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Preparation and properties of branched oligoglycerol modifiers for stabilization of liposomes

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ABSTRACT

We examined the effect on drug delivery of liposomes with surfaces that were modified with branched oligoglycerols (BGLs) and explored possible formulation advantages to increase drug exposure. BGL012 is a branched oligoglycerol derivative with a cascade-like structure of 12 glycerol units, characterized as a widely spread structure in aqueous solution. We prepared BGL-phospholipid derivatives (BGL-PEs), including BGL012, by coupling 1,2-distearoylphosphatidylethanolamine to BGLs. BGL012-PE modification of the liposomes (BGL012L) achieved a long circulation time after intravenous injection in rats. The circulation lifetime of BGL012L was almost the same as that of polyethylene glycol (PEG)-modified liposomes. The surface of BGL012L induced the formation of a fixed aqueous layer and reduced protein adsorption on the liposome surface, without strong interference with the binding reaction on the liposome. Thus, the newly synthesized branched oligoglycerol derivatives are considered to have useful hydrophilic and physical properties for modifying the liposome surface to increase drug exposure.

1. Introduction

Liposomes are small spherical particles consisting of one or more lipid bilayers with aqueous interiors (Bangham and Horne, 1964). Naturally occurring lipids have been commonly used to construct biocompatible liposomes, and such carriers have been applied to drug delivery systems to augment therapeutic efficacy and reduce encapsulated drug toxicity. However, liposomes in the conventional form are not necessarily stable in the body due to unwanted interactions with biological matter such as proteins, cells and tissues. Surface modification has been explored to inhibit biological protein adsorption on the surface of conventional liposomes, as they tend to interfere with properties such as stability in circulation, biodistribution, and targeting efficacy (Woodle and Lasic, 1992; Woodle, 1993). The most common polymer used to modify liposome surface is polyethylene glycol (PEG) (Zalipsky, 1995; Klibanov et al., 1990; Mori et al., 1991; Papahadjopoulos et al., 1991; Allen, 1992), which significantly increases stability in circulation. Doxil® is a marketed liposomal product, to which PEG modification (PEGylation) was applied for the first time, and has an impressive record of achievement (Allen, 1994; Gabizon et al., 1994).

Many other surface modification polymers have been reported to coat the surface of liposomes for the purpose of improving the stability and thus prolonging the time in blood circulation. Polyvinylpyrrolidone (Torchillin et al., 1994), polyacrylamide (Kono et al., 1999), polyglycerols (Maruyama et al., 1994), polyamino acid-based biodegradable polymers (Metselaar et al., 2003) and methyl and ethyl polyoxazolines (Woodle et al., 1994) have been investigated to date. Interestingly, while reports of these polymers have shown some ability to provide prolonged circulation, none proven to be equal to PEG have been identified. Regarding the molecular structure of these polymers, linear types of polymers were used for treating the liposome surfaces in each of these studies.

Nemoto et al. (1992, 1995, 1999) reported the branched structure of polyglycerol polymers. Novel branched oligoglycerols (BGLs) were originally developed to solubilize hydrophobic molecules via covalent modification of multiple glycerol units,

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since water solubility is a common requirement of medicinal compounds. Some BGL derivatives were designed to have functional groups also capable of protein modification. Since the structure of BGLs were different from those of PEG or linear type polymers, the properties of modified proteins exerted biological activity without significant loss of function (Nemoto et al., 2007; Yamaguchi et al., 2008).

The aim of this study was to synthesize BGL-lipid derivatives to modify liposomes and investigate the liposomal behavior of these liposomes. The BGL-modified liposomes (BGLLs) were characterized in terms of their physicochemical properties. In addition, we investigated the pharmacokinetic profiles of the BGLL *in vivo*.

2. Materials and methods

2.1. Materials

L-α-Phosphatidylcholine (soy-hydrogenated) (HSPC) and *N*-[carbamoyl-(polyethylene glycol) methyl ether]-1,2-distearoyl-sn-glycerol-3-phosphoethanolamine (PEG-PE) were purchased from NOF corporation (Tokyo, Japan). PEG 2000 (average molecular weight 2000) was used for PEG-PE. Biotin-conjugated lipid, *N*-(cap biotinyl)-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine sodium salt (BCPE) was purchased from Avanti Polar Lipids (Alabaster, AL). Synthesis of BGL006-PE, which has six glycerol units, was described previously (Yamaguchi et al., 2008), and BGL012-PE with 12 glycerol units was synthesized according to the procedure described in the supplement to this manuscript.

Doxorubicin (DOX) is product of Kyowa Hakko Kirin Co., Ltd. Streptavidin was obtained from Molecular Probes (Eugene, OR). Bovine serum albumin-fluorescein isothiocyanate conjugate (BSA-FITC) and palladium hydride were from Sigma–Aldrich (St. Louis, MO). All other materials were of analytical grade and used without further purification.

2.2. Equipment

A TOA-T26 pH meter (DKK-TOA Corp., Tokyo, Japan) was used for all pH measurements. UV–vis analysis was performed using a UV-3310 spectrophotometer (Hitachi, Ltd., Tokyo, Japan) and fluorescence analysis was performed on an F-4500 fluorescence spectrometer (Hitachi, Ltd., Tokyo, Japan). Differential scanning calorimetry (DSC) was conducted using a DSC Q1000 calorimeter (TA Instruments, Newcastle, DE). Zeta potential and particle size analysis were performed on a Zetasizer Nano series Nano ZS (Malvern Instrument, Malvern, UK).

2.3. Animals

Randomly bred Crl: CD (SD) rats were obtained from Charles River Laboratories, Inc. (Yokohama, Japan). Six-week-old male rats weighing 150–180 g were used for the evaluation of pharmacokinetics. The rats had free access to water and feed throughout the study period. This study was reviewed and approved by the Institutional Animal Care and Use Ethics Committee of Kyowa Hakko Kirin Co., Ltd. prior to study.

2.4. Preparation of polymer-modified liposomes

HSPC liposomes were prepared by hydrating the lipid in 100 mM citrate buffer (pH 4.0). HSPC in the buffer was extruded through Nuclepore® polycarbonate membrane filters (Whatman, Brentford, UK) with pore sizes of 0.4 μm (four times) and 0.1 μm (10 times) successively at 70 °C. The resulting mixture was diluted with 100 mM citrate buffer (pH 4.0) to give a liposome solution with a lipid concentration of approximately 62.5 mg/mL.

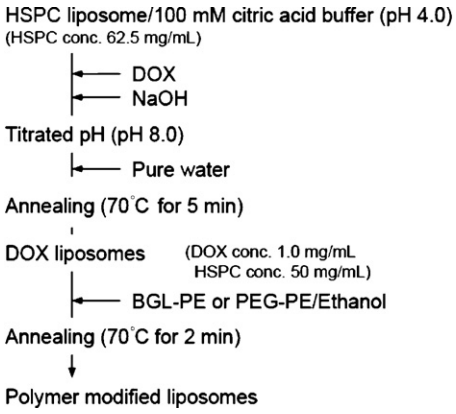

Fig. 1. Schematic diagram for preparation of polymer-modified liposomes.

DOX incorporation into liposomes was achieved by a conventional pH gradient method. Efficiency of DOX encapsulation is dependent on the drug-to-lipid ratio of the incubation mixture. Based on results of optimization experiments, a DOX-to-lipid weight ratio of 0.02 was chosen for liposome preparation. Exterior pH of the liposomal preparation was then titrated to approximately 8.0 with 1.0 mol/L NaOH, to create a pH gradient (acidic inside) across the liposomal membrane. Pure water was then added to adjust DOX concentration to 1.0 mg/mL and the mixture was heated at 70 °C for 5 min to encapsulate DOX into the liposomes. A heat-treated solution of PEG-PE was added to the DOX-encapsulating liposomes with a ratio of either 6.7 or 15.0 mol% PEG-PE in the presence of 4.0% (v/v) ethanol. The mixture was heated at 70 °C for 2 min and stored at 5 °C until use (Uster et al., 1996; Yamauchi et al., 2005). BGLLs were prepared following the same procedure as that described for PEG liposomes (Fig. 1).

The amount of DOX incorporated in the liposomes was assessed as follows: the liposomes were diluted 10-fold with water and centrifuged in an ultracentrifuge (Optima™ XL-A, Beckman, Fullerton, CA) for 1 h at 110,000 × *g*. The quantity of encapsulated DOX was determined by measuring UV absorption of the supernatant at 497 nm. After correcting for background effects, absorbance was converted to a DOX encapsulation ratio using the following Eq. (1):

$$\text{Encapsulation ratio}(\%) = \left(\frac{A-B}{A}\right) \times 100 \quad (1)$$

In the formula, *A* is the total (incorporated and unincorporated) amount of DOX in the liposome suspensions after correction of background effect, and was measured by disrupting liposomes with 80% isopropanol solution (10-fold volumes). *B* is the amount of DOX in the supernatant obtained after ultracentrifugation of the liposome suspension (unincorporated DOX).

Concentrations of lipids in liposomal preparations were determined using a phosphorus assay (Wako Pure Chemical Industries, Tokyo, Japan). The mean size of the liposome vesicles was measured by dynamic light scattering (Zetasizer Nano ZS).

2.5. Differential scanning calorimetry

Thermodynamic analysis was performed using a differential scanning calorimeter (DSC). Liposome suspensions were placed onto calorimeter sample pans and thermograms were recorded at a heating rate of 1 °C/min. The transition temperature (T_m) was recorded as a peak of the endothermic transition. Enthalpies were calculated based on the areas under heat capacity peaks.

2.6. Assessment of the thickness of the fixed aqueous layer

Zeta potential (V) of the liposomes was measured using electrophoretic light scattering (Zetasizer Nano ZS). In this study, we calculated the fixed aqueous layer thickness (FALT) using the Gouy–Chapman theory, in which the zeta potential $\psi(L)$ is the electrostatic potential at the position of the slipping plane L (nm) and is expressed as the following Eq. (2):

$$\ln \psi(L) = \ln A - \kappa L \quad (2)$$

In the formula, A is a constant and κ is the Debye–Hückel parameter ($\sqrt{C}/0.3$ for monovalent salts, in which C is the molarity of electrolytes). When zeta potentials are measured at differing salt concentrations (0, 1.25, 5, 10, 20, 30, and 40 mM sodium chloride) and plotted against κ, the slope L gives the position of the slipping plane or thickness of the fixed aqueous layer in nm units (Shimada et al., 1995; Sadzuka et al., 2002, 2006). Using this method, the thickness of the fixed aqueous layer of each liposome was estimated.

2.7. Assessment of serum protein adsorption on liposomes

The amount of serum protein adsorption on the liposome surface was determined by measuring BSA-FITC (model protein) bound to the liposomes using fluorescence spectrophotometry (Han et al., 2006). Briefly, the liposome suspension (1.0 mL, 10 mM lipid concentration) and BSA-FITC solution (0.5 mL, 0.02 mg/mL) were mixed and incubated at 37 °C with gentle stirring. At selected times (1, 12, 24, and 48 h), samples (0.3 mL) were taken and centrifuged at 18,000 × g for 40 min. The liposomal pellet was washed with distilled water and the suspension was re-centrifuged. The washing step was repeated twice to ensure that all free BSA-FITC was eliminated. After washing the liposomal pellet, the liposomal fraction was suspended in distilled water, and the BSA-FITC remaining in the liposome fraction was measured by fluorescence as an indicator of adsorbed protein. The emission and excitation wavelengths used were 510 and 490 nm, respectively.

2.8. Liposome agglutination by streptavidin

Surface binding functionality of the liposomes was evaluated using a liposome agglutination assay (Klibanov et al., 1990; Maruyama et al., 1994), based on the biotin–avidin association that occurs when biotinylated liposomes are mixed with a solution containing streptavidin. The liposomes used in this experiment were prepared from HSPC, BCPE, and various amounts of BGL-PE or PEG-PE. After preparation of DOX liposomes (Fig. 1), BCPE dissolved in 4.0% (v/v) ethanol was added at a final concentration of 2.5 mol% (relative to the concentration of liposomes), and the mixture was heated at 70 °C for 2 min. BGL-PE (6.7 or 15.0 mol%) or PEG-PE (6.7 or 15.0 mol%) was then added to the resulting mixture followed by an annealing step under the same condition to obtain final liposome solution. In a microcuvette, the liposome solution (50 μL, 1.0 mg/mL lipid concentration) was mixed with PBS (0.5 mL) containing streptavidin (5 μg). The increase in turbidity was monitored at 440 nm.

2.9. Pharmacokinetics of doxorubicin encapsulated in BGL-modified liposomes

Liposome suspensions in citrate buffer (100 mM, pH 6–7) were administered to SD rats via the tail vein at a dose equivalent to 1/1000 body weight (ca. 0.35 mg/kg of DOX and 17.5 mg/kg of HSPC). At selected time intervals (5, 20, 60, 180, 240 and 1440 min), blood samples (250 μL) were taken from the tail vein and the plasma was separated by centrifugation (8000 × g, 10 min, 4 °C). Samples (80 μL) of plasma were added to isopropyl alcohol (720 μL), vortexed for 30 s, and then put on ice for 15 min. The mixture was centrifuged (8000 × g, 10 min, 4 °C) and the supernatant was collected. The concentration of DOX in the supernatant was measured by UV absorption at 497 nm. The area under the concentration versus time curve (AUC) was calculated using the trapezoidal rule.

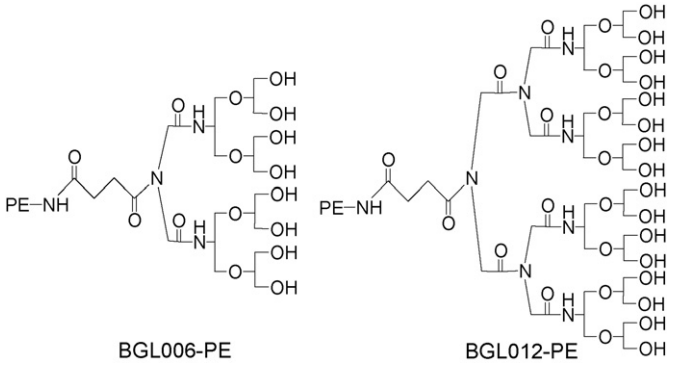

Fig. 2. Characteristic of branched oligoglycerol derivatives.

3. Results

3.1. Characterization of BGL-modified liposomes

The BGLs were coupled to L-α-distearoylphosphatidylethanolamine (DSPE) to obtain novel water-soluble BGL-PE polymers (Fig. 2). BGLLs were then prepared according to the procedure established for PEGylated liposomes (Yamauchi et al., 2005). BGLLs were compared to their PEG-modified counterparts using DOX as a model drug. The results of composition and physicochemical properties are summarized in Table 1. Diameters of the liposomes containing 6.7 and 15.0 mol% of BGL012-PE were determined as 113.9 and 118.4 nm, respectively. These diameters were higher than those of the unmodified liposomes, but similar to those of PEG-modified liposomes (PEGL). The encapsulation efficiency with DOX was higher for BGL012-modified liposomes (BGL012L) (89.3%) than that for PEGL (49.0%) when 15.0 mol% of polymer-lipids was employed for liposome preparation. Zeta potentials were measured for individual liposomes, but no significant changes were observed. The fixed aqueous layer thickness, based on zeta potential obtained using the interfacial electrochemical technique (Shimada et al., 1995), was 2.0 nm for both 6.7 and 15.0 mol% BGL012Ls. This was significantly different from those obtained for PEGL. Phase transition temperature (T_m) and enthalpy were determined for both

Table 1
Physicochemical properties of various polymer-modified DOX-containing liposomes.

Liposomes	Size (nm)	E.E.[a] (%)	Zeta potential[b] (mV)	FALT[c] (nm)
Unmodified HSPC	107.4	99.0	0.3	–[d]
PEG-PE 6.7 mol%/HSPC	116.8	95.1	−40.2	3.9
PEG-PE 15.0 mol%/HSPC	116.0	49.0	−47.2	4.3
BGL012-PE 6.7 mol%/HSPC	113.9	96.5	−52.2	2.0
BGL012-PE 15.0 mol%/HSPC	118.4	89.3	−43.4	2.0

[a] Encapsulation efficiency.
[b] Zeta potential was measured at 0 mol% NaCl concentration.
[c] FALT were calculated from zeta potential data at various NaCl concentration (0, 1.25, 5, 10, 20, 30, 40 mM) and κ.
[d] –: not determined.

Table 2
Transition temperature and enthalpy of various polymer-modified DOX-containing liposomes.

Liposomes	T_m[a] (°C)	Enthalpy[b] (J/g)
Unmodified HSPC	52.2	42.2
PEG-PE 6.7 mol%/HSPC	52.7	32.1
PEG-PE 15.0 mol%/HSPC	53.3	25.6
BGL012-PE 6.7 mol%/HSPC	51.8	40.6
BGL012-PE 15.0 mol%/HSPC	51.5	36.2

[a] T_m; phase transition temperature.
[b] Enthalpy is given in J/g total lipid in the sample.

types of liposomes by DSC measurement (Table 2). HSPC liposomes (unmodified) exhibited a main transition at 52.2 °C with an enthalpy of 42.2 J/g, similar to previous observation (Ogiso et al., 1995). Enthalpy change of BGL012L was significantly different from those of PEGL. The data demonstrated that BGL012L had intermediate enthalpy between unmodified liposomes and PEGL, and the enthalpy change in the BGLL was significantly lower than that in the PEGL.

3.2. Assessment of serum protein adsorption on liposomes

The PEG modification of liposomes prevents interactions with proteins and prolongs circulation in the bloodstream. To assess stability of the liposomes in the blood, FITC-labeled BSA was used as a model of serum protein in the bloodstream. We evaluated the protein adsorption on the liposome surface with time. The results are shown in Fig. 3. In the case of unmodified HSPC liposome, fluorescence intensity increased with time, indicating adsorption of the BSA-FITC on the liposome surface. However, the fluorescence intensities generated from both PEGL and BGL012L were lower and kept constant throughout the incubation period. The adsorption level of BSA was similar between BGL012L and PEGL. This result shows that protein adsorption on the HSPC liposome surface can be suppressed by the BGL012 modification. Moreover, liposomes with 15.0 mol% of BGL012 modification were more efficient in reduction of protein adsorption than with 6.7 mol% of BGL012 modification. Therefore, the adsorption level was likely to be dependent on the degree of modification by BGL012.

3.3. Liposome agglutination by streptavidin

To determine whether BGL012 behaves as a steric barrier on the liposome surface, the inhibition effect of BGL012 on liposome

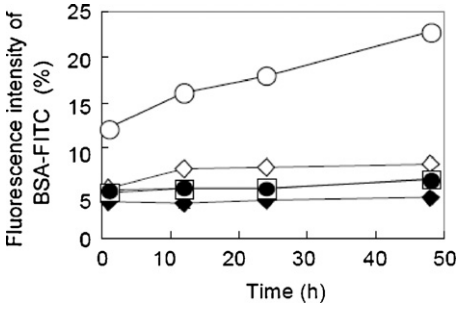

Fig. 3. Change in fluorescence intensity of BSA-FITC adsorbed on the liposome surface. Among the total amount of BSA-FITC in the liposome suspension, fluorescence intensity of the BSA-FITC (%) bound to the liposome surface was measured. Data is shown as mean ± SD ($n = 3$). Unmodified HSPC liposome (control) (○) and modified liposomes containing BGL012-PE 6.7 mol%/HSPC (◊), BGL012-PE 15 mol%/HSPC (♦), PEG-PE 6.7 mol%/HSPC (□) and PEG-PE 15 mol%/HSPC (●) liposomes are plotted. Data was taken at 37 °C in phosphate buffered solution.

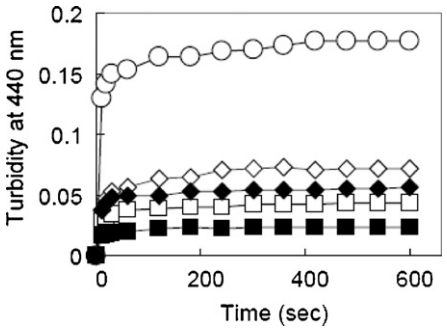

Fig. 4. Change in turbidity at 440 nm when streptavidin was incubated with various liposomes modified with BGL or PEG and biotin simultaneously. Increase in turbidity (absorbance at 440 nm) was periodically measured for unmodified HSPC (control) (○), BGL012-PE 6.7 mol%/HSPC (◊), BGL012-PE 15 mol%/HSPC (♦), PEG-PE 6.7 mol%/HSPC (□) and PEG-PE 15 mol%/HSPC (■) liposomes.

agglutination was measured by liposome agglutination assay. This assay takes advantage of the fact that the agglutination of liposomes containing BCPE is mediated by streptavidin, and thus the increase in turbidity compared to control liposomes directly reflects the degree of binding activity on the liposome surface (target binding effect). An increase in turbidity of the assay mixture was measured using absorbance at 440 nm (Fig. 4). A significant increase in turbidity was observed for unmodified HSPC liposome (control), but the polymer-modified liposomes showed different behaviors reflecting that the steric barrier was generated on the liposome surface. The binding inhibitory effect was dependent on the amount of modified polymer loaded onto the surface of the liposomes. Interestingly, agglutination of BGL012L was about two times higher than that of PEGL under equilibrated conditions. These results suggest the function of BGL012 as a weak steric barrier against liposome agglutination.

3.4. Pharmacokinetics of DOX encapsulated in BGL-PE liposomes

An animal study was performed in rats to investigate the pharmacokinetic profile of BGL012L in comparison with PEGL. DOX was used as a model drug to determine the liposomal concentration in blood. The DOX concentration was determined by fluorescence photometry at predetermined time points. The effects of BGL012 and PEG in prolonging the circulation half-life of the liposomes are shown in Table 3 and Fig. 5. $AUCs_{0-24\,h}$ (24 h concentration–time profiles) of BGL012L and PEGL were similar and 30 times greater than that of unmodified liposomes, respectively. On the other hand, BGL006 (a branched polymer with six glycerol units, Fig. 2)-modified liposomes (BGL006L) were not stable in blood. $AUC_{0-24\,h}$ for BGL006L was 10 times lower as measured from DOX concentration in blood than that for BGL012L (data not shown). Therefore the stability of liposomes was dependent on the number of branch units of BGL.

Table 3
Comparisons of AUC among BGL-PE liposomes.

Liposomes	$AUC_{0-24\,h}$ (μg/mL × h)
Unmodified HSPC	1.8 ± 0.9
BGL012-PE/HSPC 6.7 mol%	53.1 ± 27.6
PEG-PE/HSPC 6.7 mol%	45.7 ± 3.6

AUC was calculated as described in Section 2.9 "*Pharmacokinetics of doxorubicin encapsulated in BGL-modified liposomes*". The data was derived from experiments in three animals.

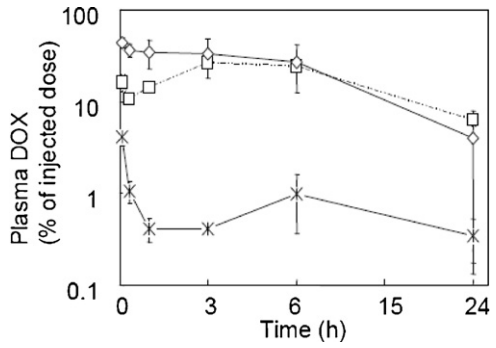

Fig. 5. *In vivo* circulation lifetimes of different polymer-modified liposomes. Unmodified HSPC liposome (control) (*), modified liposomes containing 6.7 mol% BGL012-PE/HSPC liposome (◊) and 6.7 mol% PEG-PE/HSPC liposome (□). Data were plotted as the mean ± standard deviation of three mice.

4. Discussion

PEG is a linear type polymer that exhibits considerable conformational flexibility and hence provides more effective steric barrier property for surface modification of liposomes when compared to rigid polymers. BGL is, on the other hand, likely to spread widely in a transverse direction and cover the liposome surface efficiently as consequence of its cascade-like branched glycerol units. Therefore, we assume that BGL012 may provide unique features to the surface function of liposomes. Here, we have shown that BGL012L has a similar pharmacokinetic profile to that of PEGL in rat (Table 3 and Fig. 5) and has a better encapsulation efficiency of DOX as well as a better accessibility to the ligands (Table 1 and Fig. 4). These results suggest that BGL012 is capable of altering the distribution of liposomes significantly and may have another positive impact on therapeutic efficacy. Thus, the properties of BGL012 may suggest additional advantages over the PEG in liposomal applications. Here we summarize the manner in which BGL012 modifies the structure and properties of HSPC liposomes, and discuss how these assumptions help to explain the mechanism whereby BGL012 increases the blood circulation time of the BGL012L.

First, BGL012L was found to have higher encapsulation efficiency of DOX than that of PEGL. This is one of the major advantages of BGL012L. In the case of PEGL, the breaking of liposomes occurs during the preparation and the DOX leakage gradually occurs from liposomes after complete encapsulation. Nicholas et al. (2000) reported a very dramatic change in the encapsulation efficiency of compounds in proportion to the loading amount of pegylated lipid, suggesting that the addition of pegylated lipid markedly reduced the amount of encapsulated compounds in the liposomes, especially under the condition of 15 mol% PEG-PE content. The high encapsulation efficiency of BGLL can be explained as follows: Calorimetry is used primarily to investigate the structure of lipid bilayers in liposomes. As enthalpy is known as a parameter of the physical stability of lipid bilayers, extent of the enthalpy change of BGLL and PEGL can be attributed to the reduction of encapsulation efficiency. As shown in Table 2, enthalpy of BGL012L was lower than that of the unmodified liposomes, but the extent of enthalpy decrease in BGL012L was less than that of PEGL. At the time of the modification of BGL-PE or PEG-PE, enthalpy reduction might have caused the change in stability of lipid bilayers and resulted in the release of DOX from the liposomes. Our data suggests that PEG-PE involves more significant impact on the stability of lipid bilayers than BGL-PE. The widely spread structure of BGL012, having a low mobility and less effect on the lipid bilayers may provide an increase in encapsulation efficiency of DOX. In addition, Kenworthy et al. (1995) and Hristova and Needham (1995) reported that enthalpy change was dependent on both the molecular weight and the concentration of PEG-lipid used for PEGL. The loss of enthalpy with increasing PEG lipid concentration was explained by conversion of the bilayer phase to a self-association phase which has a disrupted mixture of PEG-lipid and lipid bilayers. Our data implies that, due to the small enthalpy change, BGL012 does not give a significant effect on the structure or stability of lipid bilayers, and thus BGL012 would be more resistant to self-association.

Secondly, Sadzuka et al. (2002, 2006) reported that the retention of liposomes in the blood *in vivo* was well correlated with the fixed aqueous layer thickness (FALT) on the liposomes. These studies showed that the FALT was one of the most important physicochemical factors to explain the pharmacokinetic proper-

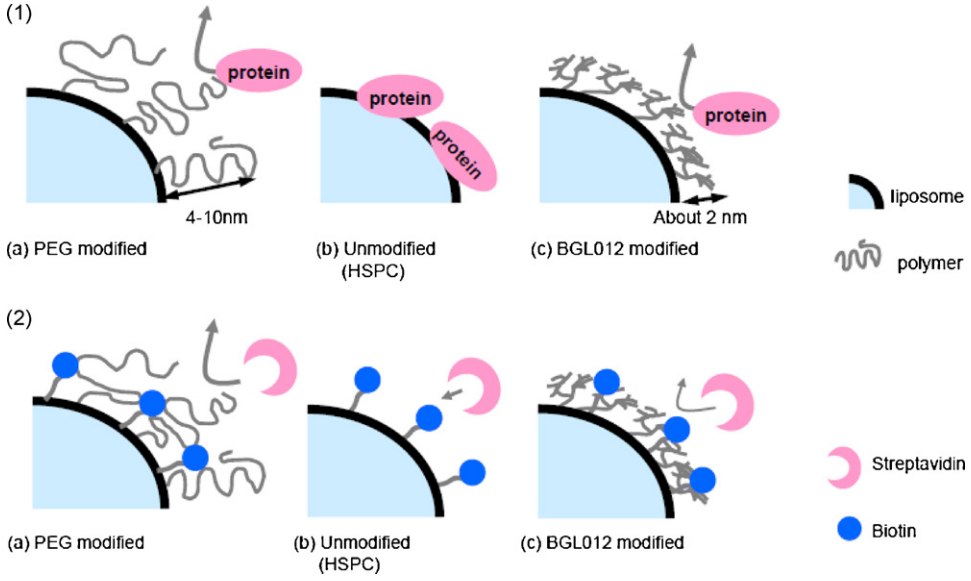

Fig. 6. (1) Schematic representation of inhibitory effect of BGLL and PEGL on the protein binding on the surface of liposomes. (2) Streptavidin-induced agglutination of BGLL and PEGL containing BCPE moiety. (a) PEGL, (b) unmodified liposome (HSPC liposome) and (c) BGL012L.

ties of PEGL (typically 3–4 nm FALT). An increase of the FALT led to improvements of the escape ability from the reticuloendothelial system (RES), long circulation in the blood, and attenuated protein adsorption on the liposomes in the blood. In the case of BGL012L, we experimentally confirmed the FALT on the liposome surface (Table 1): the FALT layer of BGL012L, approximately 2 nm, was thinner than that of PEGL. It was almost equal to the theoretical value of BGL012 stable conformation in molecular structure (2.1 nm length). We assumed that this thin FALT of BGL012L would have lower steric barrier effect and more protein adsorption on the liposome surface compared to PEGL. Therefore, we examined this hypothesis by physicochemical analyses of the liposome surface. One of these experiments was an investigation to determine whether the BGL012 modification exhibited steric barrier effect. We used a BSA-FITC protein adsorption assay of the liposome surface. The results indicated that BSA-FITC adsorption on BGL012L was unexpectedly reduced to comparable levels to those of PEGL (Fig. 3). Another experiment was an investigation of surface binding effects of BGL012 using liposome agglutination assay. The data demonstrated that BGL012L had higher binding properties to the specific ligands than PEGL (Fig. 4), even though significant reduction of the bindings was observed. This means that BGL012L has a better accessibility to the specific ligands, while the liposomes can achieve longer circulation lifetime without unwanted adsorption of serum proteins when compared to PEGL. This observation can be explained by a schematic representation of BGL012L in comparison with PEGL and unmodified liposomes (Fig. 6).

We hypothesized that the cascade-like branched structure of the BGL012 on the surface of liposomes gave the thin FALT. This thinner and more rigid layer is likely to be associated with the improved circulation in blood. This higher surface binding effect of the BGL012L may presumably be new functionality on the liposome surface. One of the important uses of liposomes with prolonged circulation time is ligand-directed liposomal targeting. Mori et al. (1991) showed that the inclusion of PEG in immunoliposomes resulted in a lower level of target binding, presumably due to its strong steric barrier effect of PEG that prevents the antibody/antigen interaction. Instead, the presence of BGL012 on the liposome surface would improve the attenuated interaction of antibody to antigen, and enhance the target binding of immunoliposomes. BGL012 seems to be one of the optimal modification approaches for target binding of immunoliposomes.

In conclusion, we synthesized BGL-lipid derivatives (BGL012-PE), and BGL012 was shown to exhibit new functions on the liposome surface based on its unique widely spread branched structure with cascade-like glycerol units. BGL012-modified liposomes (BGL012L) had an aqueous thinner FALT and their stability was not significantly affected by BGL modification. We have shown various advantages of BGL012L, such as a better encapsulation efficiency of DOX, a better accessibility to specific ligands, and a longer circulation lifetime in blood as compared to PEG-modified counterpart. As this report only provides information on physicochemical and pharmacokinetic properties of BGLL, we intend to focus our next study on evaluating therapeutic efficacy of drug-encapsulated BGLL using animal models. Our results also provide valuable information that can be used to design new BGLs as carriers of drug delivery systems alternative to PEG.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2010.03.031.

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