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# Targetability of novel immunoliposomes modified with amphipathic poly(ethylene glycol)s conjugated at their distal terminals to monoclonal antibodies

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**Abstract**

Distearoyl-*N*-(3-carboxypropionoyl poly(ethylene glycol) succinyl)phosphatidylethanolamine (DSPE-PEG-COOH) was newly synthesized and used to prepare novel immunoliposomes carrying monoclonal antibodies at the distal ends of the PEG chains (Type C). Liposomes were prepared from egg phosphatidylcholine (ePC) and cholesterol (CH) (2:1, m/m) containing 6 mol% of DSPE-PEG-COOH, and a monoclonal IgG antibody, 34A, which is highly specific to pulmonary endothelial cells, was conjugated to the carboxyl groups of DSPE-PEG-COOH to give various amounts of antibody molecules per liposome. Other immunoliposomes with PEG coating (Type B) or without PEG coating (an earlier type of immunoliposome, Type A) were prepared for comparison. The average molecular weight of PEG in Type B or C immunoliposomes was 2000. Type B and Type C liposomes without antibodies showed prolonged circulation time and reduced reticulo-endothelial system (RES) uptake owing to the presence of PEG. These three different types of 34A-immunoliposomes with 30–35 antibody molecules per vesicle were injected into mice to test the immunotargetability to the lung. The efficiency of lung binding of 34A-Type B was one-half of that of 34A-Type A, though a large amount of 34A-Type B remained in the blood circulation for a long time, suggesting that the steric hindrance of PEG chains reduced not only the immunospecific antibody-antigen binding, but also the RES uptake. The degree of lung binding of 34A-Type C was about 1.3-fold higher than that of 34A-Type A, indicating that recognition by the antibodies attached to the PEG terminal was not sterically hindered and that the free PEG (i.e., that not carrying antibody) was effective in increasing the blood concentration of immunoliposomes by enabling them to evade RES uptake. The latter phenomenon was confirmed by using nonspecific antibody-Type C immunoliposomes (14-Type C), which showed a high blood level for a long time. Our approach provides a simple means of conjugating antibodies directly to the distal end of PEG which is already bound to the liposome membrane, and should contribute to the development of superior targetable drug delivery vehicles for use in diagnostics and therapy.

*Keywords:* Liposome; Immunoliposome; Antibody; Poly(ethylene glycol); Drug delivery system

## 1. Introduction

Drug delivery to specific cells by immunoliposomes represents a potentially attractive mode of therapy. However, though immunoliposomes are effective in specific binding to target cells in vitro, their targeting efficiency in vivo is relatively low. Studies in vivo have revealed that coating liposomes with antibody (Type A in Fig. 1) leads to enhanced uptake of the immunoliposomes by the reticuloendothelial system (RES) [1,2] and the immunotargetability depends on the antibody density on the surface [3]. Thus, highly efficient target binding and a relatively

Abbreviations: CH, cholesterol; DF, deferoxamine; DSPE, distearoyl phosphatidylethanolamine; DSPE-PEG, distearoyl-*N*-(monomethoxy poly(ethylene glycol) succinyl)phosphatidylethanolamine; DSPE-PEG-COOH, distearoyl-*N*-(3-carboxypropionoyl poly(ethylene glycol) succinyl)phosphatidylethanolamine; ePC, egg phosphatidylcholine; Mes, 2-(*N*-morpholino)ethanesulfonic acid hemisodium salt; NGPE, *N*-glutaryldistearoylphosphatidylethanolamine; PEG-OSu, monomethoxy poly(ethylene glycol) succinimidyl succinate; PEG-2OSu, poly(ethylene glycol) bis(succinimidyl succinate); SUV, small unilamellar vesicle; 34A, rat monoclonal $IgG_{2a}$ antibody; 14, rat monoclonal $IgG_{2a}$ antibody, a subtype-matched nonspecific control antibody to 34A.

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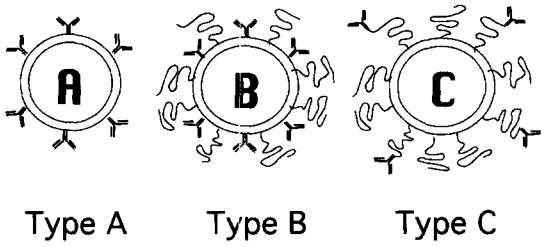


Fig. 1. Schematic illustration of immobilization of antibody on liposomes. Type A: PEG-free immunoliposomes with antibody covalently linked to the short anchor NGPE; Type B: PEG-immunoliposomes with antibody covalently linked to NGPE; Type C: new type of PEG-immunoliposomes with antibody attached to the distal terminal of DSPE-PEG-COOH.

low level of RES uptake of the immunoliposomes are apparently mutually exclusive.

Recently, long-circulating liposomes have been prepared by coating the liposome surface with ganglioside GM1 (GM1) or amphipathic poly(ethylene glycol) (PEG) [4–8]; this coating allows the liposomes to evade RES uptake and remain in the systemic circulation for a long period of time, which should maximize targeting. We have shown that incorporation of ganglioside GM1 (GM1) can significantly enhance the target binding of immunoliposomes, whereas amphipathic PEG with an average molecular weight of 5000 actually inhibits it [9–11]. These results can be explained in terms of their steric barrier activity, which is measured by a liposome agglutination assay. Surface PEG polymers (Type B in Fig. 1) hinder the binding of liposome-bound antibodies with target antigens, while GM1 presents a much weaker steric barrier. The presence of GM1 on liposome surface is apparently sufficient to prevent rapid uptake by macrophages, without hindering antibody-antigen binding. Thus, the elevated blood concentration of liposomes containing GM1 kinetically enhances the target binding of immunoliposomes [9], but the same effect provided by amphipathic PEG does not lead to an enhanced target binding. Nevertheless, PEG remains of interest because of its ease of preparation, relatively low cost, controllability of molecular weight and linkability to lipids by a variety of methods as compared with GM1 molecules.

It has been proposed by us [10] and Klibanov and Huang [12] that antibodies could be attached to the distal ends of PEG chains which are already bound to the liposome membrane (Type C in Fig. 1). Since the binding of the antibody to the target cell should not be sterically hindered by the PEG chains in this situation, the immunoliposomes should show more efficient target binding. Herein, to test this hypothesis, we have synthesized novel distearoyl phosphatidylethanolamine derivatives of PEG with terminal COOH groups for the preparation of Type C immunoliposomes. The targetability and biodistribution of Type C immunoliposomes were studied in mice in comparison with those of Type A and B immunoliposomes. The monoclonal antibody 273-34A (34A), specific for the pulmonary endothelial cells of mouse [13], was used in this study. 34A recognize surface glycoproteins, gp112, which are expressed exclusively and abundantly on the lumenal surface of the pulmonary capillary vessel wall in mouse lung [14]. It has been shown that 34A-immunoliposomes (Type A) gain direct access and bind efficiently to the lung target in vivo [3,13], so this model should allow a convenient test of our hypothesis.

Blume et al. [15] have recently coupled plasminogen to liposomes using a similar technique. However, little is known about antibody-mediated liposomal targeting in this system.

## 2. Materials and methods

### 2.1. Materials

Egg phosphatidylcholine (ePC), distearoylphosphatidylethanolamine (DSPE), monomethoxy poly(ethylene glycol) succinimidyl succinate (PEG-OSu), and poly(ethylene glycol) bis(succinimidyl succinate) (PEG-2OSu) were kindly donated by Nippon Oil and Fats (Tokyo, Japan). The number-average molecular weight ($M_n$), weight-average molecular weight ($M_w$) and polydispersity ($M_w/M_n$) of PEG-OSu and PEG-2OSu were measured by means of gel permeation chromatography (Shodex KF801, KF803 and KF804 combination columns). The values of $M_n$ of PEG-OSu and PEG-2OSu were 2219 and 2230, respectively and their polydispersities were 1.03 and 1.04, respectively. Cholesterol (CH), triethylamine, and glutaric acid anhydride were purchased from Wako Pure Chemicals (Osaka, Japan). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), *N*-hydroxysulfosuccinimide (S-NHS) and IODO-GEN were obtained from Pierce (Rockford, IL). $^{67}GaCl_3$ was kind gift from Dr. Umeda (Teikyo University), and $^{125}I$ was obtained from New England Nuclear Japan (Tokyo).

*N*-Glutaryldistearoylphosphatidylethanolamine (NGPE), which was used for preparation of Type A and B immunoliposomes, was synthesized by the method of Weissig et al. [16]. Distearoyl-*N*-(monomethoxy poly(ethylene glycol) succinyl)phosphatidylethanolamine (DSPE-PEG) was synthesized as described [8].

### 2.2. Monoclonal antibody

Rat monoclonal $IgG_{2a}$ antibody 34A and rat monoclonal $IgG_{2a}$ antibody 14, a subtype-matched nonspecific control antibody, were prepared as described [13]. All antibodies were isolated from Fischer F344 rats after immunization with Balb/c mouse lung homogenate. Antibodies were radiolabeled with $^{125}I$, using the IODO-GEN method, to a specific activity of $(2–5) \cdot 10^5$ cpm/$\mu$g.

### 2.3. Synthesis of DSPE-PEG-COOH

Distearoyl-*N*-(3-carboxypropionoyl poly(ethylene glycol) succinyl)phosphatidylethanolamine (DSPE-PEG-COOH) was synthesized by a modification of the method used for DSPE-PEG [8]. In brief, 1 ml of 5% DSPE in $CHCl_3$/methanol (3:1 v/v) was added to 9.5 ml of 10% PEG-2OSu in $CHCl_3$, followed by addition of 20.5 μl of triethylamine. The reaction mixture was stirred vigorously overnight at 35°C under protecting from light. Full conversion of the primary amino group in DSPE was confirmed by the negative ninhydrin reactivity after separation of the products by TLC. Phospholipid phosphorus assay [17] showed the appearance of a new phosphate-positive spot at a higher $R_f$ value than that of DSPE. A small amount of water was added to the evaporated reaction residues to form micelles. The DSPE-PEG-COOH micelles were dialyzed for 5 days against water using a dialysis bag with large pores (Spectra-por CE 300000 MWCO, Spectrum Medical) and then lyophilized. The yield of DSPE-PEG-COOH was approx. 180 mg.

### 2.4. Preparation of immunoliposomes

The plain liposomes for preparing Type A or Type C immunoliposomes were composed of ePC/CH (2:1, m/m) with 6 mol% of NGPE or DSPE-PEG-COOH, respectively. The plain liposomes for Type B were composed of ePC/CH (2:1, m/m), with 6 mol% of DSPE-PEG and 6 mol% of NGPE. As a tracer, $^{67}$Ga bound to DF (Desferal®, Ciba-Geigy, Japan) was used ($^{67}$Ga-DF). Small unilamellar liposomes (SUV, 90–130 nm in diameter) were prepared by the reverse-phase evaporation method [18] followed by extrusion (Lipex Biomembranes, Canada) through two stacked Nuclepore filters (0.1 μm). Normal saline was used as the liposomal aqueous phase. The extruded liposomes were then chromatographed to remove unencapsulated $^{67}$Ga-DF on a Bio-Gel A-1.5m column (Bio-Rad), which was equilibrated and eluted with Mes buffer (10 mM Mes/150 mM NaCl, pH 5.5). The liposome fraction was diluted to 10 μmol lipid/ml with Mes buffer. The average size of the liposomes was measured with a Nicomp 370 HPL submicron particle analyzer (Particle Sizing Systems, Santa Barbara, CA).

Coupling of antibodies to the plain liposomes to prepare Type A and B immunoliposomes was performed as described [10]. The Type C immunoliposomes were prepared similarly. Briefly, to 300 μl of the plain liposomes (3 μmol lipids) in Mes buffer, 120 μl of 0.25 M EDC in water and 120 μl of 0.25 M S-NHS in water were added, and the mixture was incubated for 10 min at room temperature. The mixture was neutralized with 1 M NaOH to pH 7.5. The desired amount of antibody with a trace amount of $^{125}$I-labeled antibody was then added and the whole was incubated for 8 h at 4°C with gentle stirring. The immunoliposomes were separated from the unbound protein on a Bio-Gel A-15m column preequilibrated with saline. Peak fractions containing immunoliposomes eluted in the void volume were collected, pooled, and diluted to an appropriate volume with saline. The lipid concentration and the coupling efficiency of antibody were estimated by the phosphorus assay [19] and the radioactivity of $^{125}$I, respectively. The average number of antibody molecules per liposome was calculated by using the above values and the following assumptions: molecular weight of antibody is 150 000; the average number of phospholipid molecules per liposome is estimated by the method of Enoch and Strittmatter [20].

### 2.5. Biodistribution studies

Immunoliposomes were injected into Balb/c mice (6- to 7-week-old male mice, Sankyo, Tokyo) via the tail vain. Approx. 200 μl of immunoliposomes was injected into

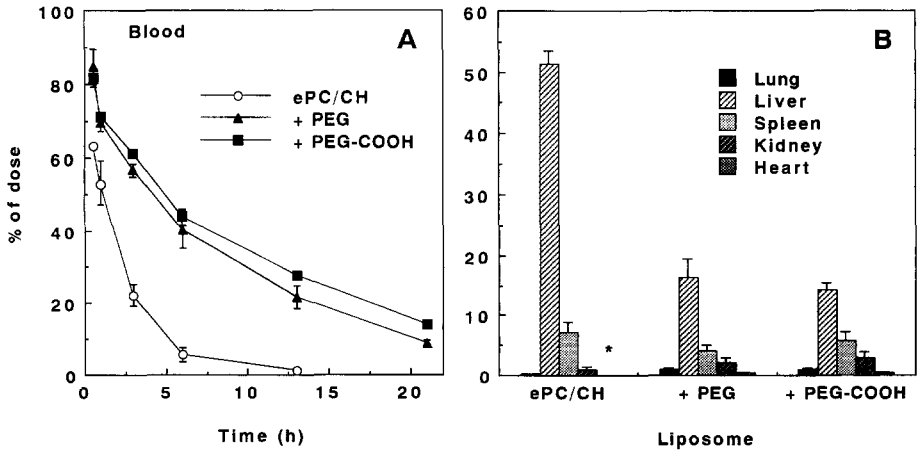


Fig. 2. Blood clearance (A) and biodistribution at 6 h (B) of liposomes after intravenous injection. Liposomes (90–130 nm average diameter) composed of ePC/CH (2:1, m/m), ePC/CH/DSPE-PEG (2:1:0.19, m/m) or ePC/CH/DSPE-PEG-COOH (2:1:0.19, m/m) were labeled with $^{67}$Ga-DF. Data are expressed as mean ± S.D. ($n = 3$). * is 0.1 ± 0.03 (% of dose in heart).

each mouse, and at intervals, mice were lightly anesthetized and bled by eye puncture. Blood was collected and weighed. Mice were killed by cervical dislocation and dissected. Organs were collected, weighed, and analyzed for $^{67}$Ga radioactivity in a gamma counter. The results are presented as the percentage of the total injected dose for each organ. The total radioactivity in the blood was determined by assuming that the total volume of blood was 7.3% of the body weight [21]. Values for organs were corrected for blood contamination [22].

## 3. Results

### *3.1. Biodistribution studies of DSPE-PEG-COOH-containing, antibody-free liposomes*

DSPE-PEG derivatives with a reactive group (carboxyl group) at the distal terminal of PEG chains (DSPE-PEG-COOH, Fig. 2) were synthesized by hydrolysis of distearoyl-*N*-(monosuccinimidyl succinoyl poly(ethylene glycol) succinyl)phosphatidylethanolamines. The purified DSPE-PEG-COOH showed a single spot of $R_f$ 0.31 on TLC with $CHCl_3/MeOH/H_2O$ (70:30:5, v/v).

To test whether DSPE-PEG-COOH can prolong the circulation time of liposomes, $^{67}$Ga-DF-labeled SUV (diameter 90–130 nm) of three different compositions were prepared and injected into Balb/c mice via the tail vein: ePC/CH (2:1, m/m), ePC/CH/DSPE-PEG (2:1:0.19, m/m) and ePC/CH/DSPE-PEG-COOH (2:1:0.19, m/m). As shown in Fig. 2, liposomes composed of ePC/CH (2:1 molar ratio) were rapidly cleared from the blood (panel A) and were found almost entirely in the liver (panel B). Addition of DSPE-PEG or DSPE-PEG-COOH to ePC/CH liposomes greatly prolonged the liposome residence in the circulation (Fig. 2). The values of $t_{1/2}$ for liposome blood clearance were estimated to be 1.5 h, 5.6 h and 6.4 h for ePC/CH liposomes and liposomes containing DSPE-PEG and DSPE-PEG-COOH, respectively. Thus, DSPE-PEG-COOH significantly enhanced the blood residence time of the liposomes, regardless of the presence of the terminal carboxyl group. There were no marked differences in tissue distribution between liposomes containing DSPE-PEG and DSPE-PEG-COOH (Fig. 2B).

### *3.2. Immunotargetability and biodistribution of type A, B or C immunoliposomes in mice*

To test the effect of both the position of the antibody and the steric hindrance of PEG chains on the behavior of immunoliposomes in vivo, three different types (Fig. 1) of 34A-immunoliposomes or 14-immunoliposomes with similar numbers of antibody molecules per liposome were prepared and their targetability and biodistribution were evaluated in mice. The characteristics of all immunoliposomes used in this experiment are summarized in Table 1. PEG content in the lipid bilayer was set at 6 mol% of the total lipid for Type B and C, respectively, to facilitate direct comparison with previous results. In this experiment, Type A, B and C 34A-immunoliposomes contained approx. 35, 30 and 30 antibody molecules per liposome, respectively. In the case of 14-immunoliposomes, Type A, B and C had approx. 21, 22 and 15 antibody molecules per vesicle, respectively. Liposome aggregation was not observed.

Lung binding of the three types of 34A-immunoliposomes was examined 30 min and 6 h after i.v. injection and was expressed as percent of injected dose (Fig. 3). Type A 34A-immunoliposome with an average of 35 antibody molecules per liposome accumulated 42.5% of the injected dose in the lung at 30 min. Type B 34A-immunoliposomes which were prepared by incorporating DSPE-PEG with an average molecular weight of 2000 into Type A immunoliposomes showed a lower level of target binding and a significantly higher blood level than those of Type A. 6 h after injection, 43.7% of the dose of Type B immunoliposomes remained in the blood, a value much higher than that of the Type A immunoliposomes. In the case of Type C 34A-immunoliposomes with 30 antibody molecules per vesicle, the degree of target binding to the lung was 56.6% of injected dose at 30 min, 1.3-fold higher than that of Type A. Furthermore, Type C immunoliposomes were better retained in the lung than the Type A immunoliposomes at 6 h after injection (Fig. 3): Type C 34A-immunoliposomes showed about 29% dissociation of the bound immunoliposomes at 6 h, whereas Type A 34A-immunoliposomes showed 41% loss of the bound immunoliposomes over the same time period.

The 14 rat monoclonal antibody is a nonspecific anti-

Table 1
Characteristics of the three types of immunoliposomes

| | 34A-Immunoliposomes | | | 14-Immunoliposomes | | |
|---|---|---|---|---|---|---|
| | Type A | Type B | Type C | Type A | Type B | Type C |
| PEG content (mol% of total lipid) | 0 | 6 | 6 | 0 | 6 | 6 |
| Mean diameter (nm) | 121 | 111 | 122 | 97 | 105 | 118 |
| Initial antibody: lipid ratio (w/w) | 1:6 | 1:6 | 1:5 | 1:4 | 1:3 | 1:5 |
| Conjugation efficiency (%) | 35.6 | 31.8 | 24.8 | 15.2 | 20.2 | 11.6 |
| Number of antibody molecules per liposome [a] | 35 | 30 | 30 | 21 | 22 | 15 |

[a] The average number of antibody molecules per liposome was estimated by Ref. [20]. See details in the text.

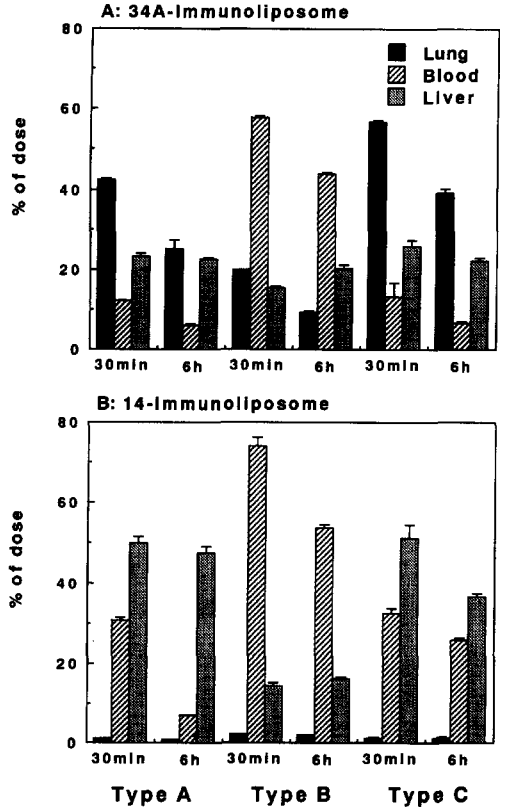


Fig. 3. Immunotargetability and biodistribution of 34A- and 14-immunoliposomes at 30 min and 6 h after intravenous injection. Type A, B and C 34A-immunoliposomes contained approx. 35, 30 and 30 antibody molecules per liposome, respectively. Type A, B and C 14-immunoliposomes contained 21, 22 and 15 antibody molecules per liposome, respectively. Data are expressed as mean ± S.D. ($n = 3$).

body, but its subtype is the same as that of the 34A antibody. By using this nonspecific antibody in the three different types of immunoliposomes, we should be able to estimate whether the steric barrier imposed by PEG is responsible for prolongation of the circulation time and avoidance of RES uptake of immunoliposomes. As shown in Table 1, the three types of 14-immunoliposome were prepared with similar numbers of antibody molecules (15–20 antibodies/liposome). A high liver accumulation of Type A and C 14-immunoliposomes was observed, whereas liver uptake of Type B was low (Fig. 3). Type B and C 14-immunoliposomes showed high blood levels up to 6 h after injection, whereas Type A showed a low blood level at 6 h. These results suggest that although the liver is still the major organ for immunoliposome (Type A) uptake, the presence of PEG on the immunoliposome surface (Type B) interferes sterically with the liver uptake, prolonging the circulation time. Linkage of antibodies to the PEG terminal (Type C) reduced the blood level and increased the liver uptake compared with those of Type B.

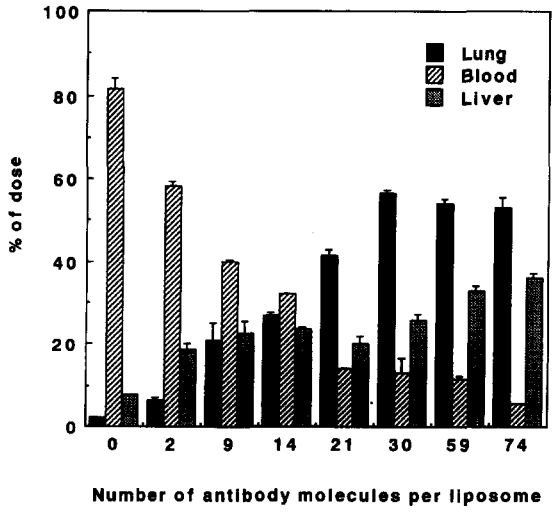


Fig. 4. Effect of the number of antibody molecules per liposome on the biodistribution of 34A-Type C immunoliposomes. Liposomes (200 μg of lipid) labeled with $^{67}$Ga-DF were injected into mice via the tail vein. Percent of injected dose ± S.D. ($n = 3$) in each organ was measured 30 min after injection.

### 3.3. Target binding of 34A-immunoliposomes as a function of antibody content

The efficiency of Type C 34A-immunoliposome binding to the target was evaluated as a function of the antibody content. A series of Type C 34A-immunoliposomes was prepared with various initial antibody-to-lipid ratios. The final number of antibody molecules per liposome varied from 0 to 74, but the average size of immunoliposomes stayed approximately the same, 90–130 nm in diameter. It is clear from the data in Fig. 4 that the efficiency of lung targeting was dependent on the antibody content of the immunoliposomes. Liposomes containing small numbers of antibody molecules per liposome accumulated in the lung at low levels and were retained in the blood at high levels, whereas liposomes linking an average of 74 antibody molecules per liposome on the PEG terminals accumulated 53% of the injected dose in the lung and

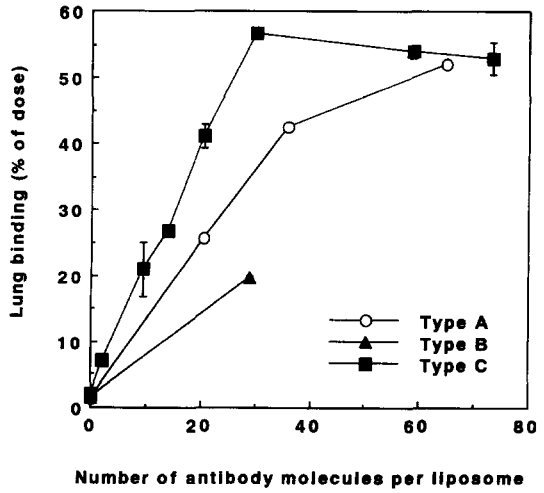


Fig. 5. Comparison of target binding to the lung among the three types of 34A-immunoliposomes with various numbers of antibody molecules per liposome. Lung binding (% of dose) was measured 30 min after injection.

their blood resident amount was only 7% of the injected dose. Lung binding reached a plateau at about 30 antibody molecules per liposome; further increase in antibody content only resulted in the increased liver uptake. Thus, antibody density is an important factor for target binding. At low numbers of antibody molecules per liposome, such as 2 and 9 molecules, free PEG favors evasion of RES uptake of the liposomes, resulting in high blood residence of Type C 34A-immunoliposome together with a low efficiency of target binding due to the low antibody content.

A portion of data in Fig. 4 is plotted in Fig. 5 to compare the degree of target binding of the three types of 34A-immunoliposomes to the lung as a function of the antibody content. Type C showed higher immunotargetability than Type A and B at low antibody content (less than 30 antibody molecules per vesicle). Thus, Type C is accumulated more effectively in the lung than the other immunoliposomes, in spite of the low antibody content. At high antibody content, Type A immunoliposomes also showed high level of target accumulation.

## 4. Discussion

We have introduced and tested a new type of long-circulating immunoliposome (Type C) which can effectively bind to the designated target site in vivo. This was achieved by the use of newly synthesized DSPE-PEG-COOH to couple antibodies (for targeting) directly to the distal terminal of PEG chains (which allow the liposomes to evade the RES and achieve prolonged circulation). The method is relatively simple and should be widely applicable.

We have previously demonstrated that 34A-immunoliposome (Type A) binding to the lung target is very rapid, and the surface density of antibody on the liposomes is an important factor determining the level of target binding of immunoliposomes [3]. Furthermore, we showed that the inclusion of GM1 in the liposomes enhanced the target binding of 34A-immunoliposomes (Type B) as a result of elevated blood concentration of the liposomes [9]. Studies in vivo revealed that the major competing process for immunoliposome target binding is uptake by the RES [9]. Long-circulating liposomes can also be obtained by incorporating PEG in place of GM1 [4–8]. However, inclusion of PEG reduced the target binding of immunoliposomes (Type B). This effect depended on the chain length of PEG, suggesting that although PEG prolongs the circulation time of immunoliposomes (Fig. 3), it sterically hinders the binding of immunoliposomes to the target sites [10,11,23,24]. Mori et al. [11] and Ahmad et al. [23,24] suggested using shorter PEG (1900 or 2000 average molecular weight), but this still tended to suppress the antibody-antigen interaction (Fig. 3). We [10] and others [12] proposed that the use of longer PEG with antibodies attached at the distal terminal of the PEG chain would afford immunoliposomes with both prolonged circulation time and good target binding (Fig. 1, Type C).

The targeting efficiency of 34A-Type C liposomes was also dependent on the number of antibodies per liposome, as shown in Figs. 4 and 5. It is noteworthy that Type C showed much higher targetability than Type A at less than 30 antibody molecules per liposome. This is probably due to the free PEG chains effectively hindering the RES uptake of liposomes, resulting in elevated blood concentration of the liposomes. Thus, the presence of free PEG-COOH (not linked to antibody) on the Type C immunoliposome surface does not interfere with the binding of the terminally linked antibody to antigen and, instead, enhances the target binding of immunoliposomes owing to its prolonging effect on the circulation time of the liposomes. The combination of using amphipathic PEG to suppress the RES uptake rate and attaching a large number of antibody molecules to the PEG terminal to enhance the target binding rate produced an unprecedentedly high level of target binding of liposomes.

Other studies have shown, however, that a coating of intact IgG molecules on liposome surfaces enhances RES uptake by an Fc receptor-mediated mechanism [1,2]. Indeed, 14-Type C showed a high liver uptake, suggesting that this phenomenon occurs in our case. Data in Fig. 4 also indicate that Type C immunoliposomes with very high 34A antibody content did not bind with the target with superior efficiency. This is probably because the rate of liver uptake of these liposomes is sufficiently high to compete with the binding with the lung target. To overcome this problem, it may be necessary to use Fab or $F(ab)_2$ fragment to avoid the Fc-receptor mediated binding of immunoliposomes. Experiments by Blume et al. [15], in which plasminogen was linked to the PEG terminal (plasminogen-Type C liposome), also resulted in a small increase in clearance of the proteoliposomes compared to plasminogen-Type B liposomes, but the plasminogen-Type C liposomes still circulated for significantly longer period of time than the plasminogen-Type A liposomes.

It should be noted, however, that the target sites for 34A-liposomes or plasminogen-liposome [15] are located at a readily accessible site, i.e., the vascular endothelial surface. For a much less accessible target, such as tumor cell surface antigens in a solid tumor, only liposomes with a strong steric barrier activity would be expected to circulate long enough to reach the target. It is thus of interest to test this new approach in a system where the antigen is in a less accessible site. We and others have demonstrated that long-circulating liposomes of small size (about 100 nm mean diameter) and rigid lipid composition showed significantly greater accumulation in solid tumor [25–27]. The capillary permeability of the endothelial barrier in newly vascularized tumors is significantly greater than that of normal organs [28]. Under these circumstances it is possible that small Type C immunoliposomes could predominantly pass through the leaky tumor endothelium by

passive convective transport. Thus, the Type C immunoliposomes with long circulation half-lives could be particularly effective for targeting solid tumors.

The observation that the conjugation of antibody directly to the PEG terminal provides excellent target binding and retention of immunoliposomes suggests that these Type C liposomes have great potential as a targeted drug delivery vehicle. They should be able to provide a high local concentration of the encapsulated drug at the target site for a prolonged period of time. Unwanted toxic effects of the drug in other tissues and cells may also be reduced. This is particularly important with the highly toxic antitumor drugs used in cancer chemotherapy.

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