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Determination of incorporated amounts of poly(ethylene glycol)-derivatized lipids in liposomes for the physicochemical characterization of stealth liposomes

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Abstract

We describe a method for determining incorporated amounts of poly(ethylene glycol) (PEG)-derivatized lipids in liposomes for the physicochemical characterization of PEG-coated liposomes. This method is based on the spectrophotometric determination of complexes of polyethers with sodium ions after their extraction as picrates into 1,2-dichloroethane, developed by Favretto for measuring levels of polyoxyethylene alkylphenyl-ether non-ionic surfactants in waste water. The same assay was applied to the estimation of PEG-derivatized lipids in liposomes and percent incorporation of PEG-derivatized lipids into liposomes was successfully determined. To prevent the interference from liposomal lipids other than PEG-derivatized lipids in this assay, liposomal samples were diluted at least to a concentration of less than 0.2 mM. The percent incorporation of PEG-lipids varied, depending on the molecular weight of PEG and anchor acyl chain length in PEG-lipids and it was suggested that the percent incorporation of PEG-lipids into liposomes would be a good parameter of quality control of PEG-liposomes in manufacturing facility and the picrate method used in the present study allows for the determination of this parameter without the need for hazardous radioisotopes.

Keywords: Liposomes; Poly(ethylene glycol); Physicochemical characterization

1. Introduction

Liposomes prepared with various lipid compositions or by different methods show great variation in physical and chemical characteristics such as particle size, size distribution, surface electric

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potential, colloid stability, the number of lamella, membrane permeability, uniformity of incorporated cholesterol which affects encapsulation efficacy of drugs and so on. When liposomes are supplied for animal experiments or clinical trials, it is very important to specify such characteristics, to ensure the liability and reproducibility of animal or human data. Poly(ethylene glycol) (PEG)-derivatized lipids are widely used as lipid components to prolong the plasma residence time of liposomes (Klibanov et al., 1990; Blume and Cevc, 1990; Lasic et al., 1991; Senior et al., 1991; Allen et al., 1991). With respect to the extended half-lives in the blood of PEG-coated liposomes, several hypotheses have been proposed (Lasic et al., 1991; Needham et al., 1992; Gabizon and Papahadjopoulos, 1988). In our laboratory, fixed aqueous layer thickness (FALT) around liposomes was determined from zeta potential measurements and PEG coating of liposomes was shown to cause an increase in FALT (Shimada et al., 1995). FALT is very important for the specification of PEG-coated liposomes, because the FALT around liposomes is shown to correlate with their biodistribution (Sadzuka and Hirota, 1997). Although percent incorporation of PEG-lipids into liposomes seems another important parameter, analytical methods useful for industrial quality control of PEG-liposomes have not been established. In this study, we aimed to modify the picrate method developed by Favretto et al. for the determination of polyoxyethylene alkylphenylether non-ionic surfactants in waste water (Favretto and Tunis, 1976). This method is based on the spectrophotometric determination of complexes of polyethers with sodium ions after their extraction as picrates, into 1,2-dichloroethane, and provided a sensitive tool for determination of PEG-lipids with PEG chains of 22, 44 and 112 oxyethylene residues and diacylglyceride anchors (PEG-DAGs). Percent incorporation of PEG-DAGs into liposomes depended on the molecular weight of the PEG and anchor acyl chains in PEG-DAGs. As it is possible to determine the percent incorporation without using hazardous radioisotopes, this technique would be useful in the quality control of PEG-liposomes in a manufacturing plant.

2. Materials and methods

2.1. Materials

Sodium nitrate and picric acid were purchased from Kanto kagaku (Tokyo, Japan). Distearoylphosphatldylcholine (DSPC), distearoylphosphatidylglycerol (DSPG), poly(ethylene glycol)-distearoylphosphatidylethanolamine with a PEG chain of 44 oxyethylene residues (PEG2000-DSPE) and monomethoxypoly(ethylene glycol)-glycerylether with PEG chains of 22, 44 and 112 oxyethylene residues (PEG-glycerylether) were generous gifts from NOF Corporation (Tokyo, Japan). Polycarbonate membrane filters were purchased from Nuclepore (California, USA). Picric acid was dried under reduced pressure over phosphorus (v) oxide for at least 3 h in a desiccator before use. All other chemicals were commercial products of reagent grade and used without further purification.

2.2. Methods

2.2.1. Synthesis of PEG-lipids

To a stirred solution of PEG-glycerylether, triethylamine (TEA) and 4-dimethylaminopyridine (DMAP) in chloroform, a solution of fatty acid anhydride (FAA) (myristic anhydride, stearic anhydride, behenoic anhydride) was added dropwise and the mixture was heated at 50–60°C under nitrogen overnight (PEG-glycerylether:TEA:DMAP:FAA = 1:3:0.5:3, mol/mol). The solvent was evaporated to dryness from the reaction mixture, and the residue was purified using a column of silica gel and eluted with chloroform-methanol (20:1 v/v). The spots on the silica gel chromatography plates were visualized with either iodine or phosphomolybdic acid. The final products were identified by both TLC and ^{1}H-NMR. The PEG-lipids synthesized above are the PEG-derivatives with diacylglyceride anchors (DAG) and referred to as PEG-DAGs. The PEG-derivatives with a dimyristoyl-glyceride, a distearoylglyceride, or a dibehenoylglyceride anchor are referred to as PEG-DMG, PEG-DSG and PEG-DBG, respectively. PEG chains of 22, 44

and 112 oxyethylene residues, are referred to as PEG1000, PEG2000 and PEG5000, respectively.

2.2.2. Preparation of sodium nitrate-picrate solution

To a solution containing 0.1 mol sodium hydroxide were added 280 g of sodium nitrate (3.3 mol) and 4.58 g of picric acid (0.02 mol) and the final volume of the solution was adjusted to 1000 ml. After sonication, the solution was filtered off and stored in a refrigerator at 5°C.

2.2.3. Preparation of standard solution of PEG-DAGs

Aqueous solutions of 50 μg PEG-DAGs per milliliter were prepared. The stock solutions were made by serial dilution and used for the preparation of calibration curves. The procedure for determining PEG-DAGs incorporation is described in Section 2.2.6.

2.2.4. Preparation of PEG-DAG incorporating liposomes

PEG-DAG incorporation into liposomes was carried out by two different methods (method A and method B). Briefly, liposome preparation by method A was as follows: DSPC (100 μmol), cholesterol (100 μmol), DSPG (60 μmol) and PEG-DAG (15 μmol) were dissolved in chloroforin–methanol (4:1, v/v), the solvent was evaporated under a stream of nitrogen gas and the resulting films were stored under vacuum for at least 1 h. The thin lipid films were hydrated with 2 ml of 10 mM Tris–HCl buffer (pH 7.4) containing 0.3 M sucrose at 60°C. To make small liposomes, the obtained multilamellar liposomes were sonicated in a bath-type sonicator for 10 min at 60°C after bubbling with nitrogen. To ensure homogeneity in size, the liposomes were extruded through two stacked polycarbonate membrane filters of 0.2 and 0.1 μm pore size, twice and three times, respectively, using a high-pressure extruder heated to 60°C. After phospholipid phosphorus assay (Ishibashi and Tabushi, 1959), the liposomes were diluted to a total lipid concentration of 10 mM with 10 mM Tris–HCl buffer (pH 7.4) containing 150 mM sodium chloride. Liposome preparation by method B was as follows: plain liposomes composed of DSPC (100 μmol), cholesterol (100 μmol) and DSPG (60 μmol) were prepared by the same procedure as previously described. PEG-DAG incorporation was conducted by incubating equal volume of the plain liposome suspension (20 mM) and a concentrated PEG-DAG micelle solution (theoretically 5.45 mol.% to total lipids) at 65°C for 1 h.

2.2.5. Separation of PEG-DAGs in the outer aqueous phase from PEG-DAG incorporating liposomes

To separate PEG-DAGs which were not incorporated into liposome membranes, 0.5 ml of the liposome suspension was centrifuged at 30 000 # *g* for 2 h. Both samples (the supernatant and the centrifugal pellet) were evaporated to dryness and adjusted to an appropriate concentration for precise determination of PEG-DAGs, by adding distilled water. It was thought that 30 000 # *g* would be large enough to make liposomes pellets, because the specific gravitation gradient between the inside and the outside of liposomes was provided; the specific gravitation of 0.3 M sucrose solution inside the liposomes is larger than that of 0.15 M NaCl solution outside the liposomes.

2.2.6. Determination of PEG-DAGs by the picrate method

In the present study, the following experimental conditions were adopted; organic to aqueous phase ratio 5:15 (v/v), concentrations of the reagents in the aqueous phase, sodium nitrate 3.33 M, picric acid 0.02 M and sodium hydroxide 0.1 M. The experimental procedure was as follows: 5 ml of sample solution was mixed with 10 ml of sodium nitrate–picrate solution and to the mixture was added 5 ml of 1, 2 dichloroethane. After vigorous shaking, the solution was centrifuged at 1500 # *g* for 10 min and the organic layer was collected and spectrophotometrically measured at 378 nm. From the calibration curve of standard PEG-lipid solution, the amount of PEG-DAGs in the sample solution was calculated.

3. Results

3.1. Synthesis of PEG-DAGs

The structures and procedure for the synthesis of PEG-DAGs are given in Fig. 1. ^{1}H-NMR data are given for PEG2000-DSG as an example of PEG-DAG identification. ^{1}H-NMR ($CDCl_3$) δ: 0. 88 (6H, t, $J = 6.4$ Hz, $CH_3(CH_2)_{16}$), 1.25 (56H, brs, $CH_3(CH_2)_{14}CH_2CH_2$), 1.56–1.66 (4H, m, $CH_3(CH_2)_{14}CH_2CH_2$), 2.30 (2H, t, $J = 6.5$ Hz, $CH_3(CH_2)_{14}CH_2CH_2$), 2.31 (2H, t, $J = 6.5$ Hz, $CH_3(CH_2)_{14}CH_2CH_2$), 3.36 (3H, s, OCH_3), 3.56–3.69 (m, oxyethylene H), 4.15 (1H, dd, $J = 11.7$, 6.3 Hz, glycerol H), 4.34 (1H, dd, $J = 11.7$, 3.4 Hz, glycerol H), 5.21–5.31 (1H, m, CH_2CHCH_2).

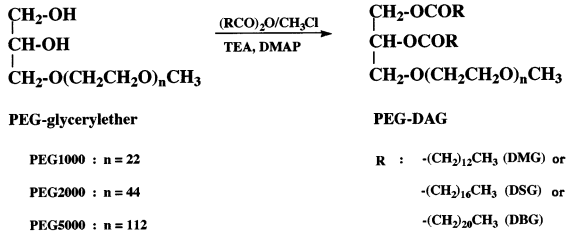

Fig. 1. Procedure for the synthesis of PEG-DAGs PEG-DAGs were synthesized according to the scheme. See text for details.

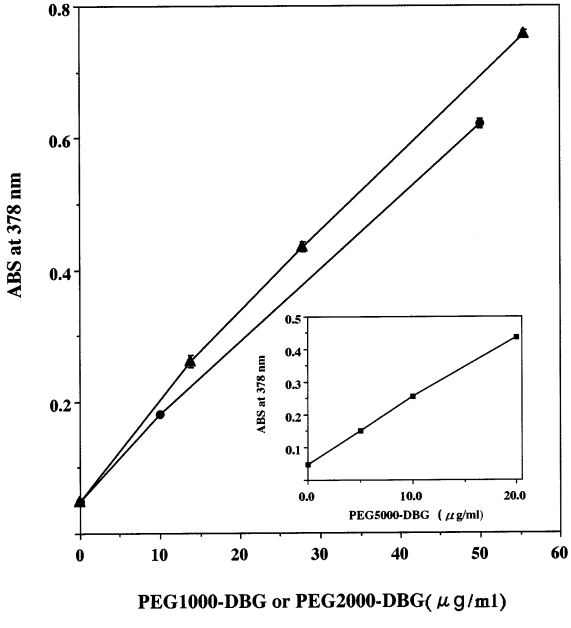

Fig. 2. Calibration curves of (●) PEG1000-DBG (▲) PEG2000-DBG (■) PEG5000-DBG. Each point in the calibration graph represents the mean values ± SD ($n \geq 3$).

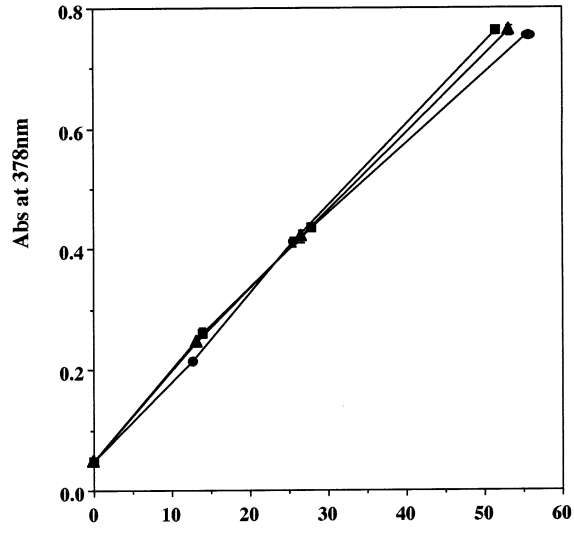

Fig. 3. Calibration curves of (●) PEG2000-DMG (▲) PEG2000-DSG (■) PEG2000-DBG Each point in the calibration graph represents the mean values ± SD ($n \geq 3$).

3.2. Calibration curves for PEG-DAGs

Fig. 2 shows the calibration curves for PEG1000-DBG, PEG2000-DBG and PEG5000-DBG obtained with the picrate method. When the concentration of PEG-DBGs was kept constant, a linear increase in the absorbance at 378 nm was observed with an increase in molecular weight of PEG in PEG-DBGs. Fig. 3 shows the calibration curves for PEG2000-DMG, PEG2000-DSG and PEG2000-DBG obtained with the same method. There was little dependency of acyl chain length in PEG2000-DAGs on the sensitivities to this reagent. Calibration curves showed a linear relationship between the absorbance at 378 nm and the concentration of PEG-DAGs in the range of 0–20 (or 0–50) μg/ml.

3.3. Interference by phospholipids

Table 1 shows the effect of liposomal lipid concentrations on the determination of PEG2000-DMG. A decrease in absorbance at 378 nm was observed only when PEG2000-DMG coexisted with more than 1.5 mM liposomal lipids. It might be that phospholipids co-existing with PEG-DAGs prevent the partition of the complexes formed between PEG and sodium ion in the organic phase. The liposomal samples containing PEG-DAGs used in the present study were adjusted to a concentration of less than 0.2 mM (lipid concentration) and therefore there was thought to be no interference by phospholipids.

3.4. Data of total recovery

For the liposomes composed of DSPC/cholesterol/DSPG/PEG2000-DSG (100:100:60:15, mol/mol) prepared by method B, the precision of the determination was tested by repetitive measurements of PEG2000-DSG contents by the picrate method. The amounts of PEG2000-DSG both in the supernatant and the centrifugal pellet was determined as described in the text (Sections 2.2.5 and 2.2.6) and the total recovery of PEG2000-DSG was calculated, assuming that the theoretical amount of PEG2000-DSG added is 1.33 mg. As shown in Table 2, the total recovery of PEG2000-DSG was almost complete.

Table 1
Effect of liposomal lipid concentrations on the determination of PEG2000-DMG

Lipid concentration (mM)	Absorbance (378 nM)	S.D.
0.01	0.310	0.006
0.04	0.312	0.005
0.08	0.304	0.006
0.20	0.301	0.010
0.50	0.303	0.009
1.50	0.296	0.007
2.00	0.287	0.004
4.00	0.268	0.003
8.00	0.213	0.004

Table 2
Results of the analysis of PEG2000-DSG-containing liposomes[a]

	PE02000-DSG content (mean ± S.D.) (mg)	Recovery (%) (mean ± S.D.)
Pellet	1.07 ± 0.015	103.4 ± 3.6
Supernatant	0.31 ± 0.056	

[a] Liposomes composed of DSPC/cholesterol/DSPG/PEG2000-DSG (100: 100:60: 15, mol/mol) were prepared as described in the text. Such liposomes were centrifuged at 30 000 # *g* for 2 h to obtain both the supernatant and the centrifugal pellet. The amount of PEG2000-DSG both in the supernatant and the pellet was determined as described in the text and the recovery of PEG 2000-DSG was calculated ($n = 3$) assuming that the theoretical amount of PEG2000-DSG added is 1.33 mg.

3.5. Determination of PEG-DAGs in either form, free or incorporated into liposomes

Figs. 4 and 5 shows the effect of acyl chain length in PEG2000-DAGs on the incorporation of PEG2000-DAGs or PEG2000-DSPE into liposomes. The longer the acyl chain length, the less PEG2000-DAG incorporated into liposomes. The same tendency was seen for liposomes prepared both by method A and method B. A striking difference was observed between the dimyristoylglyceride and distearoylglyceride (or dibehenoylglyceride) anchor. In the case of method A, for PEG2000-DMG, approximately 80% of the amount initially added was incorporated into liposomes as opposed to 57% for PEG2000-DSG. Fig. 6 shows the effect of PEG chain length in PEG-DBGs on their incorporation into liposomes. The longer the PEG chain length, the less PEG-DBG incorporated into the liposomes.

4. Discussion

Long-term stabilities of liposomal pharmaceuticals up to 2 years at room temperature should be guaranteed when they are on the market (Lasic, 1993). Stabilities include chemical and colloidal stability. The former is the stability of liposome constituents such as phospholipids, cholesterol and drug molecules. The latter includes the aggre-

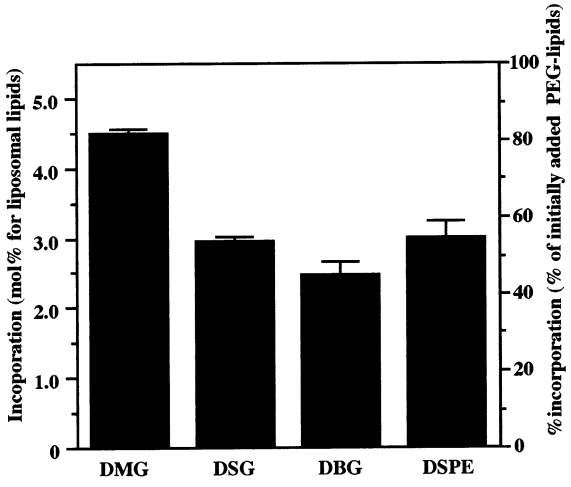

Fig. 4. Effect of acyl chain length on PEG2000-DAGs (PEG2000-DMG, PEG2000-DSG and PEG2000-DBG) incorporation into liposome membranes (method A). Liposomes composed of DSPC/cholesterol/DSPG (100:100:60, mol/mol) and additionally 5.45 mol.% PEG2000-DAGs with various acyl chains were extruded through two stacked polycarbonate membrane filters of 0.2 and 0. 1 μm pore size, twice and three times, respectively, using a high-pressure extruder heated to 60°C. After phospholipid phosphorus assay, the liposomes were diluted to a total lipid concentration of 10 mM with 10 mM Tris–HCl buffer (pH 7.4) containing 150 mM sodium chloride. Such liposome samples were centrifuged at 30 000 # *g* for 2 h to obtain both the supernatant and the centrifugal pellet. The amount of each PEG2000-DAGs in both samples (the supernatant and the centrifugal pellet) was determined as described in the text. Based on these data, the incorporation of PEG2000-DBGs in the liposome membranes was determined and expressed as the percent incorporation. Data represent the mean values ± SD ($n \geq 3$).

gation of liposomes, permeability change of the liposomal membrane and so on.

Recently, PEG-coating of liposome surfaces has been applied to drugs to prolong the circulation times of liposomes, in vivo (Unezaki et al., 1998; Moribe et al., 1998; Webb et al., 1998). Obviously, it is necessary to characterize PEG-coated liposomes as well as conventional liposomes.

At present phospholipids can be determined by many useful methods (Ishibashi and Tabushi, 1959; Stewart, 1980; Grit et al., 1991), but few methods are available for the determination of PEG-lipids (Parr et al., 1994; Uster et al., 1996; Nag et al., 1997). Bio-Rad protein assay, based on the Bradford method (Bradford, 1976), has been used for the determination of PEG-lipids in some reports (Allen et al., 1991; Ishiwata et al., 1995). However, the reagent reacted with the phospholipids and the complex with the dye was not stable (data not shown). The HPLC system may also be a convenient method for determining the amount of PEG-lipids as well as phospholipids. However, expensive apparatus would be needed to use the HPLC method.

To search for a simple analytical method of PEG-lipids without expensive apparatus and hazardous radioisotopes, determination of PEG-lipids was carried out by the picrate method employed by Favretto et al. with minor modification. The underlying mechanism of the picrate

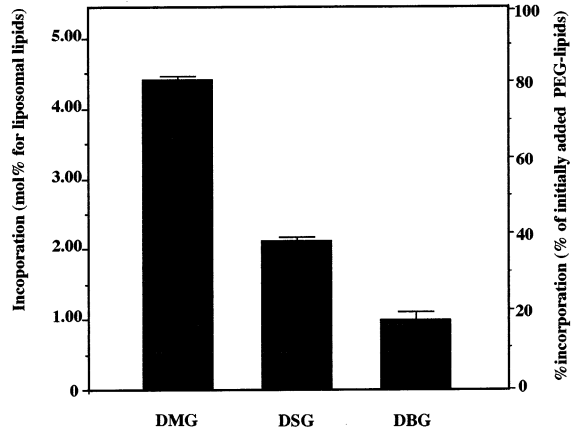

Fig. 5. Effect of acyl chain length on PEG2000-DAGs (PEG2000-DMG, PEG2000-DSG and PEG2000-DBG) incorporation into liposome membranes (method B). Liposomes composed of DSPC/cholesterol/DSPG (100:100:60, mol/mol) were extruded through two stacked polycarbonate membrane filters of 0.2 and 0.1 μm pore size, twice and three times, respectively, using a high-pressure extruder heated to 60°C. After phospholipid phosphorus assay, the liposomes were diluted to a total lipid concentration of 20 mM with 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM sodium chloride. PEG-DAG incorporation was conducted by incubating equal volume of the liposome suspension (20 mM) and a concentrated PEG-DAG micelle solution (theoretically 5.45 mol.% to total lipids) at 65°C for 1 h. Such liposome samples were centrifuged at 30 000 # *g* for 2 h to obtain both the supernatant and the centrifugal pellet. The amount of each PEG2000-DAGs in both samples (the supernatant and the centrifugal pellet) was determined as described in the text. Based on these data, the incorporation of PEG2000-DBGs in the liposome membranes was determined and expressed as the percent incorporation. Data represent the mean values ± SD ($n \geq 3$).

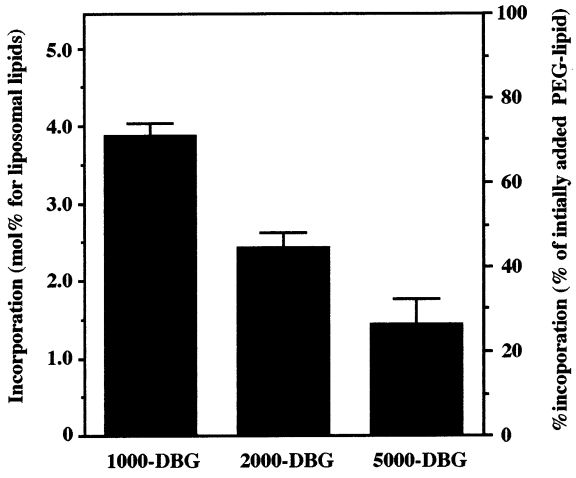

Fig. 6. Effect of PEG chain length on PEG-DBGs (PEG1000-DBG, PEG2000-DBG and PEG5000-DBG) incorporation into liposome membranes. Liposomes composed of DSPC/cholesterol/DSPG (100:100:60, mol/mol) and additionally 5.45 mol.% PEG-DBGs with various PEG chains were extruded through two stacked polycarbonate membrane filters of 0.2 and 0.1 μm pore size, twice and three times, respectively, using a high-pressure extruder heated to 60°C. After phospholipid phosphorus assay, the liposomes were diluted to a total lipid concentration of 10 mM with 10 mM Tris–HCl buffer (pH 7.4) containing 150 mM sodium chloride. Such liposome samples were centrifuged at 30 000 # *g* for 2 h to obtain both the supernatant and the centrifugal pellet. The amount of PEG-DBGs in both samples (the supernatant and the centrifugal pellet) was determined as described in the text. Based on these data, the incorporation of PEG-DBGs in the liposome membranes was determined and expressed as the percent incorporation. Data represent the mean values ± SD ($n \geq 3$).

method involves co-ordination reactions between polyoxyethylene chains and sodium ions, followed by the extraction of the complexes in the form of an ion-pair with picrate anion into an organic phase. Calibration curves for various PEG-DAGs were prepared by mixing sample solutions containing increasing concentrations of PEG-DAGs (5 ml) with sodium nitrate–picrate reagent (10 ml) and 1,2-dichloroethane (5 ml) and measuring the optical density of the organic phase (1,2-dichloroethane) at 378 nm. The complex of PEG-DAGs with picrate was stable (data not shown) and all calibration curves for PEG-DAGs obtained in the present study showed a linear relationship between the absorbance at 378 nm and the concentration of PEG-DAGs in the range of 0–20 (or 0–50) μg/ml. However, at higher concentrations of PEG-DAGs, the curves tended to deviate from the linearity. This may be due to the fact that the picric acid reagent (sodium nitrate; 3.33 M picric acid; 0.02 M used is not sufficient to react with increasing PEG-DAGs and PEG-DAGs of high molecular weight of PEG. Therefore, it was necessary to adjust the concentrations of PEG-DAGs to within 20–50 μg/ml so as to perform precise measurements. With increasing concentrations of liposomal lipids, co-existing with PEG2000-DMG, the absorbance at 378 nm in the organic phase showed a gradual decrease (Table 1). This may be overcome by diluting the liposomal samples. The liposomal samples containing PEG-DAGs used in the present study for estimation of PEG-lipids were adjusted to less than 0.2 mM (liposomal lipids) and therefore there was thought to be no interference by phospholipids.

To characterize PEG-DAG containing liposomes, percent incorporation of PEG-DAGs into liposomes composed of DSPC, cholesterol, DSPG and PEG-DAG (100:100:60:15, mol/mol) was determined using the picrate method. The reason rigid membranes of DSPC/CH/DSPG were chosen as a lipid composition of liposomes for specification is that biodistribution of liposomes of the same composition has been examined in mice. The data of such animal experiments will be published elsewhere.

To confirm the precision of the determination, we first performed the repetitive measurements of the PEG-liposomes composed of DSPC/cholesterol/DSPG/PEG2000-DSG (100:100:60:15, mol/mol) prepared by method B. In consequence, the total recovery of PEG2000-DSG from both the centrifugal pellet and the supernatant was found to be almost complete (Table 2), suggesting that this is a useful method in easily determining incorporated amounts of PEG-derivatized lipids in liposomes instead of HPLC system. Next, we compared the percent incorporation data of DSPC/cholesterol/DSPG/PEG2000-DSG (100:100:60:15, mol/mol) prepared by method B with those of the literature using the HPLC to determine the PEG-lipids concentration (Uster et al., 1996), because the lipid composition is different

but similar in that the lipid used is rigid. Besides, the methods for inserting the PEG-lipids are similar. In the literature, the plain liposomes composed of hydrogenated soy 1,2-diacyl-*sn*-glycerol-3-phosphoeholine (HSPC)/cholesterol/1, 2-dipalmitoyl-*sn*-glycerol-3-phosphoglycerol sodium salt (DPPG)/*N*-diethylenetriaminepentaacetic acid-1, 2-distearoyl-*sn*-glycero-3-phosphoethanolamine-(DTPA-DSPE)(52.4/45.4/2.0/0.2, mol/mol) are prepared by the conventional hydration method and then mixed with a concentrated micellar PEG_{1900}-DSPE(*N*-carbamoyl-methoxypoly(ethyleneglycol)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, sodium salt, 1900 Da methoxy poly(ethylene glycol) fraction) dispersion (3 mol.% to total lipid) to the desired temperature. Uster et al. have shown that insertion of PEG_{1900}-DSPE into liposomes reached the expected theoretical maximum (3 mol.%) after co-incubation of liposomes and micelles at 60°C for 1 h. On the other hand, PEG2000-DSG, which has the same hydrocarbon chains as PEG_{1900}-DSPE was incorporated with approximately 2 mol.% insertion after co-incubation of liposomes and PEG2000-DSG micelles at 65°C for 1 h. However, the insertion proceeded with approximately 3 mol.% after 3 h at 65°C (data not shown). The rate of insertion was slower for PEG2000-DSG than for PEG_{1900}-DSPE, however, the percent incorporation of PEG-lipids into liposomes reached 3 mol.% for both PEG_{1900}-DSPE and PEG2000-DSG. These results also indicate that our data of the percent incorporation will be reasonable and then the picrate method will be reliable as an analytical means of PEG-lipids.

When the polar head group was a PEG chain of 44 oxyethylene residues (PEG2000), the ranking in terms of percent incorporation was DMG > DSG > DBG, indicating that acyl chain length in PEG-DAGs is an important factor in their incorporation into liposomes. Apparently, the longer the acyl chain length, the less PEG-DAG is incorporated. For this reason, PEG2000-DSPE was thought to be incorporated to a similar extent as PEG2000-DSG. The same tendency was seen for liposomes prepared both by method A and method B. However, it is surprising that approximately 80% of PEG2000-DMG was incorporated into pre-formed liposomes in spite of the fact that incorporation of PEG2000-DMG was only on the exterior surface of the liposomes (Fig. 5). The asymmetric insertion of PEG-lipids might be thought to disrupt the membrane integrity. Therefore, the further in vitro stability test of such liposomes should be necessary. When the acyl chain was a dibehenoylglyceride anchor, in terms of percent incorporation, the PEG-DBGs ranked in the order of PEG1000 > PEG2000 > PEG5000. This result suggests that PEG-DBGs are less likely to be incorporated into liposomes as the molecular weight of the PEG increases. It was speculated that a large PEG, once incorporated into liposomes, would interfere with the approach of other PEG-DAGs and decrease the percent incorporation. The PEG-DAGs used in the present study have diacylglyceride anchors with bulky hydrophilic polar heads (PEG) and hence tend to form micelles in aqueous media. It is also possible that PEG-DAGs with high molecular weight PEGs are not incorporated into liposomes because of their high tendency to form micelles. However, this is not very likely because the PEG-DAGs used in the present study have similar critical micelle concentrations (data not shown). The results presented herein indicate that not all the PEG-lipids formulated in lipid mixtures are always incorporated into liposomes: the percent incorporation varies depending on the molecular weight of the PEG and anchor acyl chains in the PEG-DAGs. To our knowledge, no attempt has been made to characterize the percent incorporation of PEG-lipids with PEG of various molecular weights and different diacylglyceride anchors into liposomes in a systematic manner.

In conclusion, percent incorporation of PEG-DAGs into liposomes is suggested to influence the density of PEG on the liposome surfaces and hence affect the biodistribution of such liposomes. Therefore, it is concluded that the percent incorporation of PEG-lipids into liposomes is a good parameter of quality control of PEG-liposomes in a manufacturing plant and the picrate method used in this study allows for the determination without the use of hazardous radioisotopes.

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