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Effect of coupling of albumin onto surface of PEG liposome on its in vivo disposition

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Abstract

To evaluate the effect of coupling of albumin onto the surface of poly(ethylene glycol)-modified liposome (PEG liposome) on the in vivo disposition of liposome, pharmacokinetics and tissue distribution were examined after intravenous administration of rat serum albumin-modified PEG (RSA/PEG) liposome into rats. RSA/PEG liposome showed longer blood-circulating property than PEG liposome and the hepatic clearance for RSA/PEG liposome was significantly smaller than that for PEG liposome. Single-pass liver perfusion experiments also showed that the hepatic disposition of RSA/PEG liposome was much less than that of PEG liposome and that pre-treatment of liver with trypsin did not significantly reduce the hepatic disposition of RSA/PEG liposome, suggesting that RSA/PEG liposome could avoid the hepatic uptake via the receptor-mediated endocytosis. To unravel the mechanism behind the less affinity of RSA/PEG liposome to the liver, serum proteins associated on their surface were quantitatively and qualitatively assessed. The results showed that the coupling of albumin onto PEG liposome significantly reduced the total amount of serum proteins associated onto the surface, and SDS-PAGE revealed that the decrease in the association with liposomes for several serum proteins, which might have opsonic activity. From these findings, introduction of serum albumin onto PEG liposome could be useful to develop a new nanoparticulate formulation with a better pharmacokinetic property.

Keywords: Liposomes; Poly(ethylene glycol); Rat serum albumin; Hepatic disposition; Serum opsonins

1. Introduction

Although liposomes are good candidates for efficient drug carriers, the rapid clearance by the reticuloendothelial system (RES) limits their application as drug carriers to other tissues and/or cells. Early studies demonstrated that several physicochemical properties of liposomes such as size, lipid composition and surface charge, influence their pharmacokinetics after systemic administration (Allen et al., 1991a; Liu et al., 1995; Harashima et al., 1996; Li and Mitra, 1996). The serum proteins associated onto the surface of systematically administered liposomes were also suggested to influence their in vivo fate (Harashima et al., 1998; Semple et al., 1998). Among them, immunoglobulin G, complement C3 and fibronectin are well known to function as opsonins to promote the hepatic disposition of liposomes (Hsu and Juliano, 1982; Derksen et al., 1987; Wassef and Alving, 1993). In addition, it was shown that some other serum proteins such as apolipoproteins and β_2-glycoprotein I, can function as opsonins depending on the lipid composition of the liposomes (Ivanov et al., 1985; Chonn et al., 1995). On the other hand, the existences of serum components that inhibit phagocytosis of pathogens or particles, dysopsonins, were also indicated (Park and Huang, 1993). Although dysopsonins for microorganisms such as immunoglobulin A and α_1-acid glycoprotein were already reported (van Oss et al., 1974; Absolom, 1986), there is no identified serum components with dysopsonic activity for liposomes so far.

Albumin is the most abundant protein in serum, of which the concentration is approximately 5% (w/v). The functions of albumin include the transport of both endogenous and exogenous ligands such as hormones and fatty acids to tissues, the maintenance of vascular integrity and transvascular oncotic pressure

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gradient (Peters, 1975). Although albumin is also known to be one of the most abundant proteins associated with both neutral and charged liposomes (Chonn et al., 1991; Gabizon and Papahadjopoulos, 1992; Du et al., 1997), there is little information on the substantial role of albumin in the in vivo behavior of liposomes after systemic administration (Torchilin et al., 1980).

In our previous study, the pre-coating of the surface of polystyrene nanospheres (50 nm in diameter) with albumin significantly decreased their hepatic disposition, especially the uptake by Kupffer cells, which resulted in their longer blood circulation time. The results suggested that albumin might provide dysopsonin-like activity by inhibiting the subsequent association of opsonins on the surface of polystyrene nanospheres (Ogawara et al., 2004). In the present study, therefore, we investigated the effect of coupling of albumin onto the surface of PEG-modified liposomes on their in vivo disposition after intravenous administration in rats.

2. Materials and methods

2.1. Chemicals

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and rat serum albumin (RSA) were purchased from Sigma (St. Louis, MO, USA). *N*-Hydroxysuccinimide (NHS) was obtained from Pierce (Rockford, IL, USA). Egg yolk phosphatidylcholine (EPC), cholesterol (Chol) and *N*-glutaryl phosphatidylethanolamine (NGPE) were purchased from ASAHI KASEI Chemicals Industry Inc. (Tokyo, Japan), Wako Pure Chemical Industry Inc. (Osaka, Japan) and Avanti Polar Lipids Inc. (Alabaster, AL, USA), respectively. Distearoyl phosphatidylethanolamine-*N*-[methoxy poly(ethylene glycol)-2000] (PEG-DSPE) was obtained from NOF Inc. (Tokyo). [^{3}H]Cholesteryl hexadecyl ether ([^{3}H]CHE) was purchased from Perkin-Elmer Life Science Inc. (Boston, MA, USA). All other chemicals were of the finest grade available.

2.2. Animals

Male Wistar rats (Japan SLC, Hamamatsu, Japan), maintained at 25 °C and 55% of humidity, were allowed to free access to standard laboratory chow (Clea Japan, Tokyo) and water prior to the experiments. 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 "Interdisciplinary Principles and Guidelines of the Use of Animals in Research".

2.3. Liposome preparation

Liposomes were prepared according to the following procedures. EPC, Chol, NGPE and PEG-DSPE from stock solution were mixed at EPC:Chol:NGPE = 60:30:10 for non-PEG liposome or EPC:Chol:NGPE:PEG-DSPE = 55:30:10:5 for PEG liposomes with trace amount of [^{3}H]CHE. Then, the lipid mixture was dried under reduced pressure and the resultant dried lipid was hydrated in phosphate buffer (pH 5.0) under mechanical agitation. Then, the resulting multilamellar preparations were sized by repeated extrusion (15 times) through polycarbonate membrane filters (Millipore, Bedford, MA, USA) with pore size of 200 nm.

2.4. Preparation of RSA-modified PEG (RSA/PEG) liposomes

RSA was conjugated to liposomes by using carbodiimide as reported previously (Bogdanov et al., 1988; Holmberg et al., 1989; Nakajima and Ikada, 1995). To activate the carboxyl group of NGPE incorporated in liposomes, EDC (10 μmol/μmol total lipid (TL)) and NHS (10 μmol/μmol TL) were added to liposome suspension, and the mixture was further incubated for 15 min at room temperature. After 2-mercaptoethanol (5 mmol/μmol TL) was added, the mixture was applied to Sephadex G-25 (Sigma) column equilibrated with phosphate buffer (pH 5.0) and the liposome fractions were collected. RSA (0.01 μmol/μmol TL) was added to the liposome suspension and incubated for 18 h at 4 °C. Then, the liposome fractions were separated from unconjugated RSA by Sepharose CL-4B (Amersham Biosciences, Uppsala, Sweden) column equilibrated with phosphate buffered saline (PBS, pH 7.4). Particle size of liposome was determined by dynamic light scattering spectrophotometer (DLS-7000, Otsuka Electronics, Osaka), and particle sizes for non-PEG, PEG or RSA-modified PEG (RSA/PEG) liposomes in PBS (pH 7.4) were 208 ± 10, 215 ± 12 or 233 ± 15 nm, respectively. The particle size of each liposome in PBS containing rat serum (5%, v/v) was constant for up to 24 h. Quantity and quality of coupled RSA onto liposomes were evaluated by the method reported by Lowry et al. (1951) and SDS-PAGE, respectively, as described below. The amount of RSA conjugated onto the surface of PEG liposome was 19.3 ± 4.1 μg/μmol TL. SDS-PAGE under non-reducing condition revealed that RSA coupled onto the surface of liposome was exclusively in a monomeric form.

2.5. In vivo disposition experiments

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (20 mg/kg). Liposomes were injected from femoral vein at a dose of 10 μmol TL/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, and then centrifuged immediately 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 lung, kidney, heart, cerebrum, cerebellum, eyeball, muscle and lymph) were excised at 1 h after intravenous injection, and rinsed with PBS, and weighed. To solubilize organs, Soluene-350 (Packard Instrument Inc., Meriden, CT, USA) was added and incubated for 2 h at 50 °C before solubilized solution was neutralized by HCl. Scintillation medium was added to samples, and radioactivity was determined in a liquid scintillation counter (TRI-CARB® 2260XL, Packard Instrument Inc.).

Plasma concentrations of liposomes (C_p) versus time curves were analyzed by Eq. (1) using the non-linear least-squares regression program MULTI (Yamaoka et al., 1981).

$$C_p = A\,e^{-\alpha t} + B\,e^{-\beta t} \quad (1)$$

The area under the plasma concentration–time curve (AUC) was calculated by Eq. (2).

$$AUC_0^t = \int_0^t C_p\,dt \quad (2)$$

Tissue clearance (CL_{tissue}) were calculated by Eq. (3).

$$CL_{tissue} = \frac{X^t_{tissue}}{AUC_0^t} \quad (t = 1\,h) \quad (3)$$

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

2.6. Rat liver single-pass constant infusion experiments

Liver perfusion experiments were performed under single-pass conditions at a flow rate of 13 ml/min. Perfused liver was prepared according to the method reported previously (Ogawara et al., 1999a). After a stabilization period of 13 min, liposomes were continuously infused from the portal vein for 20 min at the concentration of 0.5 nmol TL/ml in the presence of serum 1% (v/v). After a 5-min wash with Krebs–Ringer bicarbonate (KRB) buffer, the liver was excised and weighed, and the accumulated amount of liposomes in the liver was evaluated by measuring the radioactivity in the liver as described 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.

2.7. Pre-treatment of liver with trypsin

Pre-treatment of the perfused liver with trypsin was conducted as reported previously (Ogawara et al., 1999b). In brief, after 5 min stabilization period of the perfused liver using KRB buffer as a perfusate, the liver was then perfused with KRB buffer containing trypsin (10 μg/ml) for 10 min. To wash the remaining trypsin in the vascular compartment, the liver was perfused with KRB buffer for another 5 min, then liposomes were perfused. It was already evidenced that the treatment with trypsin described above did affect only the receptor-mediated uptake of particles (Ogawara et al., 1999b).

2.8. Determination of amount of serum proteins associated with liposomes

Liposomes (2.5 μmol TL/ml) were incubated in rat serum (liposomes:serum = 1:1 v/v) for 20 min at 37 °C. Separation of liposomes from bulk serum proteins was achieved by Sepharose CL-4B gel filtration. Fractions of liposomes were collected, and the amount of serum proteins associated on the surface of liposomes were quantified by Lowry's method (Lowry et al., 1951) and the amount of liposomes were quantified by measuring the radioactivity. The amount of serum proteins associated with RSA/PEG liposome was calculated by subtracting the amount of RSA coupled with liposomes from total protein amount measured.

2.9. SDS-polyacrylamide gel electrophoretic analysis of serum proteins associated with liposomes

Analysis of serum proteins associated with liposomes was performed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the Mini Protean-II electrophoretic apparatus (Bio-Rad, Hercules, CA, USA) on 12.5% polyacrylamide gel (Ready Gel J, Bio-Rad). Prestained SDS-PAGE molecular weight standards (Bio-Rad) were used to estimate the molecular weights of the associated proteins. For the relative comparison of the proteins associated on the surface of different liposome preparations, the same amount of protein (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).

2.10. Statistical analysis

Statistical significance was evaluated by using Student's t-test or Dunnet's test for the single or multiple comparisons of experimental groups, respectively.

3. Results

The in vivo disposition of conventional liposome (non-PEG liposome), PEG liposome and RSA-modified PEG liposome (RSA/PEG liposome) was evaluated after the intravenous injection into rats (Fig. 1 and Table 1). The inclusion of PEG onto the surface of liposome significantly increased the AUC value about three times of conventional liposome. It was also revealed that the AUC value for RSA/PEG liposome was slightly larger than that of PEG liposome. Distribution vol-

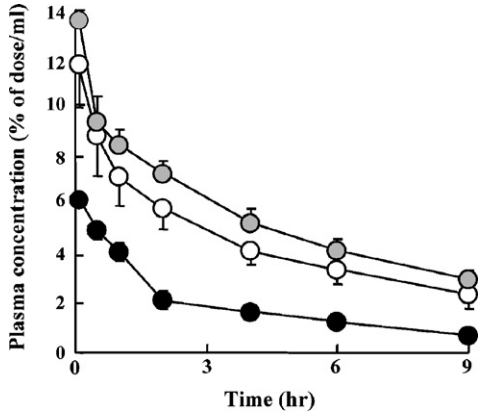

Fig. 1. Plasma concentration of conventional (non-PEG), PEG and RSA/PEG liposomes after intravenous injection into rats. Results are expressed as the mean with the bar showing the S.D. of three experiments. Keys: (●) non-PEG liposome; (○) PEG liposome; (◍) RSA/PEG liposome.

Table 1
Pharmacokinetic parameters of conventional, PEG and RSA/PEG liposomes after intravenous injection into rats

Parameter (unit)	Conventional liposome	PEG liposome	PEG/RSA liposome
AUC (% of dose/ml h)	21.9 ± 1.3	63.9 ± 10.5*	84.9 ± 13.5*
CL_{total} (ml/h)	4.6 ± 0.3	1.6 ± 0.2***	1.2 ± 0.2***
V_d (ml)	14.8 ± 0.5	8.1 ± 1.1**	6.8 ± 0.3**
k_{el} (h^{-1})	0.31 ± 0.02	0.20 ± 0.01**	0.17 ± 0.03**

Results are expressed as the mean ± S.D. of three experiments. $^{***}p<0.001$; $^{**}p<0.01$; $^{*}p<0.05$, compared with conventional liposome.

ume (V_d) and elimination rate constant (k_{el}) for PEG liposome significantly decreased compared with conventional liposome. RSA-modification tended to decrease both parameters further, although not significantly. Tissue distributions of these liposomes were also studied at 1 h after intravenous injection and it was found that liposomes mainly distributed to the liver and the spleen, and that the distribution of liposomes into other organs/tissues was negligible (less than 5% of dose). Tissue clearance calculated by Eq. (3) showed that PEG liposome had significantly smaller values both for the liver and the spleen than non-PEG liposome (Fig. 2). RSA modification further and significantly decreased the hepatic clearance of PEG liposome, but this was not the case for the spleen where both PEG liposome and RSA/PEG liposome had the similar clearances (Fig. 2).

Then, to elucidate the mechanisms behind the hepatic disposition of PEG liposome and RSA/PEG liposome, the effect of trypsin treatment on the hepatic disposition of these liposomes was investigated in the rat liver single-pass perfusion study utilizing the perfusate containing 1% serum (v/v) (Fig. 3). The hepatic accumulation of RSA/PEG liposome (2.1 ± 0.3 nmol total lipid (TL)) was found to be significantly less than that of PEG liposome (13.1 ± 0.9 nmol TL), suggesting that RSA/PEG liposome has less affinity to the liver. Pre-treatment of the liver with trypsin drastically decreased the hepatic disposition of PEG liposome to 10% of control. However, this was not the case for RSA/PEG liposome where such a significant reduction was not observed. These results suggest that the hepatic disposition of PEG liposome can be mainly ascribed to the receptor-mediated uptake, but the further modification of liposome with RSA can avoid the hepatic disposition via the receptor-mediated endocytosis.

Since the recognition of surface-associated serum opsonins by their corresponding receptors is mainly a trigger for the receptor-mediated hepatic uptake of particles, the results shown in Fig. 3 also suggest that the amount of opsonins associated on the surface of PEG liposome would be reduced by RSA-modification. Therefore, we tried to evaluate the serum proteins associated on the surface of PEG liposome and RSA/PEG liposome quantitatively and qualitatively (Figs. 4 and 5). As shown in Fig. 4, it was revealed that the amount of serum proteins associated on the surface of RSA/PEG liposome was significantly smaller than that of PEG liposome. Furthermore, the serum proteins associated on these two liposomes were qualitatively analyzed by using SDS-PAGE (Fig. 5). Although a marked qualitative change in the surface-associated serum proteins by RSA modification was not observed, the relative amount of several serum proteins (e.g., around 200, 115 and 100 kDa in apparent molecular weight) was reduced by RSA modification.

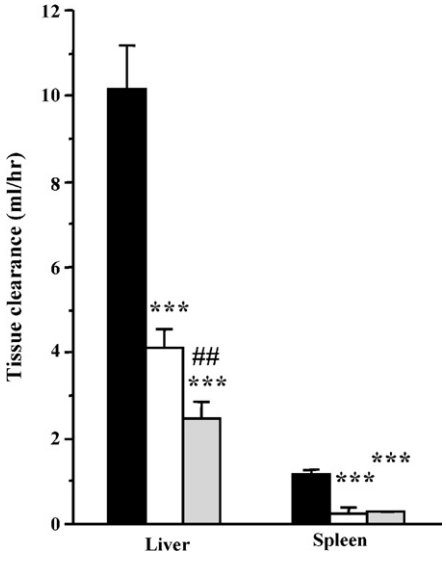

Fig. 2. Liver and spleen clearances of conventional (non-PEG), PEG and RSA/PEG liposomes. Results are expressed as the mean with a bar showing the S.D. of three experiments. (■) Non-PEG liposome; (□) PEG liposome; (▩) PEG/RSA liposome. $^{***}p<0.001$, compared with non-PEG liposome; $^{\#\#}p<0.01$, compared with PEG liposome.

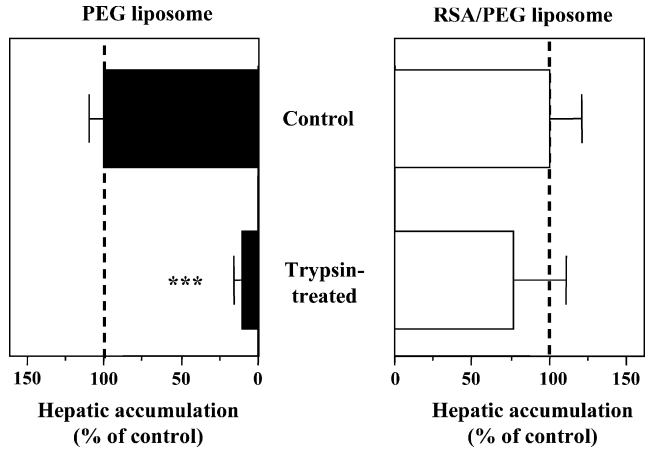

Fig. 3. Effect of pre-treatment with trypsin on the hepatic disposition of PEG liposome or RSA/PEG liposome in rat liver single-pass constant infusion experiments. Results are expressed as the mean with the bar showing the S.D. of three experiments. $^{***}p<0.001$, compared with control.

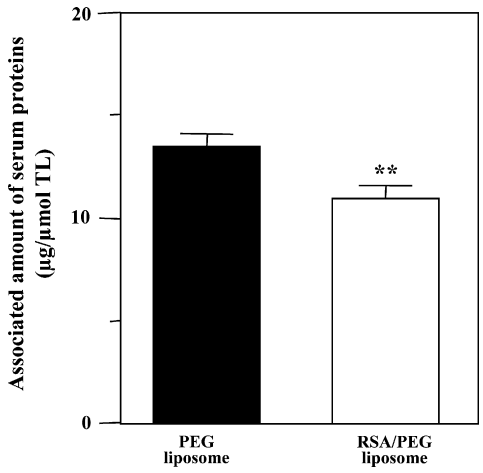

Fig. 4. Quantitative estimation of the rat serum proteins associated on the surface of PEG liposome and RSA/PEG liposome. The amount of serum proteins associated with RSA/PEG liposome was calculated by subtracting the amount of RSA coupled with liposomes from the total protein amount measured. Results are expressed as the mean with the bar showing the S.D. of three experiments. $^{**}p < 0.01$, compared with PEG liposome.

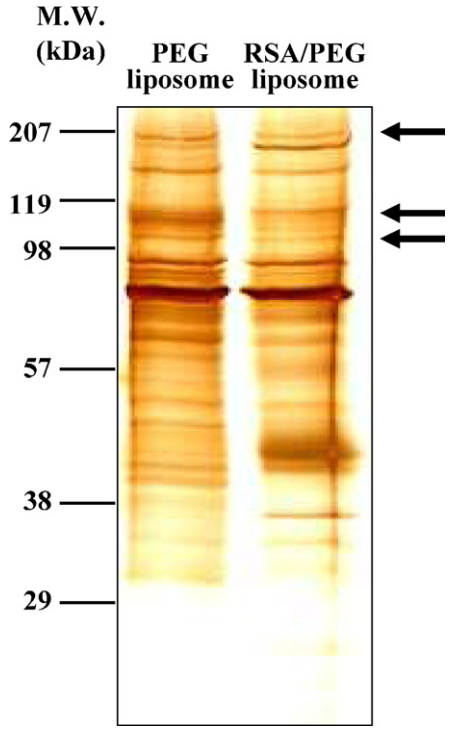

Fig. 5. Silver-stained SDS-PAGE of rat serum proteins associated on the surface of PEG liposome and RSA/PEG liposome. The same amount of protein (3 μg) was loaded onto each lane.

4. Discussion

Long-circulating particulate carriers can provide several advantages, for instance, for the development of artificial oxygen delivery or blood-pool imaging system (Weissig et al., 2000; Sakai and Tsuchi, 2006). Besides them, long-circulating particles are promising carriers for passive targeting of drugs into tumors or inflamed tissues where the integrity of the endothelial barrier is perturbed (Gabizon, 1992; Jang et al., 2003). One of the most widely-used methods for enhancing the circulation properties of liposomes is the inclusion of PEG within the membrane surface of liposomes (Blime and Cevc, 1990; Klibanov et al., 1990; Allen et al., 1991b). PEG is considered to form a hydrated shell hindering the interaction of liposomes with serum proteins, thereby greatly reducing the opsonization and uptake by macrophages.

The purpose of our present study was to investigate the effect of coupling of RSA onto the surface of PEG liposome on the in vivo disposition. Our preliminary study showed that the RSA modification significantly increased the particle size of PEG liposome with 100 nm in diameter, but not the one with 200 nm in diameter. Therefore, we utilized the liposome with 200 nm in diameter to exclude the effect of the increase in the particle size of PEG liposome by RSA modification. As a result, the modification of PEG liposome surface with albumin significantly reduced the hepatic disposition of liposomes, resulting in further prolongation of their blood circulation time. The result clearly indicates that albumin conjugated on the surface substantially influenced the hepatic handling of PEG liposome.

Chonn et al. (1992) reported that the amount of serum proteins associated on the liposomes used was inversely related to their circulation half-lives. Our present findings also revealed that RSA/PEG liposome, onto which less amount of serum proteins associated (Fig. 4), showed more prolonged blood circulation time than PEG liposome (Fig. 1 and Table 1). 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 (Needham et al., 1997; Nicholas et al., 2000; Johnstone et al., 2001). Originally, PEG incorporated into liposome forms the mushroom conformation, then PEG liposomes are thought to readily interact with serum proteins and with cell surface as well (Johnstone et al., 2001). On the other hand, a brush conformation, which is suggested to be induced by hydrostatic interactions between the surface of PEG liposome and serum proteins, can interfere with the subsequent interactions between PEG liposomes and phagocytes or other serum proteins (Mori et al., 1991; Johnstone et al., 2001). From these backgrounds, it is suggested that PEG molecules in RSA/PEG liposome would be in the brush conformation upon the introduction of RSA (Fig. 6). This could be the reason why the interaction of RSA/PEG liposome with serum proteins was significantly suppressed by the introduction of RSA (Fig. 4). Besides this speculation based on the change in the conformation of PEG molecule, other possible explanations for the reduced interaction of RSA/PEG liposome with serum proteins would be that (1) RSA introduced onto the surface of PEG liposome occupied the binding sites for the serum proteins and/or (2) RSA-modification further increased the apparent hydrophilicity of PEG liposome. However, the mechanisms behind the less association of RSA/PEG liposome with serum proteins remain to be elucidated and will be the subject of our further study.

Our previous study suggested that not only the amount but also the kind of serum proteins associated on the surface of polystyrene nanoparticles would be important determinants for their in vivo disposition (Ogawara et al., 2001; Furumoto et al.,

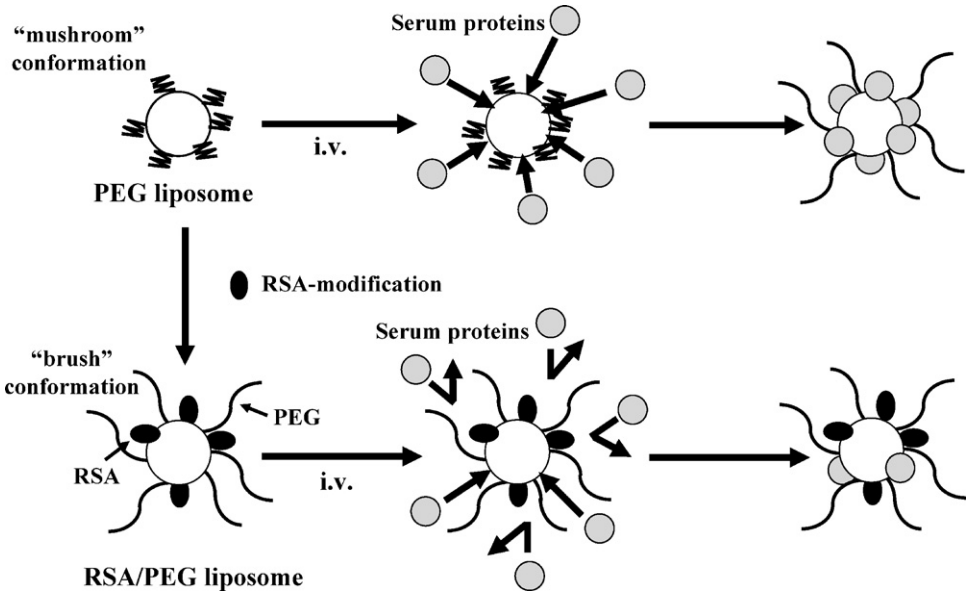

Fig. 6. Assumed mechanisms by which RSA-modification suppresses the association of serum proteins onto the surface of PEG liposome.

2002). Therefore, we performed SDS-PAGE analysis to qualitatively evaluate the serum proteins associated onto the surface of RSA/PEG or PEG liposome (Fig. 5). Although the modification of PEG liposome with RSA did not lead to a tremendous change in the kind of serum proteins associated compared with that of PEG liposomes, the association of some serum proteins (e.g., with apparent molecular weights of around 200, 115 and 100 kDa) was reduced (Fig. 5). Considering that the pretreatment of the liver with trypsin did not significantly decrease the hepatic disposition of RSA/PEG liposome (Fig. 3), some serum opsonins would be included in the proteins of which the amount adsorbed onto the surface was decreased. Western blotting will be useful to address the possible less-association of typical serum opsonins on RSA/PEG liposome and will be the subject of our further study.

In conclusion, the present study clearly indicated that the RSA modification on the surface of PEG liposome significantly decreased the hepatic disposition of liposomes, resulting in the more prolonged circulation time of PEG liposome after intravenous administration in vivo. This pharmacokinetic advantage of RSA/PEG liposome would be ascribed to the reduced association of the serum proteins including some given serum opsonins onto the surface.

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