on membrane permeability. These results mostly agreed with those of previous studies in which CDC exhibited a greater disruption of egg phosphatidylcholine (EPC)-liposome lipid bilayers than UDC at concentrations below cmc.[10,13,25] Therefore, it was suggested that, even in the case that the liposomes are not composed of EPC, CDC and UDC can exhibit similar tendencies with those observed in the previous studies,[10,13,25] and the DXR release induced by both bile salts from the PEGylated liposomes tend to be slightly higher than that from normal liposomes.

$T_{X\text{-}100}$ induced DXR-release in a dose-dependent manner, and the increased release was observed from above cmc (approx. 0.2 mM[26]) (Fig. 1c). Interestingly, the percentage of released drug of the PEGylated liposomes SL1 and SL2 were significantly increased compared with the normal liposomes L1 and L2. There were no significant changes in the particle size of the liposomes in response to the concentration of $T_{X\text{-}100}$ (Fig. 1f), but the decreased intensity and increased polydispersity index were observed at 2 mM of $T_{X\text{-}100}$ (data not shown). Thus $T_{X\text{-}100}$ can induce drug release from liposomes without complete solubilization of the bilayer, and mixed micelles and empty liposomes can be existed in the solutions.

There are no reports indicating that $T_{X\text{-}100}$ significantly and selectively enhances membrane permeability in PEGylated bilayers. Thus, to assess the effect of PEG-lipid, we prepared DXR-encapsulated liposomes with various ratios of PEG-lipid (0, 2.5, 5.0, 10 mol% (mol/mol)), and measured $T_{X\text{-}100}$-induced DXR release. The increased ratios of PEG-lipid (*i.e.*, 2.5, 5.0, 10%) caused significant increases in the rate of DXR release (Fig. 2). This result emphasizes that PEG-lipid greatly affects $T_{X\text{-}100}$-induced DXR release from PEGylated liposomes. However, a previous study indicated that giant unilamellar vesicles consisting of dipalmitoyl phosphatidylcholine (DPPC) (C16:0)/Chol with 1% PEG-lipid in the liquid-ordered phase were stable, and that no leakage of encapsulated substance was observed in the presence of high concentrations of $T_{X\text{-}100}$ by using fluorescence microscopy.[27] The difference between these results could be attributed to the fact that in this study, we used HSPC (C16:0 approx. 10%, C18:0 approx. 90%) liposomes with higher content of PEG-lipid. It was reported that the increasing the carbon chain length decreased the detergent/lipid ratios causing solubilization.[14]

Interaction Analysis by SPR The different interactions between each detergent and the lipid bilayers were considered one of the factors leading to different effects on membrane permeability and solubilization, as described above. SPR can easily capture lipid vesicles on chips in cases where alkane groups have been introduced to the dextran matrix. Thus, this method, using drug-bilayer interaction analysis, has been applied to predict intestinal permeability of drugs as an alternative to parallel artificial membrane permeability assay (PAMPA).[22,23] We, therefore, attempted to observe the interactions between detergents and lipid bilayers. That is, the more detailed information than DLS, how detergent interfaces or is inserted into the lipid bilayers as well as the process of solubilization such as membrane saturation with detergent and formation of mixed micelle, could be observed. Figure 3 outlines the complete sensorgram of measurement cycle and Fig. 4 shows the responses obtained after the injection of detergent. In the sensorgrams, the increasing RU corresponds to the interface or dispersion of detergent to lipid bilayers during the injection of detergent, and the decreasing RU corresponds to the dissociation of lipids or lipid particles from the sensor chip.

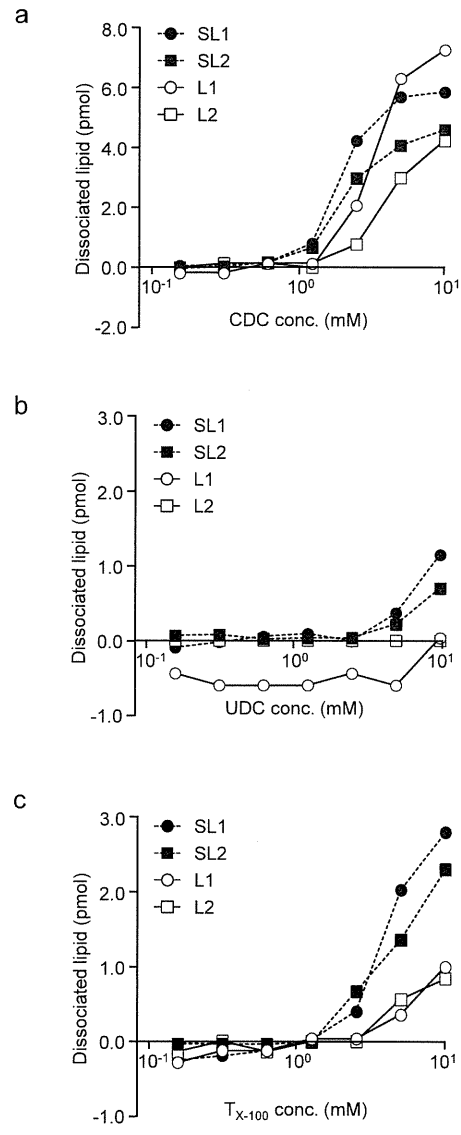

Fig. 5. Effects of Lipid Composition on Detergent-Induced Dissociation of Lipid Bilayers

Dissociated lipid (pmol) was plotted against detergent concentrations. SL1, HSPC/Chol/DSPE-PEG2000 (79/16/5 mol/mol); L1, HSPC/Chol (5/1 mol/mol); SL2, HSPC/Chol/DSPE-PEG2000 (55/40/5 mol/mol); L2, HSPC/Chol (6/4 mol/mol).

In SPR analysis, CDC exhibited the highest solubilizing ability among the 3 detergents tested and significantly induced the dissociation of lipids from 4 kinds of liposomes (Fig. 4a). Binding to the interface and incorporation of UDC into lipid bilayers occurred in a dose-dependent manner (Fig. 4b). In case of $T_{X\text{-}100}$, the binding to the normal liposomes L1 or L2 increased with higher detergent concentrations, and slight dissociation was observed. However, $T_{X\text{-}100}$ caused a significant dose-dependent dissociation in the PEGylated liposomes (Fig. 4c). Figure 5 shows the amount of dissociated lipid, an index of membrane solubilization, as a function of the concentration of each detergent. The amount of dissociated lipid was calculated from the difference between the equilibrium RU after liposome capture and that after the injection of detergent. These results correlated in part with the results for membrane

permeability (Figs. 1a–c), indicating that the lipid membrane of liposomes was partially solubilized and converted to mixed micelles in the presence of detergent, and that DXR-release was significantly increased. Therefore, it was clearly indicated that $T_{X\text{-}100}$ induces DXR release from PEGylated liposomes, because $T_{X\text{-}100}$ can exhibit higher solubilization of PEGylated liposomes *versus* normal liposomes.

The membrane solubilization induced by detergent has been evaluated by turbidity (spectral photometer) and particle size (DLS).[28] The partitioning of detergent into bilayers has been investigated using thermodynamic analyses, including isothermal titration calorimetry, differential scanning calorimeter, and ^{31}P-NMR.[29,30] To assess a more detailed mechanism of membrane solubilization, electron microscopy and dark-field microscopy have been used.[11,12] In the SPR technique used in this study, there are no standard parameters for solubilization compared with the above-indicated physicochemical approaches. However, using SPR, we observed that the detergent was either bound to or partitioned into bilayers. Subsequently, lipid bilayers were solubilized and dissociated from the chip, and it was found that the interactions of detergents with lipid bilayers differ depending on the kind of detergent. While a detailed comparison of the SPR results with the physicochemical approaches is needed, SPR may be a simple and efficient method for monitoring the solubilization and interaction of detergents with lipid bilayers because of its automated measurements.

The liquid-ordered phase of bilayers is known to be formed in the membrane consisted of saturated phospholipid, such as DPPC or distearoyl phosphatidylcholine (DSPC), and Chol. It was reported that DPPC/Chol membranes containing a high ratio of Chol (≥25 mol%) which are in lo phase were stable and resistant to solubilization by $T_{X\text{-}100}$.[15,27] In the case of CDC, it was indicated that the presence of Chol exhibited no significant effect on the induced drug release.[13] Our results mostly agreed with those of previous reports in which normal liposomes containing higher ratio of Chol were much more resistant to solubilization by $T_{X\text{-}100}$, although there were no significant differences in the permeability enhancement and solublization by CDC based on lipid composition. On the other hand, in our results, the significant differences in membrane permeability and solubilization in the presence or absence of PEG-lipid were observed when mixed with $T_{X\text{-}100}$. In general, the solubilizing ability of detergent is higher for solubilizate molecules which have higher polarity. The structure of $T_{X\text{-}100}$ is similar to that of PEG-lipid, which consists of a big hydrophilic group (a flexible polyethylene chain) and carbon chains. Therefore, while the details remain unclear, we hypothesized that PEG-lipid can easily form mixed micelle with $T_{X\text{-}100}$ because of its high polarity and structural similarity with $T_{X\text{-}100}$.

Our ultimate goal was to determine whether detergents could be applicable to *in vitro* drug-release testing of liposomal products. Because drug release from liposomes in normal buffered saline or serum/plasma takes several days, achieving a shortened test time would be a primary consideration in developing new *in vitro* release test. Our study demonstrates that the addition of detergents to test media can shorten the test time, with up to 100% release in 1 h. The lipid composition, especially the content of PEG-lipid, is a critical factor in determining the *in vivo* behavior of liposomal products. From this perspective, testing the enhancement of release with $T_{X\text{-}100}$, which can distinguish lipid compositions, may be a useful tool in developing new test methods. Further, because the purpose of evaluation is to assess quality control for efficacy and safety, biorelevance should also be considered in developing *in vitro* release tests. That is, the correlation of *in vitro* release rates with *in vivo* drug release mechanisms and behaviors of liposomal product may be required. FDA has proposed the investigation of *in vitro* drug leakage under multiple conditions in the draft guidance for generic version of doxorubicin-encapsulated liposomes (DOXIL®).[8] It is considered to appropriately characterize the drug release from liposomal products by combining different testing conditions, such as under biorelevant conditions for a given period of time to confirm the stability of liposomes, and under physicochemical stress to evaluate the physical state of lipid bilayers. Therefore, while detergent-induced drug release may not mimic the *in vivo* behavior of liposomal products, it could be useful for process control or quality control of liposomal products when using a combination of biorelevant methods.

Conclusion

Our results indicate that the differences in the membrane solubilization of PEGylated liposomes compared with normal liposomes vary depending on the detergent. $T_{X\text{-}100}$, for example, induced significant membrane permeabilization and solubilization of PEGylated liposomes compared with normal liposomes, while other detergents did not have this differential effect. Further, on the basis of SPR analysis, we suggest that this difference is due to the higher degree of solubilization exhibited by $T_{X\text{-}100}$ for PEGylated membranes than for normal membranes.

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Switzerland); Neoral® capsule, 50 mg (Product B, Lot No. S1046; Novartis Pharma K.K., Basel, Switzerland); Amadora® capsule, 50 mg (Product C, Lot No. 34006; TOYO CAPSULE Co., Ltd., Shizuoka, Japan); Cicporal® capsule, 50 mg (Product D, Lot No. EC2501; Nichi-Iko Pharmaceutical Co., Ltd., Toyama, Japan); cyclosporine capsule, 50 mg "Mylan" (Product E, Lot No. 0450RH; Mylan Seiyaku, Tokyo, Japan); and cyclosporine capsule, 50 mg "FC" (Product F, Lot No. 9C1; Fuji Capsule Co., Ltd., Shizuoka, Japan). The official CsA reference standard was purchased from the Phramaceutical and Medical Device Regulatory Science Society of Japan. Sodium taurocholate and lecithin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Cyclosporin D (CsD) (ALEXIS® Biochemicals) was purchased from Enzo Life Sciences (Farmingdale, NY, U.S.A.). Rat liver microsomes were obtained from Celsis In Vitro Technologies (Baltimore, MD, U.S.A.). β-Nicotinamide-adenine dinucleotide phosphate (NADP), glucose-6-phosphate 1-dehydrogenase (G-6-PDH), and glucose-6-phosphate (G-6-P) were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan).

Physicochemical Characteristics Eighty percent of the contents of a single capsule was placed in a test tube, and 10 mL of test medium was added. The solution was mixed by gentle inversion until the capsule contents were dispersed homogeneously in the test medium. Using this solution, 5-fold and 25-fold dilutions were prepared in different test tubes.

Preparation of Test Medium The 1st Fluid and 2nd Fluid for the dissolution test were prepared according to the Japanese Pharmacopoiea (JP)16. Fasted State Simulated Intestinal Fluid (FaSSIF) and Fed State Simulated Intestinal Fluid (FeSSIF) were prepared by the modified method reported by Galia *et al.* and Jantratid *et al.*[18,19] FaSSIF was formulated using approximately 900 mL of blank buffer, which was prepared by dissolving sodium chloride (4.01 g), sodium hydrate (1.39 g), and maleic acid (2.22 g) in 900 mL distilled water. The pH was then adjusted to 6.5. Sodium taurocholate (1.613 g) was dissolved in 50 mL of blank buffer, to which lecithin (0.15 g) was added and dissolved with heat and agitation until the solution became clear. The volume was adjusted to 1 L using the remaining blank buffer and distilled water. In the case of FeSSIF, sodium taurocholate (8.07 g) was dissolved in 50 mL of blank buffer (potassium chloride [15.20 g] and acetate [8.65 g] in 900 mL distilled water, pH 5.0), to which lecithin (2.81 g) was added and dissolved with heat and agitation until the solution became clear and yellow. The volume was adjusted to 1 L as with FaSSIF. Mixed micelles were not detected in either FaSSIF or FeSSIF when examined by a dynamic light scattering (DLS) photometer.

Absorbance To assess the degree of turbidity, the absorbance at 600 nm of each capsule sample was measured by a spectrophotometer (UV-2550/2450; Shimadzu, Kyoto, Japan) after mixing the samples with different test media.

Solubility The solubility of CsA in the dispersed solution was measured. The dispersed solution of each capsule sample was filtrated by 0.45 μm filter, and its concentration of CsA was measured by HPLC. The apparatus used for the HPLC system consisted of a constant pump (L-7200, Hitachi High-Technologies Corporation, Tokyo, Japan), a degasser (L-7610, Hitachi), an autoinjector (L-7200, Hitachi), a column oven (L-7300, Hitachi), an UV detector (214 nm) (L-7405, Hitachi), and a system controller (D-7000, Hitachi). The separation was carried out at 70°C on a Inertsil ODS-3 (100×4.0 mm i.d., 5 μm) from GL Science (Tokyo, Japan). The mobile phase consisted of water–tetrahydrofuran (5:3.6), and flow rate was 1.0 mL/min. A standard stock solution of CsA was prepared by dissolving 10 mg of CsA in 10 mL of ethanol, and stored at 4°C. A 10 μL aliquot of a sample was injected.

Particle Size Distribution The size distribution and mean diameter of particles in the capsule content samples were measured using a DLS photometer DLS-7000 (Otsuka Electronics Co., Ltd., Osaka, Japan) equipped with an He–Ne laser source (wavelength, 632.8 nm) after mixing the samples with different test media. All DLS measurements were made with a scattering angle of 90°. The neutral density filter was adjusted depending on intensity. Data were gathered with a counting period of 100 s. Histogram analysis was performed to assess the particle size distribution, and cumulant analysis was performed to calculate the mean diameter. The data between different products were statistically analyzed using a one-way analysis of variance followed by Dunnett's test.

The number of large-diameter particles (>0.5 μm) in the solution of the capsule content in 10 mL water was measured by an Accusizer 780A instrument (Particle Sizing Systems, Santa Barbara, CA, U.S.A.). This instrument is based on light extinction (LE) or light scattering (LS) that employs a single-particle optical sizing (SPOS) technique, and was equipped with an automatic dilution system. In this study, the summation mode, which is a combination of LE and LS, was applied. Duplicate measurements were made for each sample at the appropriate time point using the following conditions: data collecting time, 60 s; flow rate, 60 mL/min; injection loop volume, 1.04 mL; syringe volume, 2.5 mL; second dilution factor, 40.

Sample Preparation for Assay A 100 μL aliquot of each blood sample was transferred to a microtube. A 200 μL aliquot of internal standard (IS) solution (8.3 ng/mL of CsD in methanol–0.3 mol/L $ZnSO_4$, 7:3 v/v) was added to each tube. Tubes were tapped and vortexed for a few minutes until the pellet was completely dispersed. After centrifugation at 12000 rpm for 5 min, the supernatant was filtered by a centrifugal filter device (Ultrafree-MC, 0.22 μm polyvinylidene difluoride (PVDF); Millipore, Billerica, MA, U.S.A.). After further centrifugation at 10000 rpm for 2 min, the filtered sample was directly applied to the liquid chromatography/mass spectrometry (LC/MS) system.

Due to difficulty in obtaining reference standards of CsA metabolites, the *in vitro* metabolic reaction was performed by following the method for rat liver microsomes, and reactants containing metabolized CsA were used to confirm the LC separation of CsA and its metabolites. First, a reduced nicotinamide adenine dinucleotide phosphate (NADPH) regenerating system (NRS; 1.7 mg/mL NADP, 7.8 mg/mL G-6-P, 6.0 units/mL G-6-PDH in 2% (w/v) $NaHCO_3$) was prepared. A 50 μL aliquot of rat liver microsomes, 5 μL of 500 ng/mL CsA in acetonitrile, and 320 μL of 50 mM Tris buffer were mixed in a microtube, and then pre-incubated at 37°C for 5 min. Next, 125 μL NRS was added and the solution was thoroughly mixed. After incubation at 37°C for 60 min, 500 μL internal standard solution was added to terminate the reaction. After centrifugation at 10000 rpm for 5 min, the supernatant was filtered, as described above, and applied to the LC/MS system.

Assay for Cyclosporine A and Its Metabolites CsA and its metabolites in whole blood were measured by LC/MS

system in accordance with Koseki *et al.*,[20] with some modifications. LC/MS was performed on a Shimadzu LCMS-2010 system that includes a constant pump, column thermostat, degasser, autosampler, and quadrupole mass spectrometer (Shimazdu Corporation, Kyoto, Japan). The HPLC column was a Symmetry C8 (4.6×75 mm, 3.5 μm; Waters, Milford, MA, U.S.A.) with a guard column (Opti-Guard-min C8, 1×15 mm; Optimize Technologies, Oregon City, OR, U.S.A.). LC/MS grade water and methanol were prepared as mobile phase A and B, respectively. The flow rate was set to 0.3 mL/min and the column temperature was 80°C. A linear gradient separation was used, with 72% of mobile phase B from 0 to 1 min, then 72% to 85% of mobile phase B over 5 min, holding for 3 min, and finally 72% of mobile phase B over 6 min. The total run time was 15 min for each injection. A 20 μL aliquot of each prepared sample was injected.

The mass spectrometer was interfaced with an electrospray ionization (ESI) source used in the positive ion mode. The following parameters were retained for optimal detection of all analytes: nitrogen gas flow rate, 1.5 L/min; interface voltage, 4.5 kV; desolvation line voltage and temperature, 20.0 V and 250°C, respectively; block heater, 200°C. For the determination of CsA and its metabolites as well as CsD, the sodium adducts were measured at *m/z* 1210.9 (AM4N), *m/z* 1224.9 (CsA), *m/z* 1238.9 (CsD, IS), and *m/z* 1240.9 (AM1, AM9, AM1c) by using selected ion monitoring (SIM). Retention times for AM1, AM9, AM1c, AM4N, CsA, and CsD were 8.6, 8.9, 9.5, 10.4, 11.4, and 12.2 min, respectively. Quantification of CsA and its metabolites was achieved with a calibration curve of CsA (concentration range, 7.8–500 ng/mL). The limit of detection (signal-to-noise ratio, 3) and quantification (signal-to-noise ratio, 10) of CsA was approximately 1.5 ng/mL and 5 ng/mL, respectively.

Animal Study The animal experiments were outsourced to Charles River Japan and performed in accordance with the Guideline for Animal Experiments of Charles River Japan. Male Sprague-Dawley rats weighing 220–250 g were fasted overnight with free access to water. The content of each CsA capsule product was diluted in distilled water to obtain a CsA concentration of 1.0 mg/mL. The CsA solution equivalent of 3.5 mg/kg CsA was orally administered to rats using a stomach sonde. Next, blood samples (300 μL) were collected from the jugular vein at 0.5, 1, 2, 3, 6, 12, and 24 h using a syringe flushed with 100 mg/mL ethylenediaminetetraacetic acid (EDTA), and stored at −80°C until analysis by LC/MS. The pharmacokinetic parameters of CsA and its metabolites, such as *AUC*, C_{max}, and time to reach C_{max} (T_{max}), were estimated by non-compartmental analysis using WinNonlin (version 5.2; Pharsight Corporation, Sunnyvale, CA, U.S.A.). The data between different products were compared for statistical significance by a Kruskal–Wallis test.

Results

Physicochemical Characteristics Neoral, a microemulsion pre-concentrate formulation, is composed of lipophilic solvent, hydrophilic solvent, surfactant, and drug. Table 1 shows the difference in additive composition of CsA capsule contents among products. First, we removed the contents of each capsule and compared the physical appearances before dispersion. Product A was the oil-based formulation Sandimmune, Product B was the innovator Neoral, and Products C to F were generic formulations. As seen in Fig. 1a, Product A and Product F were yellow, Product B and Product E were slightly yellow, and Product C and Product D were almost clear. Next, we dispersed the contents of 1 capsule in 10 mL of each test medium and compared the physical appearances (Fig. 1b). For the test media, water, 1st and 2nd Fluids for the dissolution test, artificial intestinal juice, Fasted State Simulated Intestinal Fluid (FaSSIF), and Fed State Simulated Intestinal Fluid (FeSSIF) were used. For all capsules the contents were homogeneously dispersed in each test medium except for Product A. The dispersion liquid of SEDDS is typically turbid and inhomogeneous, whereas that of SMEDDS is usually nearly clear. Thus, the dispersion state of the generics was obviously different from that of Product A (SEDDS). When the capsule contents were dispersed in water, Product B and Product E produced a clear and almost clear solution, respectively; Product F produced a white solution; and Product C and D produced bluish milky solutions. Assessment using the other test media indicated the same tendency. On the other hand, when the capsule contents were dispersed in FeSSIF, all generic products produced a white cloudy solution, whereas Product A produced a clear solution.

To quantify the degree of turbidity, we measured the absorbance at 600 nm of the dispersion solutions. The absorbance of the solutions formed by dispersion in water was high and showed the following order: B≈E<C=D<F<<<A. This same tendency was observed for the other test media (Fig. 2). These results correlated with the physical appearance of the solutions, including the significantly higher absorbance of generics dispersed in FeSSIF. In the case of Product F, a slight precipitate was formed in the dispersion solution several hours after initial dispersion (data not shown). The absorbance of Products B through E in each test medium decreased with increasing dilution, and no creaming or precipitation was observed. The solubility of dispersion solution was measured and found that there were no differences between the innovator and the generics with regard to solubility.

The distribution and number of particles of the microemulsion (or emulsion) in the dispersion solution were determined. The contents of 1 capsule were dispersed in 10 mL of water, and the distribution and number of particles (>0.5 μm) were measured by the single-particle optical sizing (SPOS) method. As seen in Fig. 3a, the particle size of the oil-based formulation Product A was significantly larger than that of the other products. Whereas the particle number (>0.5 μm) of products B and E was almost the same, that of Products C and D was 5-fold larger, and that of Product F was 25-fold larger, than that of Product B, which correlates with their physicochemical appearances (Fig. 3b). These results indicated that the particle distribution of generic products in solution is wider than that of the innovator.

The mean diameter and distribution of the particles of the microemulsion (or emulsion) in the dispersion solution formed from the capsule contents were measured by dynamic light scattering (DLS) (Fig. 4, Table 2). The mean diameter of each product in water at a 5-fold dilution was as follows: Product B, 26.4 nm; E, 29.7 nm; C, 74.8 nm; D, 64.5 nm; and F, 79.2 nm. Thus, the mean diameter of the generics tended to be larger than that of the innovator. The same tendency was observed in 1st and 2nd Fluids for the dissolution test, and FaSSIF. When the capsule contents of the generics were dispersed

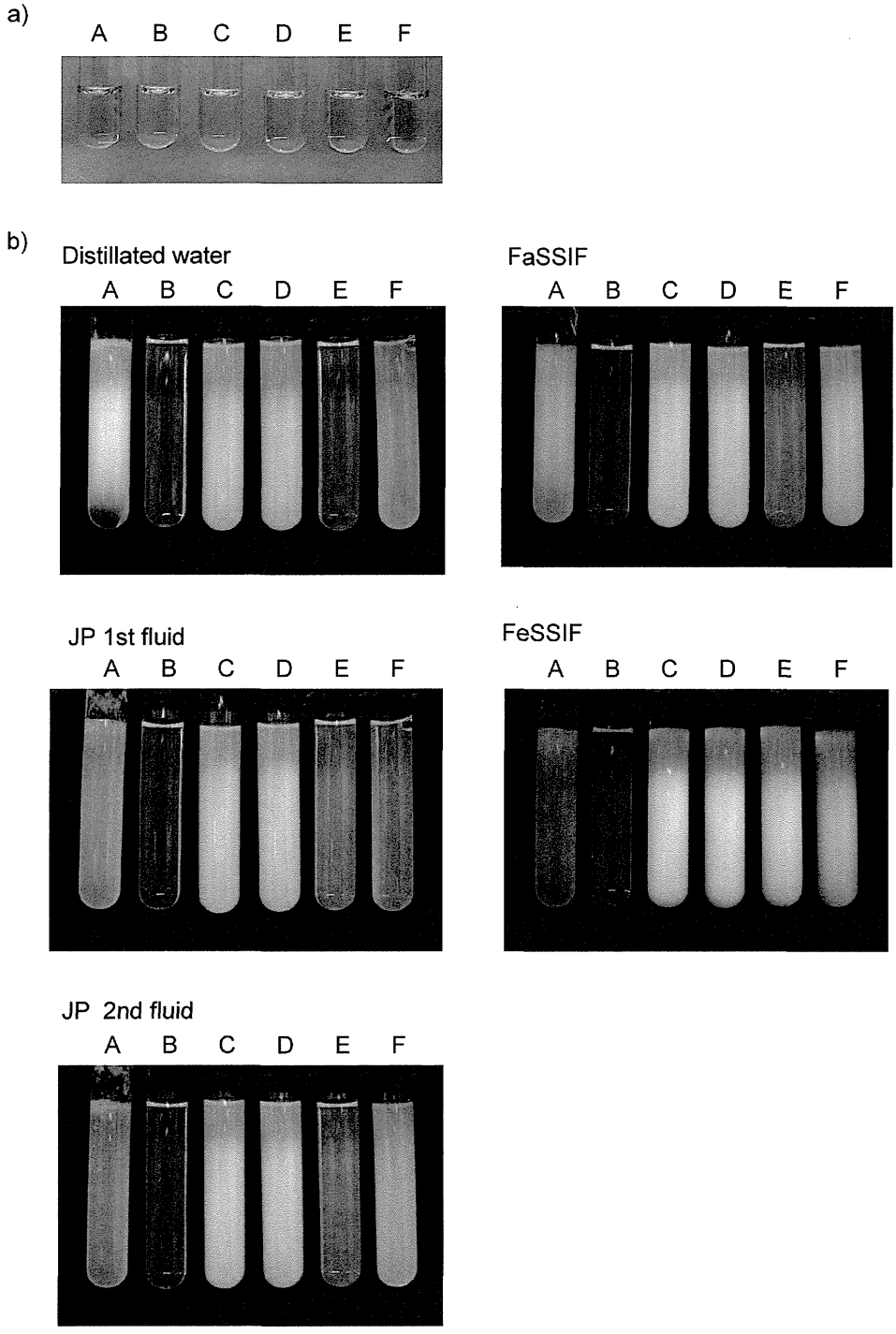

Fig. 1. Physical Appearances of (a) the Contents of Each CsA Capsule and (b) the Solution of the Contents of 1 CsA Capsule in 10 mL Water, 1st Fluid, 2nd Fluid, FaSSIF, and FeSSIF

in FeSSIF, the mean diameter of the generics increased to 100–200 nm. Among the generic products, dispersion of Product F in either the 2nd fluid or FaSSIF increased the mean diameter to 120–200 nm (Table 2), and the particle distribution expanded with increasing dilution (Fig. 4). In addition, when Product F was dispersed in either water or the 1st Fluid, the value of the mean diameter varied widely when compared with those of the other products (Table 2). These results, including those seen in Fig. 3, indicated that the particles in the dispersion solution of Product F could not be homogeneous. On the other hand, the mean diameter of the innovator, Product B, was very small (about 30 nm) in water, and in both 1st and 2nd test Fluids, and there were no changes in the mean diameter or distribution even when its capsule contents were dispersed in either FaSSIF or FeSSIF containing lecithin and taurocholic acid. This indicated that, after oral administration, a microemulsion of Product B is likely to be formed in the gastrointestinal tract. The mean particle diameter and distribution were not determined in the 1-fold and 5-fold dilutions of Product A because of the high scattering intensity. The small mean diameter of the generics in FeSSIF may be caused by multiple scattering.

Table 1. Composition of CsA Capsule Contents

	Product A	Product B	Product C	Product D	Product E	Product F
Solvent	Esterified corn oil	Glycerol esters of fatty acids	Propylene glycol esters of fatty acids	Propylene glycol esters of fatty acids	Propylene glycol esters of fatty acids	Propylene glycol esters of fatty acids
	Ethanol	Propylene glycol			Triethyl citrate	
	Corn oil	Ethanol			Sorbitan monolaurate	Ethanol
Surfactant		Polyoxyethylene hydrogenated castor oil	Polyoxyl 35 castor oil	Polyoxyl 35 castor oil	Polyoxyl 35 castor oil	Polyoxyl 35 castor oil
Other		Tocopherol	Other two components	Other two components		Other five components

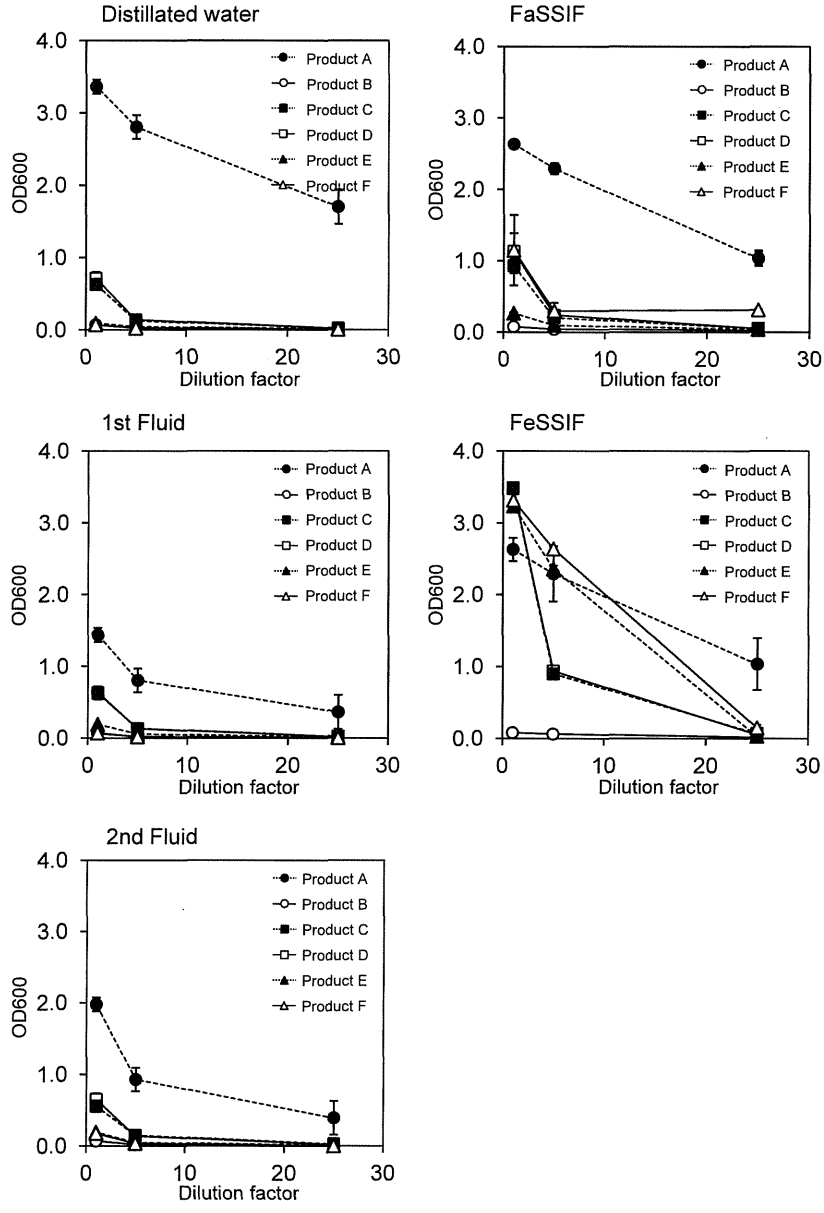

Fig. 2. Absorbance (600 nm) of the Suspension of the Contents of 1 CsA Capsule in 10 mL of Test Media and Diluted Solutions Thereof
Data are represented as the mean±S.D.

Pharmacokinetics of CsA and Its Metabolites in Rats
The results described above demonstrate that, although all generic products were dispersed in each test medium unlike Product A, the physical appearance and particle diameter of the generic dispersion solutions were different from those of the innovator Product B. Therefore, we next assessed the blood concentration profiles of CsA and its metabolites (AM1, AM9, AM1c, and AM4N) after oral administration in rats (Fig. 5). The blood concentration was measured by liquid chromatography-mass spectrometry (LC-MS) analysis, and the

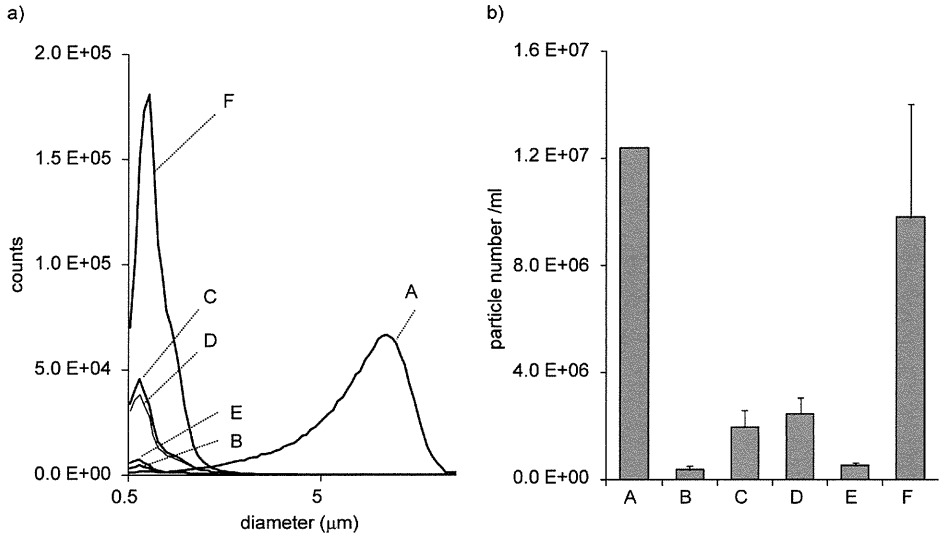

Fig. 3. Particle Distribution (a) and Particle Number (>0.5 μm) (b) of CsA Lipid Particles in Water
Each value represents the mean±S.D. (n=3).

pharmacokinetic parameters *AUC*, C_{max}, and T_{max} were analyzed as indicated in Table 3. The pharmacokinetic parameters of AM4N are not shown in Table 3 because its blood concentration in many samples was below the lower limit of quantification. The blood concentration of CsA increased rapidly, and that of its metabolites increased subsequently (Fig. 5). The C_{max} (671±95 ng/mL) and *AUC* (7194±507 h·ng/mL) of the innovator Product B was obviously higher than the C_{max} (474±60 ng/mL) and *AUC* (5839±371 h·ng/mL) of the oil-based formulation Product A (Table 3), a finding that was consistent in principle with previous reports.[21,22] The C_{max} and *AUC* of the generic products also tended to be higher than those of Product A. The C_{max} of the 4 generic products tended to be slightly lower than those of Product B. Again, however, there were no significant differences between Product B and the generic products in either C_{max} or *AUC*. Likewise, no significant differences were observed in the pharmacokinetics of CsA metabolites AM1, AM9, or AM1c. Koehler *et al.* reported that the bioavailability of a CsA generic product (Eon Labs) in rats was lower than that of Neoral, whereas the plasma AM4N level was significantly elevated in groups receiving Eon compared to that in another group receiving Neoral.[17] In our data, a significantly elevated AM4N blood level was not observed in groups treated with generic products compared with that of the group treated with Neoral, Product B. In rats, CsA undergoes first-pass metabolism by CYP3A, which is located in the gastrointestinal mucosa and in the liver. Therefore, these results suggest that the CsA contained in the generic products tested in this study was absorbed *via* the same pathway used for the CsA in the innovator. We performed the same examination again and confirmed that the bioavailability of the generic products was similar to that of the innovator. Only the T_{max} differed significantly between Product B and the generic products. This same significant delay in T_{max} of the generic products was also observed in the second experiment.

Discussion

Regions corresponding to different phases of the formulation, such as microemulsion, emulsion, micelles, or reverse micelles, are described in a ternary phase diagram according to different concentrations of each component (such as water, surfactant, and oil).[6] The variations in components, such as the presence or absence of co-surfactants/co-solvents and different types of oil, also result in the formation of different phase regions. Additive compositions of generic products of CsA are different from that of the innovator (Table 1). Actually, in this study, the physical appearance of the generic products in water was different from that of the innovator. Therefore, the phase of dispersion solution of the generic products might be near the emulsion phase, or might be a mixture of emulsion and microemulsion.

In a study of microemulsion formulation, when the optimized microemulsion pre-concentrate was dispersed in FeSSIF, the particle size remained small in the dispersion solution (20–50 nm).[23] In our study, the dispersion solution of the generics in FeSSIF was cloudy white like milk, whereas that of the innovator was clear as in water. Although the details were unclear, the formulations of the generic product could be susceptible to taurocholate and lecithin in FeSSIF, and the phase regions of their solution dispersed in FeSSIF could be shifted to another phase region. From these points, we also hypothesize that the 4 generic products are self-emulsifying formulations, but their phase states are different from those of the innovator Product B and the oil-based Product A.

The relationship between particle size and bioavailability in CsA microemulsion or emulsion formulations has been investigated in humans.[24] In this previous report, the *AUC* increased as the particle size decreased, and only the formulation whose particle size was under 100 nm exhibited a desirable bioavailability. However, the type of surfactant used for the formulation with large particles (>150 nm) was different from the formulation with small particles (<60 nm); thus, as reported in another study,[25] bioavailability can be affected not only by particle size but also by the characteristics of surfactants on the particle surface. The improved bioavailability provided by self-emulsifying formulations is believed to be due to a larger particle surface area, improved aqueous solubility of drugs, and the enhancement of intestinal membrane permeability produced by local disturbance of the cell membrane.[26] These mechanisms will be enhanced by the properties of the

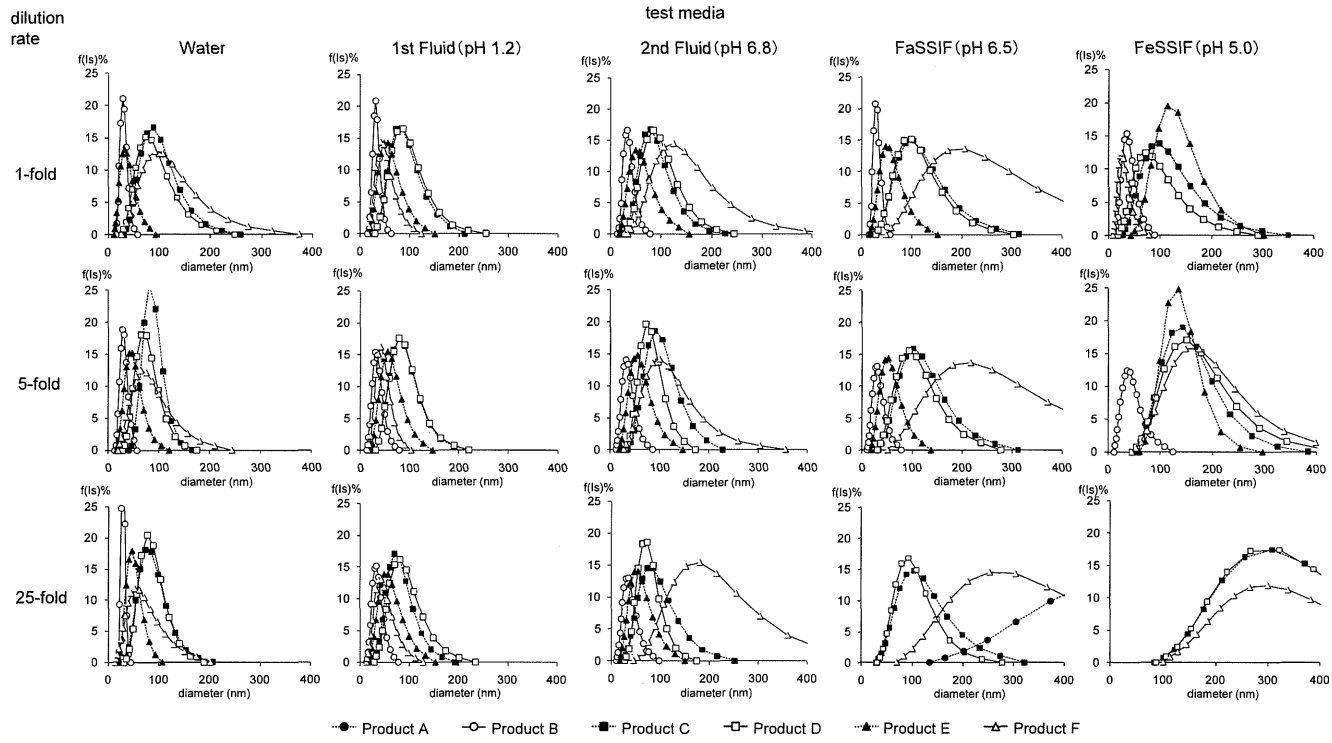

Fig. 4. Effects of Dilution and Test Media on the Size Distribution of CsA Lipid Particles

The contents of each capsule were dispersed in 10 mL of test medium, and further diluted 5-fold and 25-fold with the test medium. The size distribution of CsA lipid particles in the suspension was measured by a dynamic light scattering method.

Table 2. Particle Size of CsA Lipid Particles in Each Solution

	Dilution factor	Product A	Product B	Product C	Product D	Product E	Product F
Water	1	ND[a]	27.1±0.5	74.3±4.1[b]	68.7±3.7[b]	27.0±1.2	92.8±17.0[b]
	5	ND[a]	26.4±1.3	74.8±6.6[b]	64.5±8.7[b]	39.7±2.1	79.2±17.2[b]
	25	1423.6±1369.8	27.2±2.7	67.9±10.0[b]	65.1±6.7[b]	43.6±2.7[c]	67.7±12.3[c]
1st fluid (pH 1.2)	1	ND[a]	28.6±1.1	71.1±3.0[b]	69.8±2.0[b]	44.3±0.3[b]	80.8±61.7
	5	ND[a]	28.5±0.3	70.3±4.9[b]	72.5±1.9[b]	47.5±1.4[b]	102.3±106.1
	25	3075.2±1617.3	28.3±0.7	65.9±4.5[b]	66.7±3.8[b]	46.2±1.9[b]	43.2±0.8
2nd fluid (pH 6.8)	1	ND[a]	28.3±1.3	72.3±8.0[c]	67.6±3.0[c]	45.5±3.0	137.8±45.8[b]
	5	ND[a]	28.2±1.6	74.5±6.6[c]	68.8±2.6	45.7±2.0	121.7±62.1[b]
	25	4900.3±4128.6	29.3±2.2	68.4±4.6	66.5±2.9	46.8±2.6	197.6±88.8[b]
FaSSIF (pH 6.5)	1	ND[a]	26.5±0.9	83.4±10.9[b]	74.0±5.7[b]	42.0±1.0	136.3±23.4[b]
	5	ND[a]	25.3±1.6	84.4±7.3[b]	80.2±5.3[b]	41.3±1.3	151.4±29.5[b]
	25	388.6±65.0	ND[a]	79.5±11.5	78.7±4.5	ND[a]	204.4±23.1
FeSSIF (pH 5.0)	1	ND[a]	30.0±1.1	72.6±3.5[b]	58.7±1.1[b]	103.6±3.0[b]	22.2±3.7[c]
	5	ND[a]	29.8±0.9	122.1±4.5[b]	123.5±4.7[b]	122.4±1.7[b]	137.9±8.56[b]
	25	1995.4±2169.9	ND[a]	254.9±6.4[b]	256.9±9.0	ND[a]	264.1±18.1

Each value represents the mean±S.D. (n=3). *a*) Not determined. *b*) $p<0.01$ compared to Product B. *c*) $p<0.05$ compared to Product B.

surfactant, as will particle size. Although the oil or other components of generics are different from those of the innovator, the same type of surfactant, a polyoxyethylene castor oil derivative, is used for all of these products. Therefore, it is possible that there were no significant differences in the bioavailability of CsA between the innovator and the generics because the same type of surfactant is used.

On the other hand, the T_{max} of the generic products was longer than that of the innovator. The particle size of the innovator Product B can remain small in gastrointestinal tract, because, unlike the generic products, the particle size of the innovator Product B did not vary in any test solutions. Thus, we think that the difference in particle size between the innovator and the generics can affect T_{max}, the pre-concentrate of the generics can be dispersed homogeneously in gastrointestinal fluid with the same degree of small particle size, with sufficiently low variability to prevent differences in AUC or C_{max} at the site of drug absorption. In the bioequivalence guidelines (BE guidelines) published in Japan ("Guideline for Bioequivalence Studies of Generic Products" http://www.nihs.go.jp/drug/DrugDiv-E.html), T_{max} is generally not required to be equivalent because T_{max} is a variable parameter. The interval of administration of CsA capsules is long (about 12 h) and the maximal change in the rate of approximate $T_{1/2}$ was less than

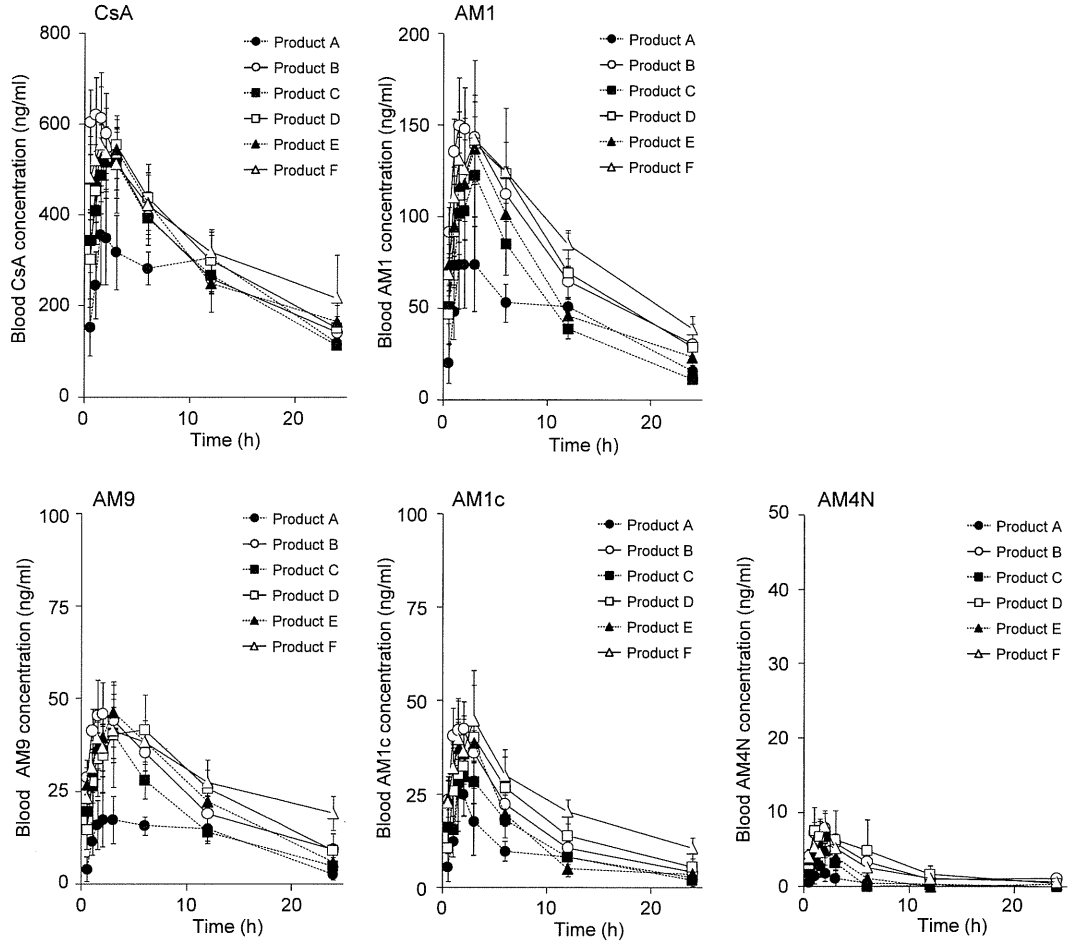

Fig. 5. Pharmacokinetics Profiles of CsA and Its Metabolites (AM1, AM9, AM1c, and AM4N) after Oral Administration (3.5 mg/kg) of CsA Lipid Particles in Water to Rats

Each point is the mean of values obtained from 5 rats, and the vertical bar represents the standard error.

Table 3. Pharmacokinetics Parameters of CsA after Oral Administration of CsA Lipid Particles to Rats

	Product A	Product B	Product C	Product D	Product E	Product F
CsA						
T_{max} (h)	7.00±2.14	1.10±0.21[a)]	2.30±0.44[a,b)]	2.90±0.87[a,b)]	2.70±0.89[a,b)]	2.85±1.01[a,b)]
C_{max} (ng/mL)	474±60	671±95	559±74	611±197	565±69	615±107
AUC (h·ng/mL)	5839±371	7194±507	6625±541	7454±2185	7105±721	7653±1502
AM1						
T_{max} (h)	7.00±2.14	3.15±1.55	2.70±0.30	3.80±0.97	2.70±0.89	3.17±0.79
C_{max} (ng/mL)	106±45	164±21	127±18	161±42	135±35	164±19
AUC (h·ng/mL)	1033±112	1675±99	1188±101	1798±472	1509±170	1743±212
AM9						
T_{max} (h)	7.00±2.14	3.10±1.55	3.00±0.84	4.80±0.73[b)]	3.00±0.84	4.30±1.49[b)]
C_{max} (ng/mL)	30±4	52±8[a)]	44±6[a)]	50±13[a)]	47±5[a)]	48±6[a)]
AUC (h·ng/mL)	278±27	519±56[a)]	414±26[a)]	604±142[a)]	566±30[a)]	607±107[a)]
AM1c						
T_{max} (h)	4.10±1.99	1.35±0.18	2.90±0.81[b)]	3.40±0.68[b)]	1.80±0.34[b)]	2.85±0.81[b)]
C_{max} (ng/mL)	29±7	46±8	33±4	45±17	39±6	50±9
AUC (h·ng/mL)	197±38	336±36	261±42	401±100[a)]	290±34	478±100[a)]

Each value represents the mean±S.E. (n=5). *a*) $p<0.05$ compared to Product A. *b*) $p<0.05$ compared to Product B.

10% between the innovator and the generics based on our results. Therefore, the difference in T_{max} would not be predicted to have a significant effect on the dosage schedule.

Our data show that the physical appearance of the 4 tested generics in FeSSIF was clearly different from that of the innovator, suggesting the possibility of variation in CsA absorption in the intestine under fed conditions, while in our study the administration was performed under the fasted condition only.

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

Hiroko Shibata,* Chikako Yomota, Toru Kawanishi, and Haruhiro Okuda

National Institute of Health Sciences; 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan.
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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.

*To whom correspondence should be addressed. e-mail: h-shibata@nihs.go.jp

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

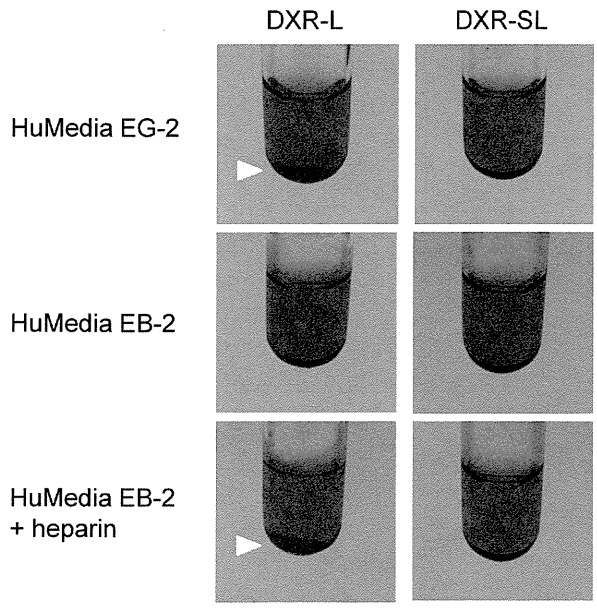

Fig. 1. Photographs of Liposomes (DXR-L and DXR-SL) Dispersed in HuMedia EG-2, HuMedia EB-2, or HuMedia EB-2 with Heparin

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

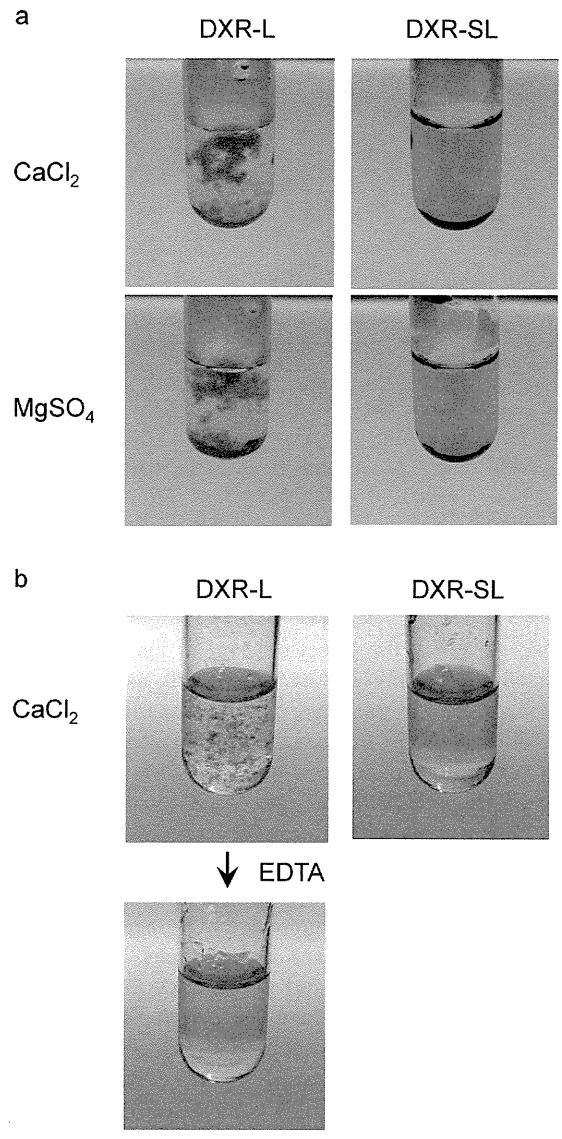

Fig. 2. Photographs of Liposomes (DXR-L and DXR-SL) Dispersed in $CaCl_2$ (100mM) or $MgSO_4$ (150mM) in the Presence of Heparin (10μg/mL) (a)

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.

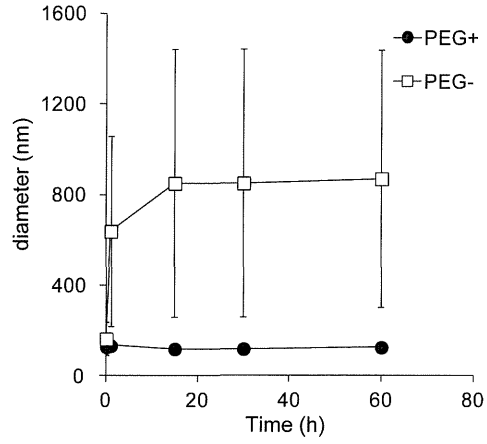

Fig. 3. The Change of the Particle Size of Liposomes (PEG +: CF-SL, PEG −: CF-L) with Time in the Presence of $CaCl_2$ (5 mM) and Heparin (10 μg/mL) at 37°C

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

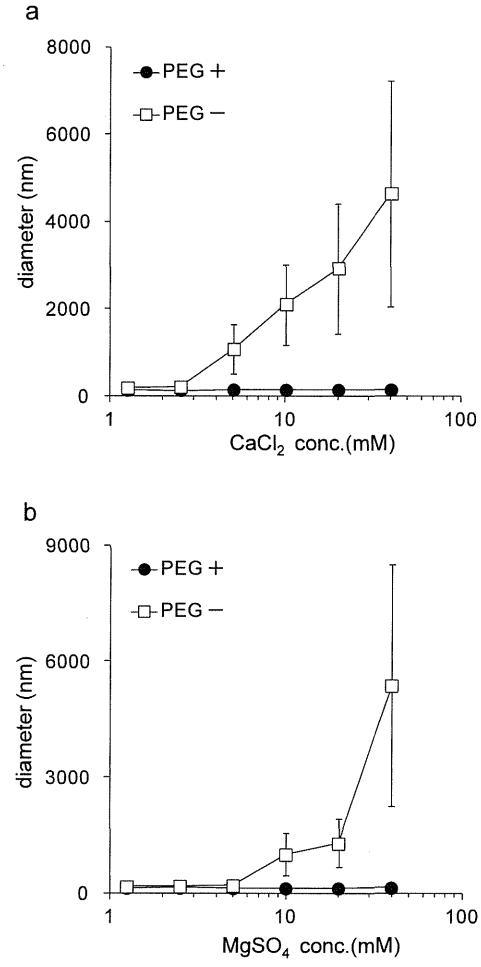

Fig. 4. The Effect of the $CaCl_2$ (a) or $MgSO_4$ (b) Concentration on the Particle Size of Liposomes (PEG +: CF-SL, PEG −: CF-L) in the Presence of Heparin (10 μg/mL)

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).

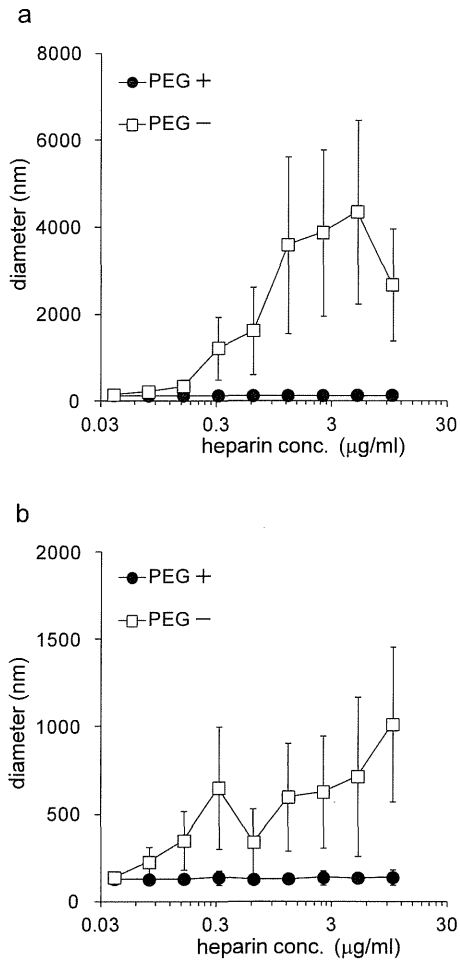

Fig. 5. The Effect of Varying Heparin Concentrations on the Particle Size of Liposomes (PEG +: CF-SL, PEG −: CF-L) in the Presence of $CaCl_2$ (10 mM) (a) or $MgSO_4$ (10 mM) (b)

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).

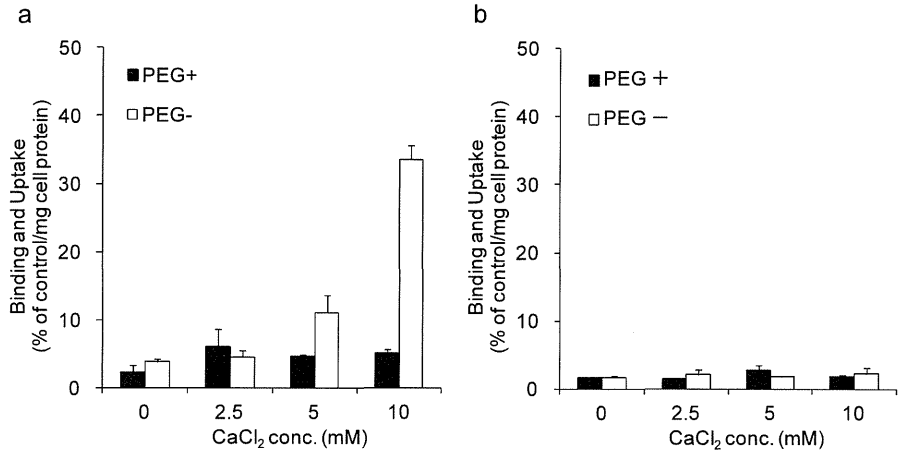

Fig. 6. Uptake and Binding Rate of Liposomes (PEG +: CF-SL, PEG −: CF-L) by Macrophages in the Presence of Ca^{2+} and Heparin (a) or in the Absent of Heparin (b)

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

北山裕貴[1)]・新村　航[1)]・四方田千佳子[2)]・斎藤博幸[1)]*

1）徳島大学大学院ヘルスバイオサイエンス研究部　製剤設計薬学分野
〒770-8505　徳島市庄町1-78-1
2）国立医薬品食品衛生研究所　薬品部
〒158-8501　東京都世田谷区上用賀1-18-1

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

Hiroki Kitayama[1)], Wataru Shinmura[1)], Chikako Yomota[2)], and Hiroyuki Saito[1)]*

1）Institute of Health Biosciences and Graduate School of Pharmaceutical Sciences, The University of Tokushima
1-78-1, Shomachi, Tokushima 770-8505, Japan
2）National Institute of Health Sciences
1-18-1, Kamiyoga, Setagaya–ku, Tokyo 158-8501, Japan

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

* Corresponding author:
Tel: +81-88-633-7267
Fax: +81-88-633-9510
E-mail: hsaito@tokushima-u.ac.jp

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