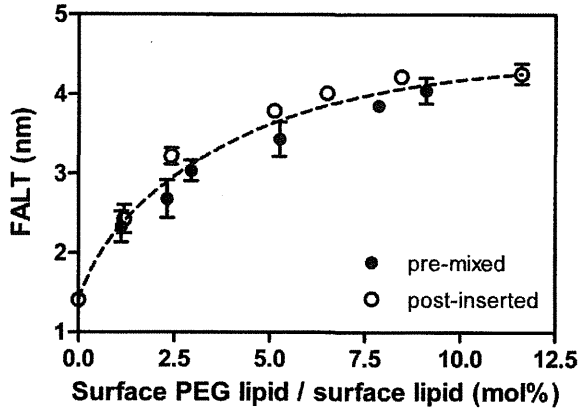

Fig. 5　Relationship of FALT with the surface coverage of liposomes with PEG lipid. Liposomes were modified with DSPE-PEG2000 in the pre-mixed (●) or post-insertion (○) methods.

PEGリン脂質のリポソーム膜への挿入効率の関係を示したが，PEGリン脂質添加量に依存してPEGリン脂質の挿入効率が低下していることがわかる．

次に，NBD基が亜ジチオン酸ナトリウム（$Na_2S_2O_4$）によって不可逆的に還元され蛍光消光する現象[17]を利用して，リポソーム膜内外層におけるPEGリン脂質の分布を測定した．NBD標識PEGリン脂質を用いてpre-mixed法及びpost-insertion法でそれぞれ調製したPEG修飾リポソームの消光実験結果をFig. 3に示す．$Na_2S_2O_4$を外水相に添加するとリポソーム膜最外層に存在するNBD標識PEGリン脂質のみが消光を受けるため，蛍光強度の減少率からリポソーム膜内外層におけるPEGリン脂質の分布を求めることができる．Pre-mixed法で調製したリポソームにおいては約50%のPEGリン脂質が，post-insertion法では100%に近いPEGリン脂質が，リポソーム膜外層に存在することが確認された．一方，リポソームリン脂質膜をNBD-PEで標識し同様の実験を行ったところ，PEG未修飾リポソーム（plain）とPEG修飾リポソームのいずれにおいても約50%のリン脂質がリポソーム膜外層に存在することが示され，リポソームが一枚膜（unilamellar）であることが確認された．

さらに，post-insertion法でのリポソーム膜に対するPEGリン脂質の挿入効率について，インキュベーション温度の影響を検討した（Fig. 4）．PEGリン脂質のリポソーム膜への挿入効率は50～60℃以上で一定値を示した．DSPC/cholesterol膜系のゲル-液晶相転移が50℃付近に存在することが報告されていることから[19]，この結果は，リポソーム膜へのPEG脂質の挿入効率がリポソーム膜の相状態に依存していることを示唆する．また，post-insertion法ではリポ

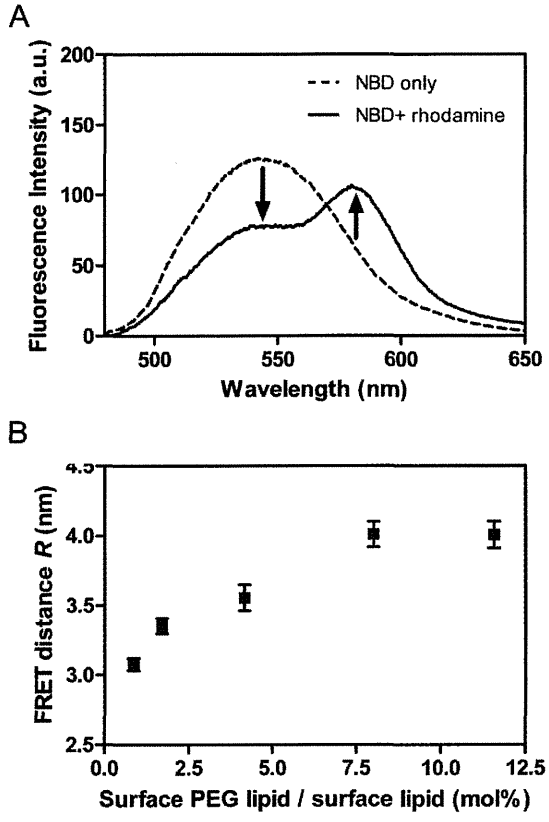

Fig. 6 (A) FRET between DSPE-PEG2000-NBD and rhodamine-PE in PEG-modified liposomes. Fluorescence emission spectra excited at 460 nm for NBD-labeled (dashed line) and NBD/rhodamine-labeled (solid line) PEG-modified liposomes. Liposomes were modified with DSPE-PEG2000 (1.0 mol% to total lipids) in the post-insertion method. (B) Change in FRET distance with increasing amount of surface PEG lipid.

ソーム膜調製後にPEGリン脂質を添加するため，PEGリン脂質はリポソーム膜外層にのみ存在すると予想されるが，post-insertionを行う際の温度条件によってはリポソーム膜内層側にPEGリン脂質が移行していることがNBD消光実験より確認された．特に80 ℃のような高温でpost-insertionを行うと15 %程度のPEGリン脂質が内膜層に移行しており（Fig. 4），高温でのpost-insertionには注意が必要であることが示唆された．これらの実験結果から，post-insertionの温度条件としては50～60 ℃が適当と考えられた．

以上の検討により，post-insertion法及びpre-mixed法で調製したPEG修飾リポソームについて，PEGリン脂質のリポソーム膜への挿入効率および内外膜への分布挙動が明らかとなった．そこで，post-insertion法とpre-mixed法で調製したPEGリポソーム膜表面でのPEGリン脂質の被覆率とFALTとの関係を比較したところ，FALTの表面PEGリン脂質濃度に対する依存性は両修飾法で同じであることが示された（Fig. 5）．すなわち，PEGリポソームの表面物性は，修飾法の違いにかかわらずPEG鎖の表面被覆率によって制御されていることが明らかとなった．

3.3 FRETによるリポソーム膜表面PEG鎖のコンフォメーション変化の観測

Fig. 5に示されたPEGリン脂質のリポソーム表面濃度の増加に伴うFALTの増大は，リポソーム膜表面でのPEG鎖コンフォメーションがマッシュルーム型からブラシ型へ変化するためと考えられている[20]．これは，PEGリン脂質濃度の増加がPEG鎖の三次元的な横方向への広がりを抑制し，縦方向に伸長することにより水和層が増大するためである[21]．このPEG鎖のコンフォメーション変化を検出するため，リポソーム膜中のリン脂質頭部をrhodamine（アクセプター分子）で，DSPE-PEG2000のPEG鎖末端をNBD（ドナー分子）で標識し，NBDからrhodamineへのFRETを観察した．

Fig. 6Aは，PEGリン脂質を1.0 mol%添加した際のDSPE-PEG2000-NBDとrhodamine-PEの蛍光スペクトルを示す（リポソーム膜表面脂質に対する表面PEGリン脂質濃度は約1.7 mol%）．NBDの励起波長460 nmで励起すると，PEG鎖末端のNBD基からリポソーム表面のrhodamine基へのFRETが起こり，NBDの蛍光極大波長である540 nm付近での蛍光強度の減少とrhodamineの蛍光極大波長である580 nm付近での蛍光強度の増大が観察された．そこで，540 nm付近におけるNBD蛍光強度の減少度からFRET効率Eを，さらにその値を用いてドナー-アクセプター分子間平均距離Rを求めた．この場合の励起エネルギー移動効率とドナー-アクセプター分子間平均距離はそれぞれ$E = 0.39$，$R = 3.5$ nmであった．

Fig. 6Bは，PEGリン脂質の表面濃度の増加によるRの変化を示す．これより，PEG修飾率の増加がリポソーム膜面からPEG鎖末端までの平均距離の増大をもたらすことが明らかとなった．また，この変化はFig. 5の固定水和層長と同様な増加挙動を示し，PEG修飾リポソームの表面固定水和層長の増大が表面PEG鎖のコンフォメーション変化に起因することを示唆した．Pre-mixed法により調製したDSPE-PEG2000修飾リポソームの場合，全脂質に対して4～5 mol%（表面脂質あたり約2～2.5 mol%に相当）程度のPEG脂質濃度でマッシュルーム構造からブラシ構造へのPEG鎖コンフォメーション変化が起こるといわれている[20,21]．Fig. 5やFig. 6Bで見られる低濃度側でのFALT及びRの増加はPEG鎖のコンフォ

メーション変化を反映し，高濃度側でプラトーに達するのはPEG鎖のほとんどがブラシ構造を形成しているためと考えられる．

4. 結　言

本研究では，PEG修飾リポソームの表面物性として表面固定水和層長を求めるとともに，蛍光標識PEGリン脂質を用いたリポソーム膜表面でのPEG被覆率およびPEG鎖コンフォメーションに関する蛍光分光学的測定から，post-insertion法で作製したPEG修飾リポソームの物理化学的特性評価を行った．

PEG修飾リポソームの固定水和層長の変化をリポソーム膜表面に存在するPEGリン脂質濃度当たりで比較したところ，リポソーム膜最外層におけるPEGリン脂質被覆率と固定水和層長との相関はpre-mixed法とpost-insertion法でほぼ同じであることが示され，修飾法の違いにかかわらずPEGリポソームの表面物性がPEG鎖の表面被覆率によって制御されていることが明らかとなった．さらに，PEG修飾リポソームの固定水和層長の増大はPEG鎖のコンフォメーション変化に起因することが示唆された．

従来のリポソームPEG修飾法であるpre-mixed法は，リポソーム膜内層にもPEGリン脂質が存在するため，内水相容積を狭め薬物封入率を低下させるだけでなく，リポソームに内封した薬物の漏出性を増大させるという問題点が報告されている[22]．今回の結果は，生産効率や拡張性に優れたpost-insertion法が，pre-mixed法と同等の表面物性を有するPEG修飾リポソームの調製法であることを示した．

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