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Effect of surface properties of liposomes coated with a modified polyvinyl alcohol (PVA-R) on the interaction with macrophage cells

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Abstract

The purpose of this study was to investigate the effect of a polymer coating using modified polyvinyl alcohol (PVA-R) on the interaction between liposomes and macrophage cells (J774 cells). The PVA-R-coated liposomes, which were labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocianin perchlorate (DiI) as a fluorescence reagent, were prepared with the conventional hydration method followed by extrusion and surface modification with PVA-R. The effects of polymer coating on the interaction with J774 cells were evaluated by using flow cytometry and confocal laser scanning microscopy (CLSM). When liposomes with or without PVA-R coating were incubated with J744 cells, the fluorescence emission intensity of DiI from J774 cells was significantly smaller than in the case of non-coated liposomes. This result indicates that decreased interaction of PVA-R-coated liposomes with macrophage cells could be well detected using flow cytometry and CLSM. These *in vitro* tests explained the differences in blood circulation of polymer-coated liposomes having different lipid formulations in rats.

Keywords: Liposomes; Macrophage; J774 cells; Polymer coating; Flow cytometry

1. Introduction

A wide variety of drugs has been incorporated in or associated with liposomes, one of the excellent colloidal drug carrier systems for intravenous (i.v.) injection (Gregodiadis, 1988; Lasic and Papahadjopoulos, 1998). Drug carrier systems must be retained in the bloodstream for a long time to allow for passive targeting of the drug. It has been demonstrated that surface modification of liposomes with polymers leads to longer circulation in the blood. The polymer coating method involves simply mixing a liposome suspension and a polymer solution, without chemically linking the polymers to the lipid molecules. We have reported the feasibility of coating the surface of liposomes with modified hydrophilic polymers such as polyvinyl alcohol or polyacrylic acid with hydrophobic anchor(s) (PVA-R or PAA-R). The particle size of PVA-R-coated liposomes remained unchanged in the presence of serum, while non-coated ones aggregated in the serum. This phenomenon might relate to the steric hindrance effect of the PVA-R layer on the surface of liposomes (Takeuchi et al., 1994, 1998).

Flow cytometry is a method used widely in biological and medical research that is based on the detection of a fluorescent marker that binds to specific cell sites and thereby enables the measurement of various characteristics of individual cells (e.g., size, shape, and fluorescent intensity) suspended in a fluid stream. Flow cytometry has powerful analysis functions, enabling the evaluation of cells or particles at an extremely rapid rate, up to 40,000 events per second, making this technology ideal for the reliable and accurate quantitative evaluation of cell populations and even for the selection of specific cells (Shapiro, 1995; Ibrahim and van den Engh, 2003; Hai et al., 2004; Sato et al., 2006).

Several experiments have been carried out to investigate the interaction between PEGylated liposomes and macrophages *in vitro* (Allen et al., 1991; Daleke et al., 1990; Zeisig et al., 1996). J774 cells are known to be a representative model of resident macrophages fixed in the sinusoids of the RES. It was reported that PVA-R-coated liposomes showed prolonged circulation time with reduced uptake by RES (Takeuchi et al., 2000). However, no investigation of the interaction of PVA-R-coated liposomes with J774 cells has been reported. In this study, we investigated the interaction of PVA-R-coated liposomes with macrophage cells under various conditions *in vitro* using flow cytometry to detect the fluorescence intensity of a fluorescent

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marker of liposomes adsorbed by or incorporated into J774 cells.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (EPC, COATSOME NC-50) was purchased from Nippon Oil and Fats Co., Ltd., Japan. Cholesterol was obtained from Sigma Chemical Co., Ltd. 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocianin perchlorate (DiI), used as a fluorescent reagent of lipid, was purchased from LAMBDA, Austria. Polyvinyl alcohol having a hydrophobic anchor ($C_{16}H_{33}S$–) at the terminal of the polymer (PVA-R) was supplied by Kuraray Co., Ltd., Japan. The degree of polymerization of PVA-R was 480. The derivative of hydroxypropylmethylcellulose having hydrophobic moieties ($C_{18}H_{37}$) in the molecules (HPMC-R) was supplied by Sankyo Chemical Co., Ltd., Japan. The molecular weight and the substituting ratio of long alkyl chain of HPMC-R were ca. 200,000 and 1.58 mol/mol, respectively. Murine macrophage J774 cells (Riken Cell Bank, Japan) were used for evaluating the interaction with liposomes. All other chemicals were commercial products of reagent grade.

2.2. Preparation of polymer-coated liposomes

Size-controlled liposomes with various lipid compositions were prepared using the hydration method followed by extrusion. The lipid mixture containing DiI (0.4% mol) was dissolved in a small amount of chloroform in a 100 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 (MLVs) were formed by adding phosphate buffered saline (PBS) to the flask followed by vortexing at 60 °C. The MLVs were then extruded 30 times using an extruder (LiposoFast®-Pneumatic, Avestin, Inc., Ottawa, ON, Canada) equipped with a polycarbonate membrane filter (pore size: 0.1 or 0.2 μm, Nuclepore®) to produce small unilamellar vesicles. The particle size of non-coated liposomes used as controls was maintained at 100–130 nm.

For preparation of polymer-coated liposomes, an appropriate amount of PVA-R or HPMC-R was dissolved in PBS at pH 7.4. An aliquot of the liposome suspension was mixed with the same volume of polymer solution of various concentrations (0–4%, w/v). The final polymer concentration in the liposome suspension was 0–2% (w/v). The mixed solution was incubated at 10 °C for 60 min.

2.3. Evaluation of physicochemical properties of liposomes

The particle size and size distribution of the liposomes were measured with a dynamic light scattering method using Zetasizer (Malvern Co.). An aliquot of each liposomal suspension was diluted with a large amount of distilled water. Table 1 shows the particle size of the polymer-coated liposomes.

Table 1
Particle size of polymer-coated liposomes

Polymer	Polymer concentration (%, w/v)	Lipid composition (molar ratio)	Particle size (nm)
Non	–	EPC:Chol = 9:1	127.1
		EPC:Chol = 5:5	136.5
PVA-R	0.5	EPC:Chol = 9:1	161.2
	0.5	EPC:Chol = 5:5	187.3
	1	EPC:Chol = 5:5	179.4
	2	EPC:Chol = 5:5	182.2
HPMC-R	0.25	EPC:Chol = 5:5	188.8
	0.5	EPC:Chol = 5:5	208.7
	1	EPC:Chol = 5:5	549.9

2.4. Cell culture and preparation

J774 murine macrophage cells were maintained as an adherent culture in humidified atmosphere (5% CO_2/95% air) at 37 °C in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 IU/mL penicillin, and 100 IU/mL streptomycin (GIBCO). We plated 6×10^5 J774 cells in 12-well plates (Becton Dickinson) for flow cytometry study and 3×10^5 J774 cells in the Lab-Tek chamber slide system (Nunc) for confocal laser scanning microscope study. After 48 h of adherence, a part of the old medium was replaced with the DiI-labeled liposomes in the new medium (final lipid concentration: 1 mg phospholipid/mL) and incubated at 37 °C.

2.5. Evaluation of the interaction of liposomes with flow cytometry

At appropriate intervals, old culture medium was removed and then J774 cells were washed three times with ice cold PBS and dissociated from plates using a cell scraper. The resultant cell suspension was filtered with a 41 μm filter (Millipore), diluted 5-fold with PBS, and analyzed with a flow cytometer (Becton Dickinson, FACSCalibur™) using CellQuest software (Becton Dickinson). The laser was an air-cooled argon ion laser emitting at 488 nm, thus allowing two light scatter parameters and three fluorescence channels to be measured. Fluorescence signals of DiI were detected using a bandpass filter at 585 ± 21 nm (fluorescence-2 channel). We counted 10,000 events for blank cells and for cells with liposomal formulation under the same conditions. The resultant data was displayed as the fluorescent intensity obtained from the analysis using CellQuest software. All data are shown at a revised value that was calculated by subtracting the fluorescent intensity of control cells from that of cells with DiI-labeled liposomes.

2.6. Observation of the fluorescence of DiI with a confocal laser scanning microscope

After incubation at 37 °C for 2 h, J774 cells were washed three times with ice cold PBS and then fixed with paraformaldehyde at 4 °C for 10 min. The coverslips were placed on a drop of SlowFade® Light Antifade (Molecular Probes). Each cell

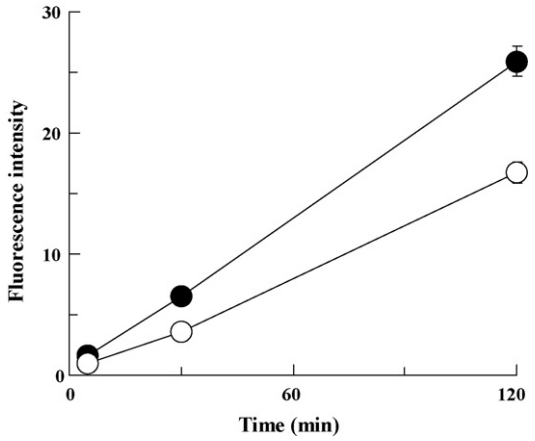

Fig. 1. Effect of PVA-R coating on the interaction with J774 cells ($n = 3$). Lipid composition of liposomes: EPC:Chol = 9:1. ●: non-coated liposomes, ○: PVA-R-coated liposomes.

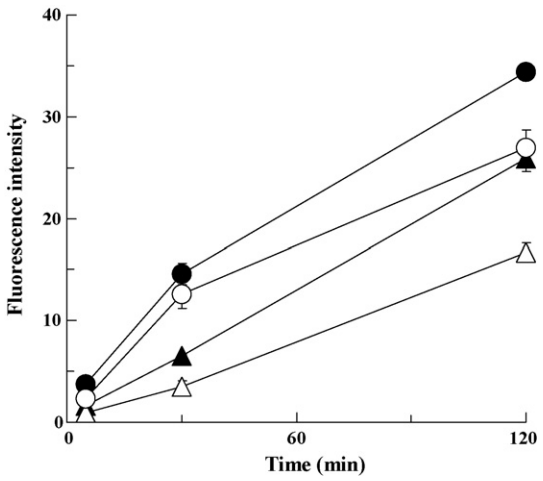

Fig. 3. Time courses of the fluorescence intensity of DiI in J774 cells that interacted with liposomes having different cholesterol content ($n = 3$). Lipid composition of liposomes: EPC:Chol = 5:5 (circles) or 9:1 (triangles). Closed symbol: non-coated liposomes, open symbol: PVA-R-coated liposomes.

sample was placed on the confocal laser scanning microscope (CLSM, LSM510, Zeiss) and observed at an excitation wavelength of 550 nm and an emission wavelength of 570 nm.

3. Results

The fluorescence intensity of DiI in J774 cells after incubating with either conventional liposomes or PVA-R-coated ones is shown in Fig. 1. At the initial stage, J774 cells themselves show negligible fluorescence emission, since there is little or no interaction between J774 cells and DiI-labeled liposomes. The fluorescence intensity of DiI detected from J774 cells gradually increases as a function of incubation time. PVA-R-coated liposomes show lower intensity than do non-coated ones throughout the incubation period.

Fig. 2 shows the CLSM photographs of J774 cells after the treatment with non-coated or PVA-R-coated liposomes for 120 min. The localized DiI molecules in J774 cells were clearly visualized by CLSM study. On the CLSM images, the red emission of DiI molecules in the system with non-coated liposomes was greater than that with PVA-R-coated ones. This finding corresponds to the result of flow cytometry, as shown in Fig. 1.

The DiI-labeled liposomes having different cholesterol contents with or without PVA-R coating were incubated with J774 cells under the same lipid dose. The interaction profiles of the liposomes were evaluated by measuring the fluorescence intensity of DiI detected from J774 cells with flow cytometry (Fig. 3). The liposomal formulation of EPC55, in which the cholesterol amount was higher than that of EPC91, shows the higher fluorescence intensity, leading to a stronger interaction of EPC55 throughout the incubation period. The PVA-R coating suppressed the interaction with J774 cells regardless of the cholesterol content.

Even though non-coated EPC55 was coated with PVA-R, a higher fluorescence intensity of DiI was detected from J774 cells than EPC91, suggesting that the interaction of PVA-R-coated EPC55 with J774 cells is higher than that of non-coated EPC91. The DiI-labeled liposomes coated with different PVA-R concentrations were incubated with J774 cells, and the fluorescence intensity of DiI after incubation with three types of PVA-R-coated liposomes for 30 min is shown in Fig. 4. This figure reveals that the fluorescence intensity of DiI detected from J774 cells decreased with increasing the PVA-R concentration, indicating that the PVA-R concentration was related to the interaction with J774 cells.

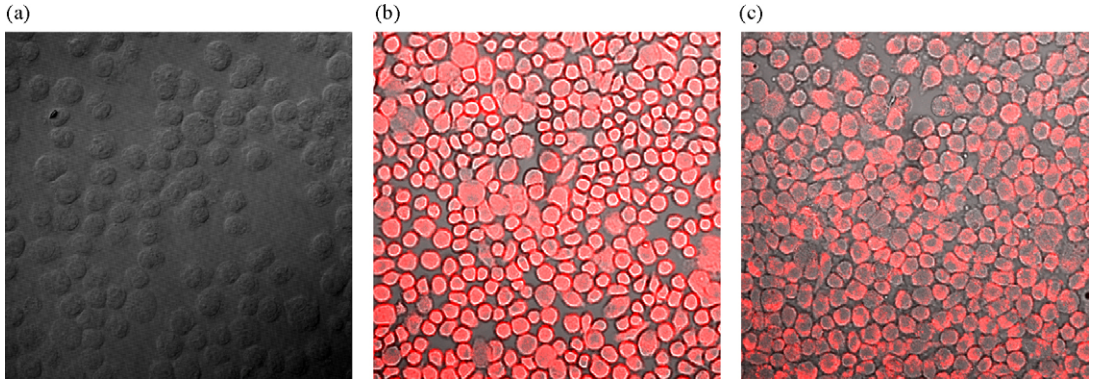

Fig. 2. CLSM images of DiI spots detected from J774 cells; (a) control, (b) non-coated liposomes, (c) PVA-R-coated liposomes.

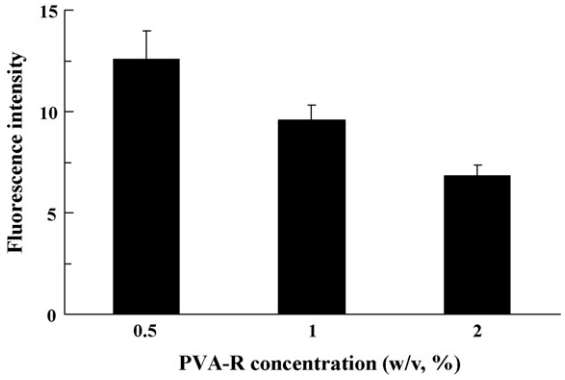

Fig. 4. Effect of PVA-R concentration in coating on the interaction with J774 cells ($n = 3$). Lipid composition of liposomes: EPC:Chol = 5:5. Incubation time: 30 min.

The DiI-labeled liposomes coated with different types of polymer were incubated with J774 cells, and the fluorescence intensity of DiI after incubation with different polymer-coated liposomes for 30 min is shown in Fig. 5. The fluorescence intensity of HPMC-R-coated liposomes decreased with an increase in HPMC-R concentration, indicating a similarity with the PVA-R system, in which the increase of polymer concentration suppressed the molecular interaction with the J774 surface. The fluorescence intensity of DiI detected from J774 cells in the systems with 1% PVA-R- and 0.5% HPMC-R-coated liposomes was estimated as 9.55 ± 0.79 and 8.51 ± 0.76, respectively.

4. Discussion

The liposomes used in the present study were coated with PVA-R or HPMC-R. The existence of a modified hydrophilic polymer coating layer was assumed for liposomal formulations with an increase in particle size of polymer-coated liposomes. It has been reported that the coating mechanism of a modified hydrophilic polymer could be explained by an anchoring of the hydrophobic moiety of PVA-R or HPMC-R to the lipid membrane of the liposomes (Takeuchi et al., 1998).

Figs. 1 and 2 demonstrate the effect of PVA-R coating on the interaction with J774 cells. It has been reported that macrophages participate in phagocytosis of particles via the Fc, complement, mannose or lectin receptors found on their surface *in vivo*. Liposomal endocytosis *in vitro* differs in this type and therefore liposomes must bind to the cell surface and be taken up by nonspecific mechanism (Miller et al., 1998). The transfer of DiI molecules from liposomes to J774 cells was induced by either an adsorption of the liposomes onto the cell surface or an incorporation of the liposomes into the J774 cells. Thereafter DiI molecules in liposomes transferred to J774 cells, showing a fluorescence emission that can be detected with flow cytometry or confocal laser scanning microscopy. As shown in Fig. 2, the fluorescence emission of DiI can be observed, which confirmed the transformation of DiI molecules from liposomes to J774 cells. The difference of interaction between liposomal formulation and J774 cells could be quantitatively evaluated by comparing the fluorescence intensity of DiI detected with flow cytometry.

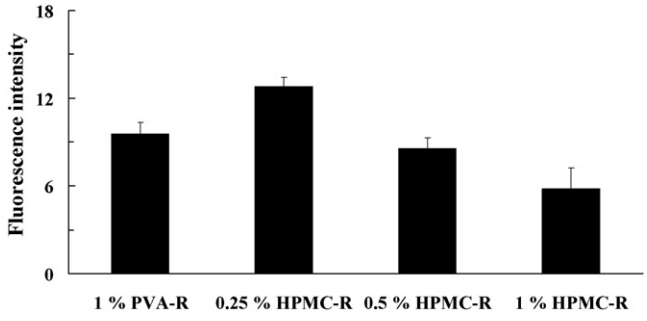

Fig. 5. Effect of the type of modified hydrophilic polymer on the interaction with J774 cells ($n = 3$). Lipid composition of liposomes: EPC:Chol = 5:5. Incubation time: 30 min.

The polyethylene glycol (PEG) chain of liposome surfaces has a steric hindrance effect on the interaction of the liposomes with various molecules; this effect is also considered to reduce the interaction of liposomes with cells (Kuhl et al., 1994; Zeisig et al., 1996; Ishiwata et al., 1998). We have reported that a steric PVA-R layer could affect the interaction of liposomes with proteins, inhibiting protein adsorption in the presence of calf serum (Takeuchi et al., 1998). Present results suggested that the PVA-R layer has a steric hindrance effect on the interaction with J774 cells, and the introduction of a PVA-R layer on the liposomal surface could inhibit the interaction of the liposomal core and J774 cell in the same manner as occurs with PEG-coated liposomes. Consequently, the low fluorescence intensity of DiI was attributed to the weak interaction with J774 cells, which was suppressed by the existence of PVA-R on the surface of liposomes.

Fig. 3 shows the DiI-labeled liposomes having different cholesterol contents with or without PVA-R coating were incubated with J774 cells under the same lipid dose. The PVA-R coating would be completed by the anchoring of its hydrophobic moiety to the lipid membrane. The amounts of PVA-R that interacted with EPC55 and EPC91 liposomes were estimated as 74.2 and 32.6%, respectively, using the previously reported method (Takeuchi et al., 2000). Since coating efficiency is affected significantly by cholesterol content, a more hydrophobic lipid membrane might facilitate the anchoring of PVA-R. It was expected that a sufficient amount of PVA-R on the surface of liposomes could change the surface properties sufficiently to suppress the interaction with J774 cells. However, the interaction of PVA-R-coated EPC55 with J774 cells was higher than that of non- or PVA-R-coated EPC91 in spite of the higher coating efficiency of PVA-R to EPC55 than EPC91. This result would be due to the excess amount of cholesterol in liposomal formulation of EPC55. Shin et al. reported that the excess amount of cholesterol formulated to the liposomes might form clusters in the resultant liposomes (Shin and Freed, 1989). The surface might be heterogeneous if the clusters have been formed on the surface of the EPC55. We confirmed that PVA-R-coated EPC55 has as great a PVA-R layer as that of PVA-R-coated EPC91, since the particle size increased from 130 to 160–190 nm, as shown in Table 1. However, we assumed it would be difficult for the PVA-R molecules to coat the liposomal surface homogeneously due to the cluster structure of cholesterol, resulting in the less

steric hindrance effect on the interaction with J774 cells. It is important to achieve a homogeneous coating with PVA-R on the liposomal surface to avoid the uptake of the PVA-R-coated liposomes by macrophage cells.

Fig. 4 shows the fluorescence intensity of DiI after incubation with three types of PVA-R-coated liposomes for 30 min. Although the particle size of each liposome remained unchanged, as shown in Table 1, the amount of resultant coating layer on the surface of EPC55 might have increased when the PVA-R concentration of the coating solution was increased (coating amount of 0.5, 1 or 2% PVA-R-coated liposomes is 45.4, 82.2 6r 136.4 mg PVA-R/mmol lipid). Harigai et al. reported that steric hindrance of the PEG chain on the liposome surface depends on the surface content of the PEG chain and also on the character of the interacting molecules themselves, e.g., the structure, molecular size, charge, and macroscopic structure of molecules if the molecules are polymeric molecules (Harigai et al., 2001). Therefore, the polymer amount on the liposomal surface could affect the behavior of liposomes in the interaction with macrophage cells. When a low concentration of PVA-R was used to coat the surface of EPC55, the cholesterol cluster in the liposomal membrane of EPC55 induced the heterogeneous coating of PVA-R. On the other hand, the present study results suggested that increasing the PVA-R concentration in the coating could overcome the problem of the heterogeneous coating of PVA-R. Therefore, the PVA-R amount on the liposomal surface was also an important factor for controlling the interaction with macrophage cells.

The fluorescence intensity of DiI after incubation with different polymer-coated liposomes for 30 min is shown in Fig. 5. If the hydrophobic moieties of HPMC-R were incorporated into the surface of the liposomes, the formation of a steric coating layer would be expected as well. We have already reported that the amount of HPMC-R coated on the liposomal surface was significantly higher than that of HPMC. The coating amount of HPMC-R on the liposomes was 101.1 mg/lipid mmol, which was 5.1-fold larger than that of HPMC at a polymer concentration of 0.5% (w/v) (Takeuchi et al., 2001). The present study indicated that HPMC-R-coated liposomes also showed suppressed interaction with J774 cells. When the concentration of HPMC-R in the coating increased, the fluorescence intensity detected from J774 cells incubated with HPMC-R-coated liposomes decreased, in the same manner as that of PVA-R-coated liposomes. These results suggest the existence of a coating layer with HPMC-R on the surface of the liposomes. However, as shown in Table 1, it was difficult to control the particle size of HPMC-R-coated liposomes within the submicron range if the concentration of HPMC-R in the coating increased. HPMC-R-coated liposomes form aggregates with an increase of the HPMC-R concentration, and aggregation leads to more rapid clearance from the bloodstream than with smaller particles such as PVA-R-coated liposomes. Therefore, PVA-R-coated liposomes were considered to be a more effective drug carrier than HPMC-R-coated ones.

It is known that the clearance mechanism of liposomes from the bloodstream is complicated. Chonn et al. reported that the values of protein-binding ability and half-life observed *in vivo* strongly suggest that blood proteins play a significant role in determining the *in vivo* fate of liposomes (Chonn et al., 1992). Harashima et al. reported that a complementary system was activated and that it enhanced hepatic uptake, depending on the size of liposomes (Harashima et al., 1994). Furthermore, Dams et al. reported the remarkable pharmacokinetics of repeated injections of PEG liposomes in rats and rhesus monkeys: the circulatory half-life of a second dose of radiolabeled PEG liposomes dramatically decreased when administered from 5 days up to 4 weeks after a first injection (Dams et al., 2000). The selective binding of IgM to the second-injected PEGylated liposomes and the subsequent complement activation by IgM resulted in the accelerated clearance and enhanced hepatic uptake of these second-injected PEGylated liposomes (Ishida et al., 2006). Although a comprehensive study of the interaction with macrophage cells should be undertaken to understand the behavior of liposomes in the body, the present study may provide useful information to predict roughly the uptake by RES at the initial stage after i.v. administration.

5. Conclusion

The interaction of liposomes with J774 cells could be suppressed by coating the liposomal surface with polyvinyl alcohol having a hydrophobic moiety at the end of the molecule (PVA-R). The interaction with J774 cells is affected by combining some factors of the liposome such as the lipid composition, the PVA-R amount on the liposomal surface, or the type of polymer for modifying the liposomal surface. The results suggest that the steric property of the PVA-R layer suppressed the interaction with J774 cells as well as the adsorption of serum protein.

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