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# Prolongation of residence time of liposome by surface-modification with mixture of hydrophilic polymers

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ABSTRACT

The objective of this study is to evaluate the biodistribution characteristics of liposomes surface-modified with the mixture of polyethylene glycol (PEG) and polyvinyl alcohol (PVA) as a drug carrier for passive targeting of drugs. The liposomes (egg phosphatidylcholine:cholesterol = 55:40, molar ratio) modified with both PEG and PVA (4:1 molar ratio) (PEG4%/PVA1% liposome) provided the largest AUC, which could be attributed to the smallest hepatic clearance of the liposomes. The liver perfusion studies clearly indicated that lower hepatic disposition of PEG4%/PVA1% liposome was ascribed to the decrease in its hepatic uptake via receptor-mediated endocytosis. Furthermore, the amounts of whole serum proteins and of opsonins such as complement C3 and immunoglobulin G adsorbed on PEG4%/PVA1% liposome were significantly smaller than those on the liposome solely modified with PEG (PEG5% liposome). On the other hand, several proteins were adsorbed at larger amount on PEG4%/PVA1% liposome than PEG5% liposome, and the protein identification by LC–MS/MS suggested that some of those proteins including albumin might function as dysopsonins. The decrease in the adsorbed amount of several opsonins and the increase in the adsorbed dysopsonins would be responsible for its lower affinity to the liver and long residence in the systemic circulation of PEG4%/PVA1% liposome.



## 1. Introduction

Liposomes, mainly made from naturally occurring phospholipids, are biocompatible vehicles. Liposomes can entrap both hydrophilic and hydrophobic drugs in their aqueous internal compartment or within their membrane bilayer, respectively, and hence it can protect the entrapped drug from external destructive condition such as light, pH and enzymes. Therefore, liposomes are considered to be one of the advantageous candidates of drug carriers (Lian and Ho, 2001; Torchilin, 2005). In spite of these merits, their rapid clearance by the reticuloendothelial system (RES) limits their application as drug carriers to other tissues and/or cells (Poste et al., 1982; Senior, 1987; Allen et al., 1991). Various strategies have been developed in order to avoid RES uptake, including the modifying of the liposomal surface with natural polysaccharides such as mannan, pullulan, amylopectin and dextran (Sihorkar and Vyas, 2001). Besides these approaches, long circulating liposomes were developed by the incorporation of ganglioside GM1, phosphatidylinositol or lipid-conjugated polyethylene glycol (PEG) onto the surface (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; Allen and Hansen, 1991). Among them, many studies have demonstrated that PEG-modified liposome exhibits its prolonged blood circulating property by inhibiting adsorption of various opsonins such as immunoglobulin G (IgG) and complement-related components (Banerjee, 2001; Ishida et al., 2002). PEG liposome has been widely used in an attempt to achieve a passive targeting of drugs due to its easy preparation, relatively low cost and its multiple linkability to other lipids (Allen et al., 1991; Maruyama et al., 1999). Lately, the feasibility of modifying the surface of liposomes with polyvinyl alcohol (PVA) or polyacrylic acid (PAA) having a hydrophobic anchor(s) was reported, and it was confirmed that the modification of the liposomal surface with PVA could improve the physical stability of liposomes (Takeuchi et al., 1998, 2000). In addition, the blood circulation time of PVA (MW: 20,000)-modified liposome was comparable to that of PEG (MW: 2000)-liposome (Takeuchi et al., 2001). However, there has been no report examining the effect of the modification of liposomes with the mixture of different hydrophilic polymers. In order to develop the longer circulating liposomal preparations, therefore, we formulated the liposomes surface-modified with the mixture of PEG and PVA, and evaluated their biodistribution characteristics in rats. The hepatic disposition characteristics of these liposomes were evaluated in the liver perfusion experiments. In addition, to have a better understanding of their in vivo behavior, especially their hepatic uptake, the interaction of these polymer-modified liposomes with blood components was also studied.

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## 2. Materials and methods

### 2.1. Materials

Egg yolk phosphatidylcholine (EPC), cholesterol (Chol) and distearoyl phosphatidylethanolamine-*N*-[methoxy poly(ethylene glycol)-2000] (PEG-DSPE) were purchased from ASAHI KASEI Chemicals Industry Inc. (Tokyo, Japan), Wako Pure Chemical Industry Inc. (Osaka, Japan) and NOF Inc. (Tokyo), respectively. [$^3$H] Cholesteryl hexadecyl ether ([$^3$H] CHE) was purchased from PerkinElmer Life Science Inc. (Boston, MA, USA). Polyvinyl alcohol derivatives bearing a hydrophobic anchor ($C_{12}H_{25}$–S–) at the terminal of the molecule with molecular weight of 20,000 was a kind gift from Kuraray Co. (Tokyo). Trypsin from porcine pancreas was purchased from Sigma (St. Louis, MO, USA). Calcein was purchased from Kanto Chemical Co. Inc. (Tokyo). Phospholipid content of liposomes was determined using Phospholipid C-Test Wako (Wako Pure Chemical, Osaka). Rabbit anti-rat IgG polyclonal antibody or goat anti-rat complement C3 polyclonal antibody was purchased from Southern Biotech. (Birmingham, AL, USA) or from MP Biomedicals, LLC (Solon, OH, USA), respectively. All other reagents were of the finest grade available.

### 2.2. Preparation of liposomes

Small unilamellar liposomes were prepared by the hydration method reported previously (Furumoto et al., 2007). PEG2000 is almost completely incorporated into liposomes at 5 mol% of total lipid contents, but its amount incorporated is saturated over 5–7 mol% (Allen et al., 1991). Therefore, we set the total polymer content to be 5 mol%. EPC, Chol and/or PEG-DSPE from stock solution were mixed at the molar ratio of EPC:Chol = 60:40 for conventional liposome or EPC:Chol:polymer(s) = 55:40:5 for polymer-modified liposomes, respectively, the liposomes were radiolabeled by incorporating trace amount of the non-exchangeable, non-metabolizeable marker [$^3$H] CHE to follow the biodistribution of liposomes (Stein et al., 1980). Then, the lipid mixture was dried under reduced pressure. The resultant dried lipid film was hydrated with phosphate-buffered saline (PBS, pH 7.4) under mechanical agitation. The obtained liposomal suspensions were extruded through polycarbonate membrane filters (Millipore, Temecula, CA, USA) with pore sizes of 200 nm 5 times, followed by the extrusion through 100-nm filter 10 times. In order to prepare liposomes-containing PVA, furthermore, an aliquot of the liposomal suspensions were mixed with PVA polymer solution with various concentrations to give an intended final PVA content and was followed by the incubation at 10 °C for 60 min according to the method reported previously (Takeuchi et al., 1998). In the case of calcein-containing liposomes, the hydration of dried lipid film was performed with PBS (pH 7.4) containing calcein (0.2 mg/mL), then the liposomal suspensions were obtained by following the same procedure as described above. Non-encapsulated calcein was removed by gel-filtration chromatography (Sepharose CL-4B, Amersham Bioscience, Uppsala, Sweden). The amount of PVA associated onto the liposomes was estimated by the method reported previously (Takeuchi et al., 1998). In brief, 0.3 mL of liposomal suspension was ultra-centrifuged at 300,000 × *g* for 120 min. The mixture of 3 mL of boric acid solution (4%, w/v) and 0.6 mL of $I_2$/KI solution (0.05 M/0.15 M) was added to 0.05 mL of the supernatant, then the solution was diluted to 10 mL with distilled water. The polymer concentration was measured spectrophotometrically at the wavelength of 620 nm. The amount of associated PVA was calculated by subtracting the PVA amount in the solution. It was confirmed that more than 90% of added PVA was incorporated into the liposomal membranes.

### 2.3. Liposomal size distribution and zeta potential measurements

The size distribution and zeta potentials of liposomes in PBS (pH 7.4) were determined by dynamic light scattering spectrophotometer (DLS-7000, Otsuka Electronics, Osaka) and by electrophoretic light scattering spectrophotometer (ELS-6000, Otsuka Electronic), respectively.

### 2.4. In vitro release of calcein from liposome

The in vitro release of calcein from liposomes was evaluated by equilibrium dialysis method. In brief, 1 mL of liposomal suspension was mixed with 1 mL of PBS (pH 7.4) containing 10% serum (v/v) and the mixture was loaded into the membrane tube (Spectra/Por® Membrane, MWCO: 12,000–14,000, Spectrum Laboratories Inc., Breda, The Netherlands). After both ends were tightly closed, the dialysis tubes were placed into 40 mL of PBS (pH 7.4) as an acceptor medium, and were incubated at 37 °C for 12 h. The percentage of released calcein from liposomes was calculated as follows:

$$\text{release}\,(\%) = \frac{I_r - I_0}{I_{total} - I_0} \times 100$$

where $I_0$ and $I_r$ are the fluorescence intensities of calcein before and after incubation, respectively. $I_{total}$ was the florescence intensity of total calcein loaded into liposomes, which was determined after the destabilization of liposomes by 5% Triton X-100 (final concentration). The florescence intensity of calcein was measured at 490 and 520 nm for excitation and emission wavelengths, respectively.

### 2.5. Animals

Male Wistar rats (Japan SLC, Hamamatsu, Japan), maintained at 25 °C and 55% humidity, were allowed free access to standard laboratory chow (Clear Japan, Tokyo) and water. Rats weighing 220–240 g were randomly assigned to each experimental group. Our investigations were performed after approval by our local ethical committee at Okayama University and in accordance with Principles of Laboratory Animal Care (NIH publication #85-23).

### 2.6. In vivo disposition experiments

After rats were anesthetized by intraperitoneal injection of sodium pentobarbital (20 mg/kg), liposomes were injected into the femoral vein at a dose of 10 μmol total lipid/kg. Body temperature of rats was kept at 37 °C using a heat lamp during the experiment. Blood samples were withdrawn from the jugular vein at fixed time points, followed by immediate centrifugation at 4000 × *g*. The obtained plasma was collected (100 μL) and scintillation medium (Clear-sol II, Nacalai Tesque, Kyoto) was added. For the tissue distribution study, organs (liver, spleen, kidney, heart and lung) were excised at 6 h after the intravenous injection, rinsed with PBS, and weighed. To solubilize the organs, Soluene-350 (Packard Instrument Inc., Meriden, CT, USA) was added and incubated for 2 h at 50 °C before the solubilized solution was neutralized by HCl. Then, scintillation medium was added to the samples, and radioactivity was measured in a liquid scintillation counter (TRI-CARB® 2260XL, Packard Instrument Inc.).

Plasma concentrations of liposomes ($C_p$) versus time curves were analyzed by the Eq. (1) using the non-linear least-squares regression program MULTI (Yamaoka et al., 1978).

$$C_p = A \cdot \exp(-\alpha \cdot t) + B \cdot \exp(-\beta \cdot t) \quad (1)$$

The area under the plasma concentration–time curve (AUC) was calculated by the following equation:

$$\mathrm{AUC}_0^t = \int_0^t C_p \, \mathrm{d}t \tag{2}$$

Total body clearance ($CL_{total}$), elimination rate constant ($k_{el}$), distribution volume of central compartment ($Vd_c$) and distribution volume at steady state ($Vd_{ss}$) were calculated by the following equations:

$$\mathrm{CL}_{\mathrm{total}} = \frac{\mathrm{Dose}}{\mathrm{AUC}_0^\infty} \tag{3}$$

$$\mathrm{Vd}_{\mathrm{c}} = \frac{\mathrm{Dose}}{A+B} \tag{4}$$

$$k_{\mathrm{el}} = \frac{\mathrm{CL}_{\mathrm{total}}}{\mathrm{Vd}_{\mathrm{c}}} \tag{5}$$

$$\mathrm{Vd}_{\mathrm{ss}} = \left(1 + \frac{k_{12}}{k_{21}} \cdot \mathrm{Vd}_{\mathrm{c}}\right) \tag{6}$$

where $AUC_0^\infty$ means AUC value from 0 to infinity. $k_{12}$ and $k_{21}$ are first-order rate constants from peripheral to central compartment and from central to peripheral compartment, respectively. Tissue uptake clearance ($CL_{tissue}$) was calculated by the following equation:

$$\mathrm{CL}_{\mathrm{tissue}} = \frac{X_{\mathrm{tissue}}^t}{\mathrm{AUC}_0^t} \quad (t = 360\,\mathrm{min}) \tag{7}$$

where $AUC_0^t$ means AUC value from 0 to time $t$ and $X_{tissue}^t$ represents the amount of liposomes in a tissue at time $t$.

### 2.7. Single-pass liver perfusion experiments

Liver perfusion was carried out following the procedure reported previously (Furumoto et al., 2002). After the liver was stabilized by 13-min perfusion with Krebs–Ringer bicarbonate (KRB) buffer, each liposomal preparation was continuously infused at a concentration of 0.5 nmol total lipid/mL in the presence of 1% serum from the portal vein for 20 min. After 5-min wash with KRB buffer, the liver was excised, weighed and the accumulated amount of liposomes in the liver was evaluated by measuring the radioactivity in the liver as mentioned above. The serum was prepared just before use as follows: rat whole blood was collected from the carotid artery and allowed to clot at room temperature for 20 min, then centrifuged at 1500 × *g* for 20 min at 4 °C and the supernatant obtained was used. To investigate the contribution of the receptor-mediated endocytosis to the uptake of liposomes, the perfused liver was pretreated with 10 μg/mL trypsin for 10 min (Ogawara et al., 1999).

### 2.8. Quantitative and qualitative determination of serum protein associated onto liposomal surface

Aliquots of $^3$H-liposomal suspension (2.5 μmol total lipid/mL) were incubated with equal volume of fresh rat serum for 20 min at 37 °C. Then, the liposomes were separated from bulk serum proteins by Sepharose CL-4B gel filtration (Johnstone et al., 2001). Fractions of liposomes were collected, and the amount of serum proteins associated on liposomes was quantified by Lowry's method (Lowry et al., 1951) and the amount of liposomes was quantified by measuring the radioactivity. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by using the Mini Protean-II electrophoretic apparatus (Bio-Rad, Hercules, CA, USA) on 12.5% polyacrylamide gel (Ready Gel J, Bio-Rad). For the relative comparison of the proteins associated on the surface of each liposomal preparation, the same amount of protein (0.3 μg) was loaded onto the gel. The detection of proteins was performed by a silver-stain procedure by using a silver-stain kit (Daiichi Pure Chemicals, Tokyo).

After SDS-PAGE was performed as described above, proteins were blotted on cellulose nitrate membrane (Advantec, Tokyo). For the detection of complement C3 or IgG, the blots were incubated with 1:100 diluted goat anti-rat complement C3 or 1:250 diluted rabbit anti-rat IgG polyclonal antibody. As second antibodies, peroxidase-linked anti-goat polyclonal antibody (Cosmo Bio. Tokyo) and anti-rabbit polyclonal antibody (Zymed® laboratories Inc., CA, USA) were used at 1:10,000 and 1:5000 dilution in blocking buffer, respectively. The protein band was visualized with the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Buckinghamshire, UK) and the densitometric intensities of protein bands were quantified by Scion Image™ (Scion Corporation, Frederick, MD). Since SDS-PAGE was conducted under reducing condition where many small fragments can be generated from the protein of interest, the densitometric intensities of bands were integrated for each lane to semi-quantitatively evaluate the amount of C3 and IgG.

### 2.9. Sample preparation for LC–MS/MS analysis and data search

The protein bands were excised from the gel and transferred to Ependorf tubes. The gel pieces were washed twice with 50% acetonitrile/25 mM ammonium bicarbonate, washed with 100% acetonitrile and then dried in a speed vacuum concentration system (CVE-100D, Rikakikai, Tokyo). Approximately 30 μL of trypsin (20 μg/mL) in 25 mM ammonium bicarbonate was added to the dried residue and the samples were incubated overnight at 37 °C. The supernatant was transferred to a separate Ependorf tube and the peptides were further extracted from the gel pieces by incubation in 50% acetonitrile/5% formic acid (FA) for about 4 h at room temperature. The supernatants obtained from the two steps were pooled, dried by SpeedVac and dissolved in 5 μL 50% acetonitrile/0.1% FA and stored at −20 °C until use. Sample analysis was performed on Agilent 1100LC/MSD Trap XCT series system. The ionization system was Chip Cube using HPLC-Chip-Ms (Agilent Technologies, Santa Clara, CA, USA). The chip was automatically loaded and positioned into the MS nanospray chamber. The chip contained a Zobrax 300SB-$C_{18}$ (43 mm × 75 μm, 5 μm) column and a Zobrax 300SB-$C_{18}$ (40 nL, 5 μm) enrichment column. The mobile phase, the mixture of $H_2O$/0.1% FA and acetonitrile/10% $H_2O$/0.1% FA, was delivered at the flow rate of 300 nL/min. Tryptic peptides were eluted from the column into the MS using gradient elution. The capillary voltage was set to 1850 V, the flow and temperature of the drying gas were 4 L/min and 300 °C, respectively. The MS and MS/MS data were analyzed by Data Analysis software (Spectrum Mill Ver. 3.3).

### 2.10. Statistical analysis

Results are expressed as the mean ± S.D. Analysis of variance (ANOVA) was used to test the statistical significance of differences among groups. Statistical significance in the differences of the means was evaluated by using Student's *t*-test or Tukey's test for the single or multiple comparisons of experimental groups, respectively.

## 3. Results and discussion

In order to prolong the residence time of liposomes in the systemic circulation, PVA as well as PEG were employed to modify the surface of liposomes. In this study, we prepared five different

**Table 1**
Composition and physical properties of liposomes

| Liposomes | Liposomes composition EPC:Chol:PEG:PVA (molar ratio) | Particle size (nm) | Zeta potential (mV) |
|---|---|---|---|
| Naked | 60:40:0:0 | 90.8 ± 0.9 | −29.0 ± 1.2 |
| PEG5% | 55:40:5:0 | 88.5 ± 1.2 | −4.5 ± 0.8* |
| PEG4%/PVA1% | 55:40:4:1 | 91.1 ± 2.1 | 3.7 ± 0.5* |
| PEG1%/PVA4% | 55:40:1:4 | 124.2 ± 2.2* | 0.7 ± 0.8* |
| PVA5% | 55:40:0:5 | 141.0 ± 0.6* | 0.4 ± 1.0* |

Results for particle size and zeta potential are expressed as the mean ± S.D. of three experiments.
* $p < 0.05$, compared with naked liposome.

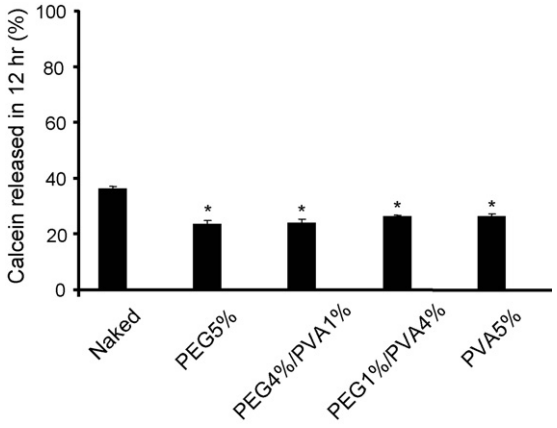


**Fig. 1.** In vitro release of calcein from surface-modified liposomal preparations. Each liposomal preparation was incubated in PBS (pH 7.4) containing 10% rat serum (v/v) at 37 °C for 12 h. Results are expressed as the mean with the vertical bar showing S.D. of three experiments. * $p < 0.05$, compared with naked liposome.

liposomal preparations including naked liposome, PEG liposome (PEG5%), liposomes modified with the mixture of both PEG and PVA (PEG4%/PVA1% and PEG1%/PVA4%) and liposome modified with PVA only (PVA5%). It is well known that PEG-DSPE is stably inserted into the lipid bilayer of liposomes (Parr et al., 1994). The 1-h incubation with 50% rat serum at 37 °C revealed that more than 90% of PVA incorporated stably remained on the liposomes, suggesting that the hydrophobic moiety of PVA derivative was also stably inserted into the lipid bilayer of liposomes as suggested by Takeuchi et al. (1998).

Since several factors such as particle size, charge and lipid composition have been reported to influence the in vivo fate of liposomes after intravenous administrations (Levchenko et al., 2002; Murao et al., 2002; Awasthi et al., 2003), the physicochemical properties of the liposomes prepared were examined (Table 1). The particle sizes for naked and PEG liposomes were found to be almost the same, while the modification of liposomes with PVA increased the particle size depending on its molar ratio. The measurement of zeta potential showed that the negative charge of naked liposome tended to be neutralized by the surface-modification either

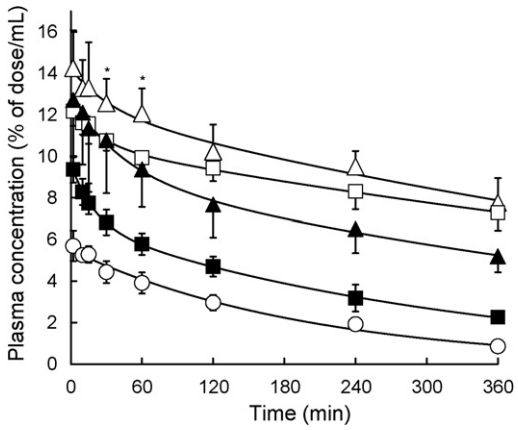


**Fig. 2.** Plasma concentration–time profile of surface-modified liposomal preparations after intravenous injection into rats. Each liposomal preparation was injected at a dose of 10 μmol total lipid/kg. Results are expressed as the mean with the vertical bar showing S.D. of three rats. Keys: –○–, Naked; –□–, PEG5%; –△–, PEG4%/PVA1%; –▲–, PEG1%/PVA4%; –■–, PVA5%. * $p < 0.05$ compared with PEG5% liposome.

with PEG, PVA or their mixture. In order to confirm the stability of prepared liposomes in the presence of serum, calcein release from each liposomal preparation was determined. As illustrated in Fig. 1, the surface-modified liposomes were characterized by the significant lower release of calcein compared to the naked liposome, demonstrating their good stability in the presence of serum.

In vivo pharmacokinetics and biodistribution of the surface-modified liposomes were investigated after intravenous administration to rats. Fig. 2 shows the plasma concentration–time profiles of the liposomes, and the pharmacokinetic parameters obtained are summarized in Table 2. As shown in Fig. 2, naked liposome was rapidly eliminated from systemic circulation with the largest values of $k_{el}$, $Vd_c$ and $Vd_{ss}$ (Table 2). On the other hand, other polymer-modified liposomes exhibited longer blood circulating properties. Among them, PEG4%/PVA1% liposome showed the smallest $CL_{total}$ and the largest $AUC_0^\infty$, which was about 12 times or 1.2 times larger than that of naked liposome or PEG5% liposome, respectively (Table 2). In addition, the PEG4%/PVA1% liposome showed significantly higher plasma levels than PEG5% liposome at 30 and 60 min after intravenous injection. Moreover, the PEG4%/PVA1% liposome provided the smallest values of both $Vd_c$ and $k_{el}$ among the liposomal preparations examined, where the small $Vd_c$ and $k_{el}$ would mean the decrease in the rapid distribution to the liver just after dosing and the delay of elimination from plasma, respectively. Tissue uptake clearances calculated for various organs would support the above consideration (Fig. 3). The hepatic clearances for the liposomes modified with polymers were significantly smaller than that for naked liposome, and PEG4%/PVA1% liposome provided the smallest value of hepatic clearance among the liposomes investigated. Furthermore, it is worth to note that PEG4%/PVA1% liposome showed significantly smaller clearances for liver (68%), spleen (38%) and lung (22% of PEG5%) than PEG5% liposome. On the other hand,

**Table 2**
Pharmacokinetic parameters of different liposomal formulations after intravenous injection into rats

| Parameters | Naked | PEG5% | PEG4%/PVA1% | PEG1%/PVA4% | PVA5% |
|---|---|---|---|---|---|
| AUC (% of dose min/mL) | 1096 ± 101 | 10755 ± 3012** | 13438 ± 4520** | 6045 ± 604 | 2267 ± 282 |
| $CL_{total}$ (μL/min) | 91.9 ± 8.3 | 9.7 ± 2.4** | 8.0 ± 2.4** | 16.7 ± 1.6** | 44.6 ± 5.3** |
| $k_{el}$ ($min^{-1}$) | 4.98 ± 0.24 | 1.21 ± 0.36** | 1.13 ± 0.36** | 2.12 ± 0.25** | 4.29 ± 0.42 |
| $Vd_c$ (mL) | 18.4 ± 1.1 | 8.1 ± 0.5** | 7.1 ± 0.9** | 8.0 ± 1.7** | 10.4 ± 0.7** |
| $Vd_{ss}$ (mL) | 18.4 ± 1.1 | 9.4 ± 0.3** | 9.5 ± 2.3** | 10.6 ± 2.3** | 14.3 ± 1.4 |

Each pharmacokinetic parameter was obtained by following the equations described in Section 2. Results are expressed as the mean ± S.D. of three rats.
** $p < 0.01$, compared with naked liposome.

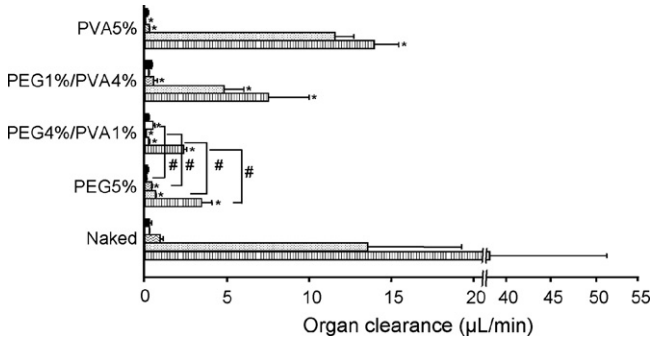


**Fig. 3.** Tissue uptake clearances of surface-modified liposomes after intravenous injection into rats. Each liposomal preparation was injected at a dose of 10 μmol total lipid/kg. Each tissue was excised at 6 h after injection. Results are expressed as the mean with the vertical bar showing S.D. of three rats. Keys: ▥, liver; ▫, spleen; ▨, lung; □, kidney; ■, heart. $^{*}p < 0.05$ compared with naked liposome; $^{\#}p < 0.05$ compared with PEG5% liposome.

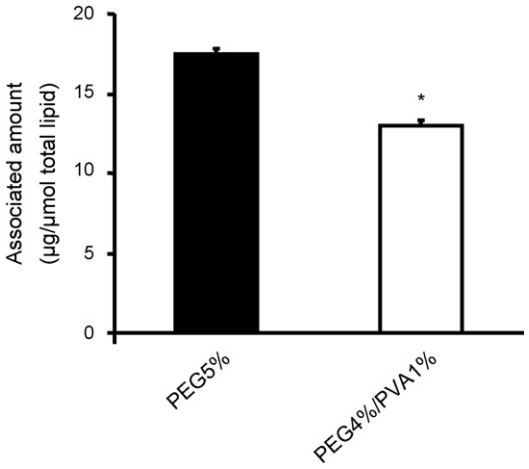


**Fig. 5.** Amount of serum proteins associated on the surface of PEG5% and PEG4%/PVA1% liposomes. Results are expressed as the mean with the vertical bar showing S.D. of three experiments. $^{*}p < 0.05$, compared with PEG5% liposome.

the renal uptake clearance was larger for PEG4%/PVA1% liposome than that for PEG5% liposome. The reason for it remains to be clarified, but the longer circulation of larger amount of liposomes might lead to the disposition of intact and/or degraded liposomes into kidney.

To clarify the mechanism behind the less affinity of PEG4%/PVA1% than PEG5% to the liver where these liposomes were mainly distributed, a single-pass liver perfusion experiment was performed by using the perfusate containing 1% serum (v/v) (Fig. 4). Naked liposome showed significantly higher hepatic accumulation ($4.3 \pm 1.2$ nmol total lipid) than the two polymer-modified liposomes. In addition, the hepatic accumulation of PEG4%/PVA1% liposome ($0.6 \pm 0.2$ nmol total lipid) was significantly lower than PEG5% ($1.9 \pm 0.3$ nmol total lipid). These results were similar to those obtained in the in vivo study (Figs. 2 and 3 and Table 2). Furthermore, the pretreatment of the perfused liver with trypsin drastically decreased the hepatic accumulation of both naked ($0.96 \pm 0.1$ nmol total lipid) and PEG5% liposomes ($0.90 \pm 0.06$ nmol total lipid). On the contrary, the same treatment did not significantly affect the hepatic accumulation of PEG4%/PVA1% liposome ($0.70 \pm 0.20$ nmol total lipid). These results clearly indicate that the modification of liposomes with the mixture of PEG4%/PVA1% can avoid the hepatic disposition via the receptor-mediated endocytosis, which, on the other hand, substantially contributes to the hepatic disposition of PEG5% liposome.

It is well known that the hepatic uptake of liposomes is largely affected by the association of serum opsonins such as IgG, fibronectin, complement components, C-reactive protein and $\alpha_2$-macroglobulin (Tsujimoto et al., 1981; Rossi and Wallace, 1983; Bonte and Juliano, 1986; Huong et al., 2001; Price et al., 2001; Ishida et al., 2006; Moghimi et al., 2006). In addition, the recognition of surface-bound opsonins by their corresponding receptors is known to be a main trigger for the hepatic uptake of particles via receptor-mediated endocytosis (Moghimi and Davis, 1994; Liu et al., 1995). Therefore, we tried to evaluate the serum proteins associated on the surface of PEG5% liposome and PEG4%/PVA1% liposome quantitatively and qualitatively. As illustrated in Fig. 5, the total amount of serum proteins adsorbed on the surface of the PEG4%/PVA1% liposome ($13.0 \pm 0.3$ μg/μmol total lipid) was significantly smaller than that for PEG5% liposome ($18.0 \pm 0.4$ μg/μmol total lipid). This result was in good agreement with the previous report demonstrating that the circulation half-lives of liposomes after intravenous administration is inversely related to the total protein amount associated on the surface (Chonn et al., 1992). It has previously been reported that the fixed aqueous layer thickness (FALT) around liposomes was increased by the surface-modification with PEG and that thicker FALT would be likely to prevent serum proteins from interacting with liposomes (Shimada et al., 1995; Zeisig et al., 1996). Moreover, it has been postulated that surface-grafted PEG would form either a mushroom or a brush conformation, depending on molecular weight and surface density of PEG on the liposomes, and that the latter conformation would build the thicker FALT than the former one (Needham et al., 1997; Nicholas et al., 2000; Johnstone et al., 2001). Sadzuka et al. (2002) reported that the surface-modification with the mixture of PEG500 and PEG2000 provided thicker FALT than the modification with either PEG500 or PEG2000, and that the liposomes modified with both PEG500 and PEG2000 revealed the lowest hepatic uptake. They speculated that PEG500 would facilitate to transform of PEG2000 from the mushroom structure into the brush one, and that the liposomes on which less amount of opsonins would be adsorbed due to thicker FALT had lower affinity to the liver (Sadzuka et al., 2002). Considering this background, we speculated that PVA alone on the surface of liposomes would be present as the mushroom (shrunk) structure with thin FALT. Then, further addition of adequate amount of PEG might facilitate the conformational change of PVA to the brush-like (extended) structure with thicker FALT, leading to the decrease in the adsorbed amount of serum proteins on PEG4%/PVA1% liposome. In the case of PEG1%/PVA4% liposome, the amount of PEG might be still insufficient to facilitate the conformation change of PVA. Sadzuka et

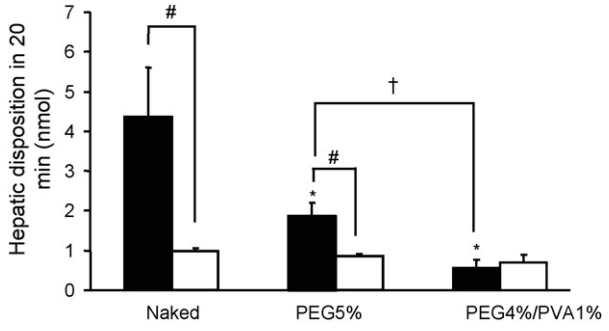


**Fig. 4.** Hepatic disposition of naked, PEG5% and PEG4%/PVA1% liposomes in single-pass liver perfusion experiments. The liver perfusion was performed for 20 min. Results are expressed as the mean with the vertical bar showing S.D. of three experiments. Keys: ■, control; □, + trypsin treatment. $^{*,\#,\dagger}p < 0.05$, compared with naked, control or PEG5% liposome, respectively.

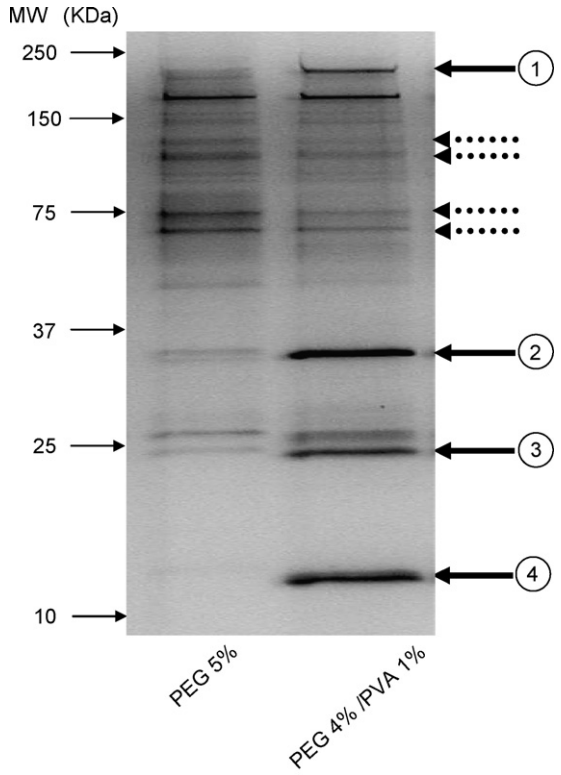


**Fig. 6.** Comparison of serum proteins associated on the surface of PEG5% and PEG4%/PVA1% liposomes. The same amount of protein (0.3 μg) was loaded onto each lane. SDS-PAGE was performed following the procedure described in Section 2, and proteins were silver-stained. Solid and dotted arrows indicate the typical proteins increased and decreased on PEG4%/PVA1% liposome, respectively, as compared with PEG5% liposome.

al. (2002) proposed that the optimal amount of PEG500 would be needed to support the brush-like structure of PEG2000 and it could also be the case with PEG/PVA liposomes. To have better understanding of the mechanisms; however, the conformational dynamics of PVA molecule on the surface have to be elucidated and will be the subject of our further study.

In order to identify the proteins adsorbed on the liposomes, SDS-PAGE was at first performed. The results revealed that there was quite large difference in the profiles of surface-associated serum proteins between PEG5% liposome and PEG4%/PVA1% liposome (Fig. 6). The proteins highlighted with dotted arrows seem to be preferentially associated onto PEG5% liposome. On the other hand, the proteins highlighted with solid arrows (15, 25, 35 and 240 kDa) are associated more onto PEG4%/PVA1% liposome. Taken the results obtained in the in vivo and liver perfusion studies together, the proteins with dotted arrows might contain opsonins enhancing the hepatic uptake of PEG5% liposome, while the proteins with solid arrows might possess dysopsonin-like activity suppressing the uptake of PEG4%/PVA1% liposome.

As discussed above, complement C3 (C3) and IgG are the major opsonins and are known to play important roles to promote the hepatic uptake of liposomes via their corresponding receptors expressed on the surface of Kupffer cells in the liver (Ishida et al., 2002). Therefore, we conducted the Western blot analysis to compare the amounts of C3 and IgG associated on the surface of PEG5% and PEG4%/PVA1% liposomes (Fig. 7). As shown in Fig. 7A and B, the semi-quantification of the densitometric intensities derived from C3 and IgG fragments revealed that these typical opsonins associated more with PEG5% liposome than PEG4%/PVA1% liposome.

Besides opsonins, it has been suggested that there are some dysopsonins in serum, which can inhibit phagocytosis of pathogens or particles. Although it was reported that immunoglobulin A and $\alpha_1$-acid glycoprotein functioned as dysopsonins for microorganisms (Van Oss et al., 1974; Absolom, 1986), there is no identified serum components with dysopsonic activity for liposomes so far. Then, we tried to identify the proteins which might act as dysopsonin for PEG4%/PVA1%, highlighted with solid arrows in Fig. 6. After SDS-PAGE was performed for proteins associated on PEG4%/PVA1% liposome, the proteins highlighted with solid arrows in Fig. 6 were subjected to LC–MS/MS system for identification and the results were summarized in Table 3. The analysis showed that albumin would be one of the serum proteins preferentially associated onto PEG4%/PVA1% liposome. Taken our previous reports that the pre-coating of polystyrene nanospheres with albumin or the coupling of albumin onto the surface of PEG liposome reduced their affinity to the liver (Ogawara et al., 2004; Furumoto et al., 2007), albumin might function as dysopsonin for PEG4%/PVA1% liposome.

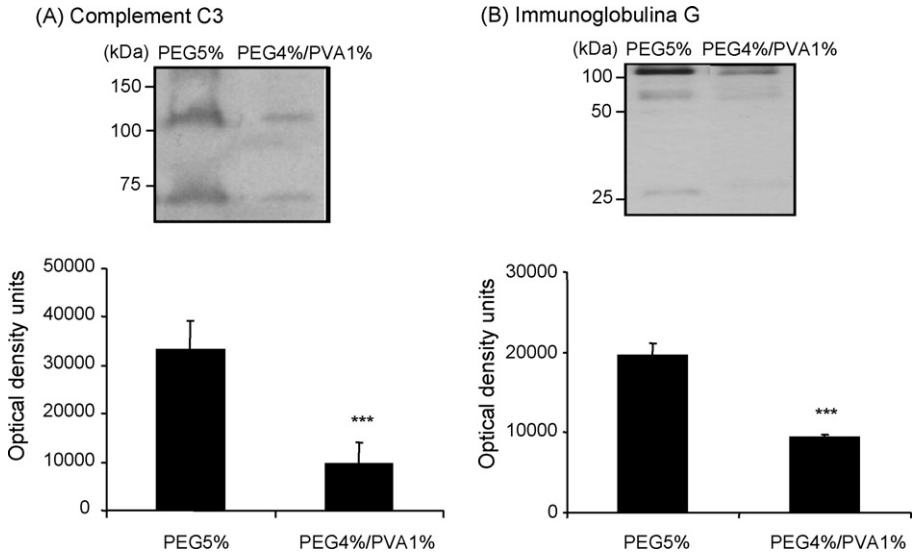


**Fig. 7.** Semi-quantification of complement C3 and immunoglobulin G associated on the surface of PEG5% and PEG4%/PVA1% liposomes by Western blot analysis. The same amount of protein (1.2 μg) was loaded onto each lane. Results of semi-quantification are expressed as the mean with the bar showing S.D. of five experiments. $^{***}p < 0.05$ compared with PEG5% liposomes.

**Table 3**
Identification of several serum proteins associated on PEG4%/PVA1% liposome at larger amount than PEG5% liposomes

| Band number | Identified proteins | Accession number | Spectrum Mill score |
|---|---|---|---|
| 1 | Apolipoprotein B | 61098031 | 43.67 |
| 2 | Apolipoprotein A-IV | 8392909 | 96.62 |
| | Albumin | 55391508 | 41.07 |
| 3 | Apolipoprotein E | 37805241 | 186.39 |
| 4 | Apolipoprotein A-I | 113997 | 97.65 |
| | Albumin | | |

LC–MS/MS data were analyzed by Spectrum Mill with NCBInr database (http://www.ncbi.nlm.nih.gov/). The following filters were used after database searching: peptide score >6, peptide% SPI > 60 and protein score > 11. Band numbers represent the proteins highlighted with solid arrows in Fig. 6.

In addition, apo A-I, A-IV, B and E were also shown to preferentially be associated onto PEG4%/PVA1% liposome. Although apoE itself is known to function as an opsonin for the uptake of particles by hepatocytes, Bisgaier et al. (1989) indicated that the co-existence of apoE with apoA-I, A-IV or B leads to the conformational change of apoE and abolishes its ability to enhance the uptake of liposomes by HepG2 cells. Therefore, the enrichment of these apolipoproteins on the surface of PEG4%/PVA1% liposome might abolish the opsonic activity of apoE. However, the mechanisms by which the specific proteins such as albumin, apo A-I, A-IV, B and E were preferentially associated onto PEG4%/PVA1% liposome are still unclear and would be investigated in the further study.

## 4. Conclusion

An incorporation of small percentage of PVA into PEG liposome (PEG4%/PVA1% liposome) improved the in vivo disposition characteristics, which could be attributed to lower hepatic distribution of PEG4%/PVA1% liposome. The decrease in the affinity to the liver would be attributed to lower amount of serum proteins including opsonins and larger amount of dysopsonins such as albumin adsorbed on the surface. These findings can form a solid basis to develop useful particulate drug carriers with better in vivo disposition characteristics. To confirm the advantage of PEG4%/PVA1% liposome, the in vivo anti-tumor activity of the liposome including some anti-tumor drug will be investigated in our next study.

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