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Research paper

Prolonged circulation time of doxorubicin-loaded liposomes coated with a modified polyvinyl alcohol after intravenous injection in rats

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Abstract

The purpose of this study was to evaluate the functions of a modified polyvinyl alcohol (PVA-R), which has a hydrophobic moiety, as a coating material for liposomes to be loaded with the anticancer drug, doxorubicin. The size controlled liposomes (egg phosphatidylcholine: cholesterol = 1:1 molar ratio) were prepared by the hydration method followed by extrusion. Drug encapsulation and surface modification with polymers (PVA and PVA-R) were carried out simultaneously using a modified pH gradient method. The existence of a thick polymer layer on the surface of the liposomes was confirmed by an increase in particle size and the amount of polymer on the liposomal surface, especially for the PVA-R-coated liposomes. The effects of polymer coating on the behavior of the liposomes in vivo were evaluated by measuring the circulation time and biodistribution of the drug after i.v. administration of the liposomal drug in rats. The PVA-R-coated liposomes showed a more prolonged circulating time for the drug with less uptake by the reticuloendothelial system after i.v. administration in rats, compared with non-coated liposomes. These results confirm that polymer possessing a hydrophobic anchor at its end, like PVA-R, is a suitable material for modifying the surface of doxorubicin-loaded liposomes to improve their stability in the circulating blood.

Keywords: Liposomes; Polymer coating; Doxorubicin; Reticuloendothelial system avoidance; Modified polyvinyl alcohol

1. Introduction

Liposomes have been extensively investigated as drug carriers for a variety of drugs including the anticancer drug, doxorubicin. The drug-loaded liposomes are usually injected intravenously for systemic application. However, there is usually rapid clearance from the circulating blood by the reticuloendothelial system (RES) located mainly in the liver and spleen [1,2], although this depends on the particle size and lipid composition of the liposomes [3,4]. This appears to be effective for the delivery of drugs to the RES, but is of no use for targeting drugs to specific parts of the body such as solid tumors other than in the RES. To avoid this disadvantage, 'stealth technology' has been developed. It has been reported that the formulation of ganglioside G_{M1}, polyethylene glycol attaching phospholipids (PEG-lip) and gluconic acid derivatives lead to a reduction of RES uptake and prolongation of the plasma half-life of liposomes administered intravenously [5–9]. A highly water-soluble polymer grafted onto the lipid bilayer of the liposomes exerts a long range mutual repulsion between adjacent bilayers and, presumably, prevents the interaction of liposomal surfaces with macromolecules, such as proteins, in the blood [10].

Polymer coating is an alternative method of modifying the surface of liposomes. It is a characteristic of the polymer coating method that the manner of coating is very simple, just mixing a liposome suspension and a polymer solution, without chemically linking the polymers to the lipid molecules. We [11] have reported the feasibility of coating the surface of liposomes with modified hydrophilic polymers such as polyvinyl alcohol or polyacrylic acid having hydrophobic anchor(s) (PVA-R or PAA-R). Previously [12], we confirmed the formation of a thick coating layer on the surface of liposomal particles and the improved physical stability of PVA-R coated liposomes.

In this study, we examined the effectiveness of PVA-R-coated liposomes as a carrier for the anticancer drug, doxorubicin, when administered intravenously in rats. After

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confirming the formation of doxorubicin-loaded, PVA-R coated and size-controlled (100 nm in diameter) liposomes composed of egg phosphatidylcholine and cholesterol in a molar ratio of 5:5, the fate of the PVA-R-coated liposomes in vivo was evaluated by measuring the circulation time and biodistribution of the drug after i.v. administration of the liposomal drug. The data suggested that coating of liposomes with PVA-R effectively improved the drug circulation time with reduced uptake of liposomes by the RES.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (COATSOME NC-10S) was purchased from the Nippon Oil and Fats Co., Japan. Cholesterol was obtained from the Sigma Chemical Co. Doxorubicin hydrochloride was kindly donated by the Kyowa Hakko Kogyo Co., Tokyo, Japan. Cholesteryl-anthracene-9-carboxylate (CA), used as a lipid marker, was purchased from LAMBDA, Austria. Polyvinyl alcohol (PVA205) and polyvinyl alcohol bearing a hydrophobic anchor ($C_{16}H_{33}$-S-) at the terminal of the polymer (PVA-R) were supplied by Kuraray Co., Japan. The degree of polymerization of PVA and PVA-R was 450, 480, respectively. Male Wistar rats (body weight 230–260 g, 11 weeks old) were obtained from Japan SLC Ltd., Japan and were maintained in standard housing. All other chemicals were commercial products of reagent grade.

2.2. Preparation of liposomes

The size-controlled liposomes with egg phosphatidylcholine and cholesterol in a 1:1 molar ratio were prepared by the hydration method followed by extrusion. In brief, the lipid mixture was dissolved in a small amount of chloroform in a 200 ml round-bottom flask and dried in a rotary evaporator under reduced pressure at 40°C to form a thin lipid film. The thin lipid film was dried in a vacuum overnight to ensure complete removal of the solvent. Multilamellar vesicles (MLV) were formed by adding a saline solution containing 50 mM citric acid to the flask followed by vortexing at 60°C and incubation for 1 h at 10°C. The MLVs were then extruded 12 times using an Extruder (Lipex Biomembranes, USA) equipped with a polycarbonate membrane filter (pore size: 1 μm or 0.4 μm, Nuclepore®) to produce small size liposomes (SUV).

For the preparation of CA-containing liposomes, a small amount of CA as a lipid marker was added to the lipid mixture before evaporation. CA-containing liposomes of diameter 100 nm were prepared by the hydration method followed by sonication (UR-200P, Tomy Seiko Co.).

Polymer-coated liposomes were prepared by mixing the resultant liposomal suspension (1 ml) with an equal amount (1 ml) of polymer solution, followed by incubation at 10°C for 1 h.

Doxorubicin was loaded into the liposomes by the pH gradient (interior acidic, pH 2.1) method [13] with some modifications. In the preparation, the liposomal suspension (1 ml) was mixed with an equal amount of doxorubicin aqueous solution, followed by adding a saline solution containing 0.2 mmol sodium carbonate (2 ml) diluted with distilled water (2 ml). This mixture was incubated with periodic mixing for 10 min at 60°C, followed by incubation for 1 h at 10°C. For the preparation of polymer-coated and doxorubicin-loaded liposomes, polymer coating and drug loading were carried out simultaneously. To coat the liposomes with polymer, a saline solution containing both 0.2–0.4 mmol sodium carbonate and an appropriate amount of PVA or PVA-R (4 ml) was used instead of the sodium carbonate solution in the drug-loading process described above.

In order to investigate the effect of temperature on the encapsulation efficiency of doxorubicin into liposomes, incubation after mixing the solutions was carried out at 10°C for 1 h instead of heating at 60°C.

2.3. Properties of liposomes

The particle size and distribution of the liposomes were measured with a dynamic light scattering method using LPA-300 equipment (Otsuka Electron Co.). An aliquot of each liposomal suspension was diluted with a large amount of distilled water.

The amount of doxorubicin loaded in the liposomes was determined by measuring the concentration of non-entrapped drug. In measuring the drug concentration, 3 ml of the liposomal suspension diluted 50 times with phosphate buffer (pH 5.0) was mixed with 0.7 ml 1 N sodium hydroxide, and the drug concentration in the samples was measured spectrophotometrically at 600 nm (UV-160A, Shimadzu), taking into consideration the turbidity of the system, which was also spectrophotometrically measured at 750 nm. The total amount of doxorubicin in the system was determined in the same manner after collapsing the liposomal structures by adding 0.06 ml 10% Triton solu-tion to the samples. The percentage of drug trapped was calculated by dividing the amount of the substance in the interior of the liposomes by the total amount in the system.

2.4. Animal experiments

All animal experimental protocols were approved by the animal welfare commission of Gifu Pharmaceutical University.

In monitoring the plasma levels of doxorubicin in rats, free or liposomal (non-, PVA- or PVA-R-coated) doxorubicin at a concentration of 2 mg/ml was injected via the tail vein at a dose of 5 mg doxorubicin/kg body weight. At appropriate intervals, blood was collected from the retro-orbital sinus. Subsequently, plasma was separated by centri-

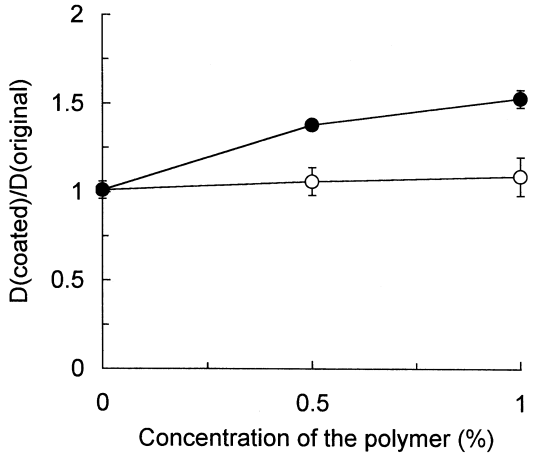

Fig. 1. Particle size change in liposomes following coating with PVA-R or PVA ($n = 3$). Lipid composition of liposomes: EPC/cholesterol =5:5. ●, PVA-R, ○, PVA.

fugation ($3000 \times g$, 10 min) and the drug concentration analyzed.

In monitoring the plasma pharmacokinetics of the carriers, CA-containing liposomes with or without polymer coating were injected into the rats at the same dose of total lipid as in the doxorubicin evaluation, followed by collection of plasma as described above.

In the drug distribution study, rats were injected in the same manner at a dose of 1 mg doxorubicin/kg body weight. At 1, 8 and 24 h after injection, the rats were sacrificed and the liver, spleen and heart were immediately removed, weighed and homogenized with citric acid buffered saline (CBS) (pH 8.0) (05 ml/g tissue).

The quantitative analysis of doxorubicin in plasma or each tissue was carried out by the HPLC method previously reported by Masuike et al. [14]. In short, the drug-in-plasma and tissue homogenates, both of which were diluted with CBS, pH 8.0, were extracted with an appropriate amount of toluene/ butanol (1:1, v/v) by vigorously mixing for 40 min in a reciprocal shaker (SR-2, TAITEC, Japan). The organic phase was then dried in a rotary evaporator (VEC-310, IWAKI, Japan) under reduced pressure at 60°C. The residues were dried in a vacuum overnight to ensure complete removal of the solvent, and dissolved in a carrier solution (0.03 mM $NH_4H_2PO_4$:MeOH = 3:7) for HPLC analysis. The samples were subjected to HPLC analysis with a fluorospectrophotometer (FP-920, JASCO, Japan) using daunorubicin as an internal standard.

To determine the quantity of the CA in plasma, 50 μl aliquots of the plasma samples were extracted with an appropriate amount of chloroform/methanol (1:1, v/v), followed by centrifugation (3,000 rev./min, 10 min). The concentration of CA was measured by the fluorescence in the resultant supernatant with a fluorospectrophotometer (Hitachi F3010).

The significance of the in vivo data was evaluated using Student's t-test and $P < 0.05$ was considered significant.

3. Results

3.1. Polymer coating of liposomes

The feasibility of polymer coating of the chosen liposomes with polyvinyl alcohol (PVA) and a modified polyvinyl alcohol (PVA-R) was evaluated by comparing the particle size of the liposomes before and after polymer coating. The liposomes used in this study were composed of EPC and cholesterol in a molar ratio of 5:5 and the particle size was 107.3 ± 8.2 nm. Mixing the liposomal suspension with the polymer solution and following incubation, i.e. the polymer coating process, led to an increase in the particle size of the liposomes. The change in particle size depended on the type and concentration of polymer used. The particle diameter of liposomes coated with PVA-R at a final concentration of 1% was 163.9 ± 12.9 nm, i.e. 1.53-fold that of the original liposomes (Fig.1). The increase in particle size of PVA-coated liposomes was relatively small. These results suggest the existence of a coating layer on the surface of the liposomes, especially for the PVA-R-coated liposomes. These results were relatively consistent with our previous observation for liposomes of dimilistoylphosphatidylcholine (DMPC)/dicetyl phosphate (DCP)/cholesterol in a molar ratio of 7:3:1 [12].

The amount of polymer coating the surface of the liposomes was estimated by measuring the polymer concentration of the supernatant in the centrifuged liposomal suspension after coating. The amount of PVA-R coating increased with the increasing concentration of PVA-R used in coating (Fig. 2). When the amount of coating was calculated on a molar basis by assuming the molecular weight of the polymer to be about 20000, the molar ratio of PVA-R to phospholipids is 1/270 at a final polymer concentration of 1 %. The value is almost the same for the DMPC/DCP/cholseterol (7:3:1) liposomes. Although only a thin coating layer was detected for PVA in measuring the particle size change, about one-third the amount of PVA relative to PVA-R was observed as the coating layer at the same polymer concentration. These differences in coating

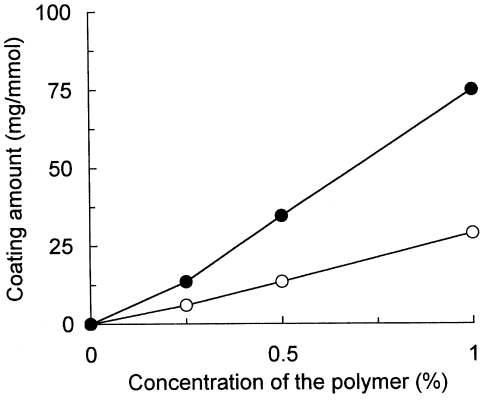

Fig. 2. Amount of PVA-R- or PVA coated onto liposomes ($n = 3$). Lipid composition of liposomes: EPC/cholesterol =5:5. ●, PVA-R, ○, PVA.

Table 1
Doxorubicin concentration in plasma of rats after intravenous administration of doxorubicin solution (sol), non-coated liposomal doxorubicin (100 nm, non-100), PVA-coated liposomal doxorubicin (100 nm, PVA100), PVA-R-coated liposomal doxorubicin (100 nm, PVA-R100), non-coated liposomal doxorubicin (300 nm, non-300) and PVA-R-coated liposomal doxorubicin (300 nm, PVA-R300)[a]

Time (h)	Conc. of doxorubicin in plasma (μg/ml)					
	sol	non-100	PVA100	PVA-R100	non-300	PVA-R300
0.5	0.15 ± 0.01	73.1 ± 2.7	76.2 ± 3.1	88.8 ± 5.2**	21.9 ± 2.0	83.0 ± 6.8
1	0.15 ± 0.00	63.5 ± 2.1	60.8 ± 5.5	79.1 ± 4.0***	22.3 ± 2.9	71.2 ± 2.0
2	0.14 ± 0.00	42.7 ± 2.8	45.3 ± 3.4	57.0 ± 3.6**	17.0 ± 4.5	46.8 ± 2.3
4	0.13 ± 0.01	28.8 ± 2.2	34.0 ± 2.0	38.2 ± 2.2**	13.3 ± 5.9	25.2 ± 3.6
8	0.13 ± 0.01	13.5 ± 0.8	16.5 ± 0.7	19.4 ± 2.3*	3.3 ± 1.7	13.8 ± 1.1
12	0.11 ± 0.00	4.1 ± 0.7	5.2 ± 0.4	7.7 ± 1.2**	1.1 ± 0.3	5.7 ± 1.3
24	0.00 ± 0.00	0.7 ± 0.0	1.1 ± 0.5	1.4 ± 0.2**	0.2 ± 0.1	0.2 ± 0.1
AUG (μg/ml h)$^{0-24}_{h}$	2.22	353.2	386.7	473.3	121.9	363.2

[a] The number of animals is given in the caption to Fig. 4. Lipid composition of liposomes: EPC:cholesterol = 5:5. Dose of doxorubicin: 5 mg/kg weight.

amount could be attributed to the different coating procedures for these polymers [12].

When doxorubicin was encapsulated into the liposomes (EPC/cholesterol = 5:5) using the pH gradient method, the encapsulation efficiency of drug was 50.3 % for the non-coated liposomes. The encapsulation efficiency was markedly improved to 93.5% by heating the system during the encapsulation of drug as described in Section 2.2. The increased temperature made the lipid membrane more fluid, which could facilitate drug transport through the lipid membrane of the liposomes. Similar encapsulation efficiency was observed for PVA-coated (93.2%) and PVA-R-coated (93.3%) liposomes using the modified pH gradient method.

Although the data presented in Figs. 1 and 2 were observed for the corresponding empty liposomes, almost the same values for the particle size change and polymer coating amount were found for the doxorubicin-loaded ones under typical conditions. Thus, we confirmed the preparation of PVA- and PVA-R-coated, and doxorubicin-loaded liposomes using the modified pH gradient method.

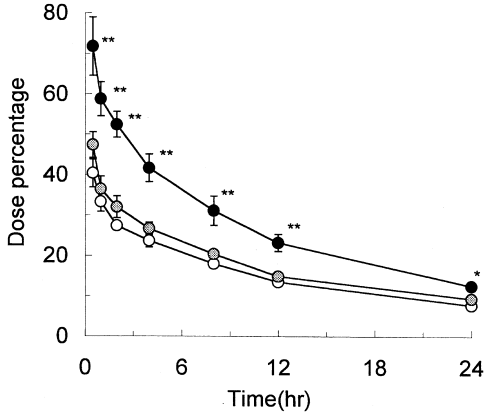

Fig. 3. Effect of polymer coating on the circulation profiles of liposomes in plasma after i.v. injection ($n = 3$). Lipid composition of liposomes: EPC/cholesterol =5:5. ●, PVA-R-coated liposomes, shaded circle, PVA-coated liposomes and ○: non-coated liposomes. *$P < 0.05$, **$P < 0.01$: significantly different from the level for PVA-coated liposome. The level for PVA-R-coated liposomes is significantly different from that for non-coated liposomes, $P < 0.01$.

3.2. Plasma pharmacokinetics

The doxorubicin solution, or that encapsulated into the liposomes with or without polymer coating, was injected into male Wistar rats via the tail vein at the same dose. The plasma pharmacokinetics of doxorubicin is presented in Table 1. In the case of i.v. administration of doxorubicin solution, the drug disappeared from the circulating blood very rapidly owing to its short half-life. In contrast, liposomal doxorubicin showed a long circulating time, although it depended on the particle size of the liposomes. When the liposomes were coated with PVA-R, the drug levels in plasma were higher than those for the non-coated liposomes at each sampling time. The effect of polymer coating on the circulation time of doxorubicin was evident from the relatively larger liposomes (approx. 300 nm in diameter), which were prepared by extruding with a membrane of 400 nm pore size (Table 1). In the case of PVA coating, the effect was not as large as that for PVA-R coating. The area under the doxorubicin concentration-time curve (AUC) from 0 to 24 h post-injection in Table 1 clearly shows the effects of polymer coating on the systemic drug circulation described above.

One possible explanation for the enhanced drug circulation with PVA-R-coated liposomes is the prolonged circulation time of the liposomes following PVA-R coating. In order to evaluate the actual circulation profiles of the carriers, CA-containing liposomes with or without polymer coating were prepared and injected into the rats in the same manner and at the same dose of total lipid as used to study the pharmacokinetics of doxorubicin. The plasma pharmacokinetics of the liposomes is shown in Fig. 3. When the

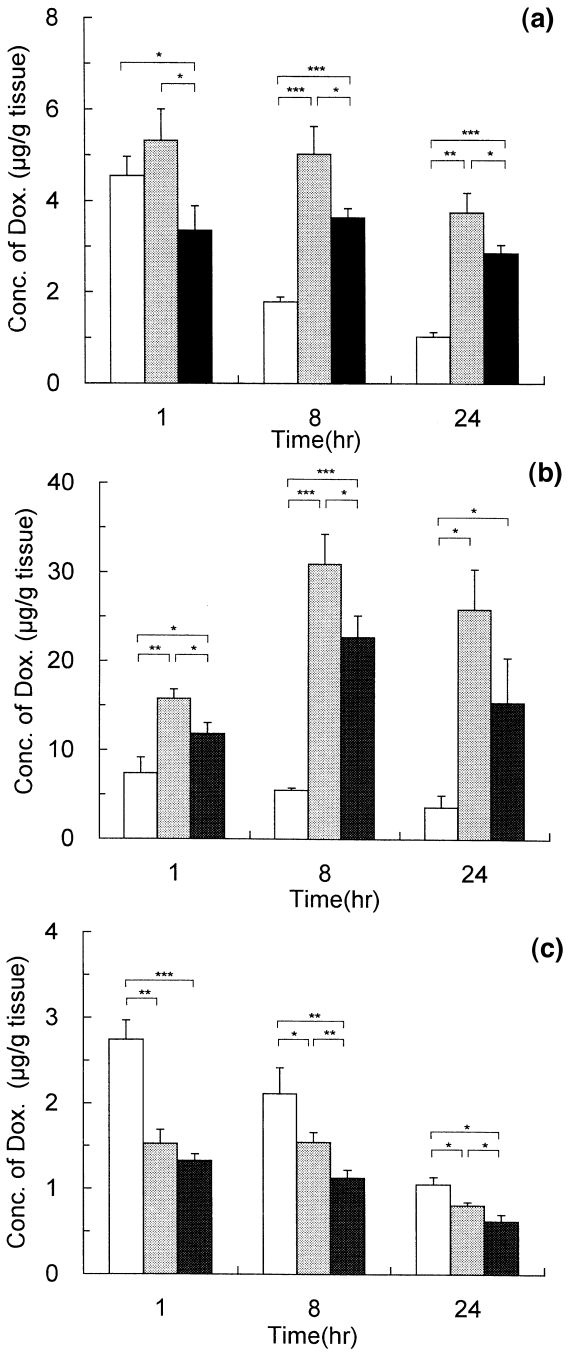

Fig. 4. Doxorubicin concentration in the tissues (a, liver, b, spleen and c, heart) of rats after intravenous administration of doxorubicin solution or liposomal doxorubicin ($n = 3$). Lipid composition of liposomes: EPC:cholesterol = 5:5. ■, PVA-R-coated liposomal doxorubicin; shaded square, non-coated liposomal doxorubicin and □, doxorubicin solution. Particle size of the liposomes: approx. 100 nm. Dose of doxorubicin: 1mg/kg weight.

CA-containing liposomes were coated with PVA-R, the liposomal circulation time became much longer than that for the non- and PVA-coated liposomes. These profiles confirm the effectiveness of PVA-R coating in improving the liposomal stability in the bloodstream.

3.3. Distribution study

The prolonged systemic circulating time of polymer-coated liposomes may be attributed to the reduction in uptake by the RES in the liver and spleen. To clarify the reduction in RES uptake following polymer coating, the drug concentrations in liver and spleen were measured at appropriate intervals after injection of liposomal doxorubicin, with or without PVA-R coating or doxorubicin solution (Fig. 4a,b). Comparing the drug concentration for PVA-R-coated liposomal doxorubicin with that for non-coated, the former was significantly lower than the latter at each sampling point. These observations confirm the reduction in RES uptake of liposomes following polymer coating with PVA-R. On administering doxorubicin solution to rats, the highest concentration of drug in liver was observed at 1 h and the concentration decreased comparatively rapidly with time. On the other hand, when doxorubicin was encapsulated into liposomes, the drug concentration in liver decreased more slowly compared with the injection of free doxorubicin. This may be due to the continuous influx of drug from the blood via RES uptake of liposomal doxorubicin.

The drug concentration in spleen after injection of liposomal doxorubicin was considerably higher than that for the doxorubicin solution. This result clearly shows the RES uptake of the particulate systems in spleen. A significant difference in drug concentration was observed between non-coated and PVA-R-coated liposomes as well as in the liver, which suggests again the inhibitory effect of the polymer coating on RES uptake. As far as the time-course profiles of drug concentration are concerned, the liposomal doxorubicin produced a maximum drug concentration at 8 h after injection, while the free doxorubicin steadily decreased with time. This can be also attributed to the continuous uptake of liposomal doxorubicin by the RES.

Drug levels in the heart as a parameter of cardiac toxicity are shown in Fig. 4c. Following administration of free doxorubicin, the drug concentrations in heart were very high at any sampling time. Formulating doxorubicin as a liposomal system significantly reduced drug distribution to the heart. At 1 h, in particular, the drug concentrations of non- and PVA-R-coated liposomes were 0.55- and 0.49-fold that of free doxorubicin. It was interesting that the drug concentration in heart following injection of PVA-R-coated liposomes was lower than that for non-coated liposomes although the corresponding drug concentration in blood was higher at any of the sampling points.

4. Discussion

In a previous paper, we confirmed that a steric coating layer was formed on the surface of liposomes composed of DMPC, DCP and cholesterol (7:3:1 molar ratio) with PVA-R. The mechanism for the formation of the thick coating

layer with PVA-R involved anchoring the hydrophobic portion of the polymer to the lipid membrane at the surface of the liposomes. When PVA was used as the coating polymer, the resulting coating layer was not thick owing to the coating mechanism, simple physical adsorption. Similar coating properties of these polymers were observed for the EPC:cholesterol (5:5) liposomes (Figs. 1 and 2).

Much attention has been paid to the reduction in RES uptake of liposomes, since Allen et al. [5] reported that the formulation of the monosialo-ganglioside (G_{M1}) in liposomal systems was effective in prolonging their blood circulation. The synthetic lipids linking with polyethylene glycol (PEG-lip) have been found to form sterically stabilized liposomes with both natural and synthetic phospholipids, which can markedly prolong the circulating time in blood after i.v. injection. The clearance and tissue distribution of the sterically stabilized liposomes following i.v. administration has been examined by a number of research groups [15]. It is well accepted that the polymer layer formed on the liposomal surface may effectively protect the liposomes from interacting with plasma proteins in the blood, leading to a reduction in RES uptake and prolonged circulating time in blood [10].

When doxorubicin in various formulations was injected into rats via the tail vein, higher retention of the drug in blood was observed for the PVA-R-coated liposomes than for the non-coated liposomes (Table 1). This result strongly suggests that the circulation time of liposomes in the bloodstream is prolonged by coating with PVA-R as well as by using PEG-lip-containing liposomes. This was confirmed by measuring the circulation time of the liposomes themselves using a lipid marker CA (Fig. 3).

The coating effect on the circulation time of doxorubicin was more marked for the relatively larger liposomes (Table 1). This is because these larger liposomes (usually more than 200 nm in diameter) disappeared rapidly from the blood circulation compared with the smaller vesicles after i.v. administration because of the enhanced RES recognition [3,4]. Measuring the doxorubicin concentration in liver and spleen after i.v. administration, a statistically significant difference in concentration was observed between PVA-R-coated and non-coated liposomes at all sampling intervals (Fig. 4). The lower concentration of PVA-R-coated liposomes suggests a reduction in the RES uptake of liposomes following coating with PVA-R.

The reduction in RES uptake of sterically stabilized liposomes, such as PEG-lip-containing liposomes, is generally attributed to the reduced binding of plasma proteins to the liposomes [10]. The coating layer of PVA-R-coated liposomes would be steric enough to prevent this unfavorable adsorption of protein under biological conditions. In considering the steric stabilizing effect of polymer coating, both the hydrophilicity and flexibility of the coating polymer layer may be responsible for reducing the RES uptake of particles. However, when the liposomes were coated with PVA, no significant improvement in the circulation time of the particles was observed (Fig. 3). Since PVA forms a coating layer on the surface of the liposomal particles (Fig. 2) and PVA is a typical hydrophilic polymer, this suggests that the flexibility of the coating layer is more important for the longer circulation of the particles in bloodstream.

An alternative explanation for the ineffectiveness of PVA-coating is the dissociation of the polymer from the liposomes when the liposomes enter the bloodstream. It is presumed that the tendency to detach depends on the coating mechanism, i.e. PVA-R is not easily dissociated from the liposomal surface because of anchoring of the hydrophobic moiety to the phospholipid bilayers, while it is relatively easy for the PVA in the coating layer to detach since simple physical adsorption is involved. This tendency may be reflected in the retention of the coated liposomes in the bloodstream.

Generally, doxorubicin tends to be taken up by heart muscle, resulting in severe toxicity [16,17]. The use of drug carriers including liposomes is expected to reduce this side-effect by controlling the distribution of the drug. In comparing the drug concentrations in heart post i.v. administration of the drug, encapsulation of the drug into liposomes was found to significantly reduce distribution to the heart (Fig. 4c). As we confirmed that the liposome particles could not be distributed to the cardiac cells, by using CA-containing liposomes (data not shown), it is suggested that the free doxorubicin leaked from the liposomes is responsible for the small amount of doxorubicin detected in the heart following the use of liposomal doxorubicin.

In conclusion, polymer coating is effective in prolonging the circulation time of doxorubicin-loaded liposomes. It was demonstrated that a polymer possessing a terminal hydrophobic anchor like PVA-R is a suitable material for modifying the surface of liposomes. Although the effectiveness of the polymer-coated liposomes is not yet as high as PEG-derivative-containing liposomes in terms of retention in the bloodstream after i.v. injection, polymer coating is expected to be a promising method for preparing drug carriers to avoid RES uptake because of its simplicity. It may be possible to enhance the effectiveness of polymer-coated liposomes by improving the liposomal formulation by optimizing factors including lipid composition, the particle size and molecular weight of the polymer.

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