ELSEVIER

Biochimica et Biophysica Acta 1279 (1996) 75–83

BBA Biochimica et Biophysica Acta

Poly(ethylene glycol)-coated anti-cardiac myosin immunoliposomes: factors influencing targeted accumulation in the infarcted myocardium

Vladimir P. Torchilin [a,*], Jagat Narula [b,d], Elkan Halpern [a], Ban An Khaw [c,d]

[a] *Center for Imaging and Pharmaceutical Research, Massachusetts General Hospital and Harvard Medical School, MGH-East, 149 13th Street, Charlestown, MA 02129, USA*

[b] *Cardiac Unit Massachusetts General Hospital, Boston , MA 02114, USA*

[c] *Division of Nuclear Medicine, Massachusetts General Hospital, Boston, MA 02114, USA*

[d] *Center for Drug Targeting and Analysis, Northeastern University, Boston, MA 02115, USA*

Received 23 May 1995; revised 1 August 1995; accepted 16 October 1995

Abstract

Biodistribution and infarct accumulation of different liposome preparations in rabbits with experimental myocardial infarction have been investigated. The influence of such parameters as liposome size, and presence or absence of poly(ethylene glycol) (PEG) and infarct-specific antimyosin antibody (AM) on liposome behavior in vivo was studied. All three variables were shown to affect liposome biodistribution, liposome size being the least significant variable. Statistical analysis of the data obtained demonstrated that of all variables, PEG coating expresses the strongest influence on the liposome blood clearance, significantly ($P = 0.0001$) increasing the mean level of blood radioactivity under all circumstances. Infarct accumulation depended upon the presence of both PEG ($P = 0.0013$) and AM ($P = 0.005$). The infarct-to-normal ratio was affected by the presence of AM ($P = 0.0002$), but the extent of the effect depended also on the presence of PEG ($P = 0.01$). Two differing mechanisms can be seen in infarct accumulation of PEG-liposomes (slow accumulation via the impaired filtration) and AM-liposomes (specific binding of immunoliposomes with the exposed antigen). Both mechanisms are supplementary in case of liposomes carrying PEG and AM at the same time. An optimization strategy is suggested for using liposomes as carriers for diagnostic (a high target-to-nontarget ratio is required) and therapeutic (a high absolute accumulation in the target is required) agents.

Keywords: Liposome; Immunoliposome; Long-circulating liposome; Antimyosin; Antibody; Myocardial infarction; Drug targeting

1. Introduction

The targeting of pharmaceuticals to the heart is aimed at two main objectives: diagnostic imaging and the delivery of therapeutics to the damaged myocardium. Liposomes have been shown to serve as convenient carriers for both diagnostic and therapeutic pharmaceuticals. Spontaneous accumulation of positively charged liposomes in the regions of experimental myocardial infarction was described by Caride in 1977 [1]. Further experiments [2–4] demonstrated that liposome accumulation in ischemic tissues is a general phenomenon and might be explained by impaired filtration in these areas, resulting in trapping of liposomes within the ischemic zones [5]. This observation led to the conclusion that drug-loaded liposomes can be used for 'passive' drug delivery into the ischemic tissues, particularly into the infarcted myocardium [6,7]. Thus, liposomes loaded with a thrombolytic enzyme, streptokinase, were able to accelerate thrombolysis and reperfusion in a canine model of myocardial infarction [8]. Liposomes with superoxide dismutase were reported to be more effective in preventing ischemic and reperfusion injuries in different tissues, including myocardium, compared to the native enzyme [9,10]. Furthermore, it was shown that liposomes loaded with sodium or calcium ions have demonstrated significant influence on the electrical activity of cultured heart cells from chicken embryos [11]. Similarly, liposomes with entrapped ATP were shown to normalize ischemic conditions in some tissues [12].

To improve the efficiency of liposomal drug delivery

* Corresponding author. Fax: +1 (617) 7267830.

SSDI 0005-2736(95)00248-0

into the ischemic myocardium utilisation of antibody-mediated liposome targeting was proposed. Antimyosin antibody [13,14] has been used for 'active' targeting of liposomes to infarcted myocardium [15]. This targeting phenomenon is based on the observation that normal myocardial cells with intact membranes do not permit extracellular macromolecules, such as antimyosin antibody, to traverse the cell membrane. However, necrotic cardiomyocytes with disrupted membranes can no longer prevent the antibody from interacting with myosin [16]. This forms a basis for targeted delivery of pharmaceuticals into the affected myocardium [17]. Antibody to canine cardiac myosin covalently coupled to liposomes has been successfully used for targeting of the experimental myocardial infarction in dogs [15]. Incorporation of radioactivity within liposomes also allowed for visualization of the necrotic myocardium [15].

However, 'passive' liposome targeting or 'active' targeting with immunoliposomes can not provide high enough accumulation of the targeting agent in the areas with limited blood supply (such as an infarct zone) due to fast sequestering of liposomes by the reticuloendothelial system (RES), resulting in insufficient contact time with the target. Some in vivo properties of liposomes, such as circulation time, can be improved by grafting their surface with certain flexible polymers [18–20]. One of the most promising approaches for increasing liposome circulation time is coating them with poly(ethylene glycol), or PEG. PEG decreases the rate of opsonization of liposomes and therefore their recognition by liver cells, which significantly increases liposome circulation time in the blood [21,22]. Moreover, long-circulating polymer-coated liposomes can be made target-specific' by co-incorporation of a specific antibody on the liposome surface, as described earlier [23]. In that study, antimyosin-modified PEG-liposomes had the abilities to recognize and bind the target, as well as circulate long enough to provide high target accumulation. The next step in this endeavor is to optimize liposome properties for the delivery of diagnostic and therapeutic agents into the damaged myocardium. For diagnostic purposes maximum target-to-nontarget ratio is essential; whereas, for therapeutic purposes the maximum absolute dose delivery of the liposomal contents into the affected tissue is more important. However, with highly toxic pharmaceuticals, high background is undesirable and low non-specific accumulation could be required. With these aims in mind, in vivo properties and biodistribution of PEG and/or antimyosin antibody-coated liposomes of different sizes were investigated in rabbits with experimental myocardial infarction.

We report here that in the rabbit myocardial infarct model, the size of the liposomes has limited influence on their in vivo behavior and accumulation at the target organ. The presence of antibody and/or PEG on the liposome surface is more critical resulting in differences which may influence whether its use should be in diagnosis or therapy.

2. Materials and methods

2.1. Materials

Diethylenetriaminepentaacetic acid (DTPA) cyclic anhydride, monomethoxy poly(ethylene glycol) succinimidyl succinate (PEG-OSu), M_r approx. 5000, 1-ethyl 3-(3-dimethylaminopropyl) carbodiimide (EDC), octyl glucoside (OG), and cholesterol were purchased from Sigma Co. (St. Louis, MO). Dioleoylphosphatidylethanolamine (PE), egg yolk phosphatidylcholine (PC), and *N*-glutarylphosphatidylethanolamine (NGPE) were obtained from Avanti Polar Lipids (Alabaster, AL). *N*-Hydroxysulfosuccinimide (HSSI) was from Pierce (Rockford, IL). Carrier-free ^{111}In as InCl3 was obtained from Amersham (Arlington Heights, IL). Mouse monoclonal antibody R11D10 or 2G4-2D7 specific for cardiac myosin heavy chains was prepared as described [16].

2.2. Methods

Synthesis of PEG-PE. The synthesis of PEG-PE was performed as described in [21]. Briefly, an aliquot of PEG-OSu was added to a solution of PE in chloroform, followed by addition of triethylamine (PEG-OSu/PE/triethylamine = 3:1:3.5, mol/mol). The reaction mixture is incubated overnight at room temperature and the chloroform is evaporated with a stream of nitrogen gas. The reaction mixture is than redissolved in 0.145 M NaCl. Unreacted PEG-OSu is rapidly hydrolyzed in the aqueous media. The resulting mixture in saline is applied to a Bio-Gel A1.5m column equilibrated with saline. Peak fractions containing PEG-PE micelles eluted in the void volume are pooled, dialyzed against water and lyophilized.

Preparation of antibodies and their fragments. Monoclonal antibodies were purified according to the standard method from corresponding murine ascites by ammonium sulfate precipitation, DEAE-cellulose anion exchange chromatography, and chromatofocusing over a pH gradient of 7.0 to 5.0 [16]. Antibody preparations were characterized by gel electrophoresis and HPLC. Fab fragments were used instead of whole antibodies in order to decrease Fc-mediated uptake of liposome-whole antibody conjugates by cells of the reticuloendothelial system (RES). The digestion of IgG and Fab purification was performed as follows: (a) 0.5 ml of the 50% slurry of immobilized papain (Pierce) were washed two times with 4 ml of digestion buffer (42 mg of cysteine/12 ml of phosphate buffer (pH 7.0)) for equilibration, and then suspended in 0.5 ml of the same buffer; (b) 0.5 ml of IgG sample (10–20 mg protein/ml) were diluted with 0.5 ml of the digestion buffer and added to the tube with immobilized papain suspension; (c) incubation proceeded for overnight depending at 37°C with intensive stirring; digestion degree was monitored with HPLC; (d) digested Fab and Fc fragments, and non-digested IgG were separated from papain

gel; (e) column with 5 ml of protein A-Sepharose (Pharmacia) was equlibrated with phosphate-buffered saline (PBS) (pH 8.0), and digested IgG sample was applied; (f) column was washed with PBS, Fab fragment was collected (purity was checked by HPLC); (g) Fc fragment was washed away with 0.1 M glycine (pH 3.0) to regenerate the column.

Antibody modification. For the incorporation into the liposomal membrane, antimyosin antibody Fab was initially modified with the hydrophobic anchor, NGPE, as described in [24]. Briefly, 0.3 mg of NGPE were dried with argon from chloroform solution and then solubilized with 0.5 ml of 0.016 M OG in 50 mM Mes. The solution was supplemented with 12 mg EDC and 15 mg of HSSI. The mixture was incubated for 5 min and then added to the solution of 2 mg of Fab in 0.1 M Hepes (pH 7.6). pH was adjusted to 8.0 with 1 M NaOH, and the mixture was incubated overnight at 4°C. The modified antibody was purified by dialysis against HBS.

Preparation of liposomes. Liposomes were prepared by the detergent (OG) dialysis from the mixture of PC and cholesterol (7:3 molar ratio). 6 mol% of PEG-PE and 1 mol% of DTPA-SA were added to the lipid mixture, which was then argon-dried, vacuumed, solubilized with OG in Hepes-buffered saline (HBS) (pH 7.4) (final total lipid concentration may vary from 5 to 20 mg/ml), and dialyzed overnight against HBS at 4°C. When necessary, 0.01 mol% of NGPE-antimyosin Fab was added to OG-solubilized lipid mixture to prepare targeted liposomes. Modification with NGPE normally allows the binding of several hundred protein molecules per single 250 nm liposome [24]. As was shown with ^{125}I-labeled antibody, in our particular case the efficacy of protein binding varied between 65 and 75% and did not depend on the presence of PEG-PE in the lipid mixture. The unbound antibody was separated on a Bio-Gel A15m column as in [23]. Liposomes obtained were sized by passing through the polycarbonate filters with pore size of 0.6, 0.4, and 0.2 μm (Nuclepore). Liposomes of two sizes were prepared: 120–150 nm (small liposomes, SL) and 350–400 nm (large liposomes, LL). Actual size and size distribution of liposomes were determined with Coulter N4 MD Submicron Particle Size Analyzer (Coulter Electronics).

For biodistribution studies, liposomes were radioactively labeled with ^{111}In via liposome-incorporated amphiphilic chelating agent diethylenetriaminepentaacetic acid-stearylamine (DTPA-SA). DTPA-SA was synthesized according to recommendations of [25] with some changes. Briefly, 1.55 g of SA and 2.5 g of DTPA cyclic anhydride were mixed with 250 ml of dry chloroform. The mixture was refluxed for 1 h, the top outlet of the system being coated with foil. Then, 3 ml of triethylamine were added, and the mixture was refluxed for an additional 48 h. Chloroform was evaporated on the rotor evaporator, it's traces were removed by incubating the flask in the water bath at 50°C. 100 ml of 0.1 M HCl were added to the dry product, and the mixture was stirred with heating at 80°C for 10 min and stored overnight at room temperature. The precipitate was separated by centrifugation, washed three times with 100 ml of 0.1 M HCl with stirring, and then lyophilized. The lyophilized product was washed twice with 100 ml of methanol, and then recrystallized twice from boiling methanol and dried.

DTPA-SA loading with ^{111}In was performed after its incorporation into liposomes via the transchelation mechanism. For this purpose, the liposome suspension (normally, 2 ml) was supplemented with 30 μl of 1.0 M citrate and incubated for 1 h with required quantity of citrate complex of ^{111}In at room temperature, and then dialyzed overnight against HBS at 4°C to remove free label.

Determination of antibody-liposome immunoreactivity by direct binding of radiolabeled antibody-liposome and antibody-liposome-polymer conjugates. Microtiter plates were coated with 50 μl of 10–50 μg/ml of dog cardiac myosin and incubated at 4°C for 12–18 h. The antigen solution was removed and the wells were filled with 1% horse serum in 0.15 M phosphate-buffered saline (pH 7.4) to saturate the remaining non-specific binding surfaces of the microtiter wells. The solution was removed after a 4 h incubation at room temperature and the wells were washed extensively with standard washing solution. To the antigen-coated wells prepared as described above, serial dilutions of ^{111}In-labeled antibody-polymer-liposome preparation were added. The maximum count used per 50 μl aliquot was $2 \cdot 10^5$ cpm. Half dilutions were made until the aliquots contained approximately 10000 cpm per 50 μl. The reaction was allowed to proceed until equilibrium, for 4 h at room temperature or overnight at 4°C. The wells were extensively washed to remove slightly-bound radioactivity, cut and counted in a γ-scintillation counter for ^{111}In activity. Binding of ^{111}In-labeled antibody-polymer-liposome conjugates was compared with the binding of corresponding antibody directly labeled with ^{111}In via DTPA technique.

Experimental myocardial infarction in rabbits. Rabbits (New Zealand White rabbits, 2–3 kg) were anesthetized with Ketamin (70–75 mg/kg) and Xylazine (7–7.5 mg/kg). Right femoral artery cut-down was performed to establish a blood pressure line and for arterial blood sampling. The right femoral vein was catheterized to allow intravenous medication. A tracheostomy was performed, and ventilation was instituted through an endotracheal tube with a Harvard Rodent Ventilator (model 683). After artificial respiration, the anesthesia was switched to 3 ml (19.5 mg) pentobarbital infusion per hour. A left thoracotomy was performed and the anterior descending coronary artery was occluded with a silk suture placed through the myocardium with an SH-needle. After 40 min, the snare was released to allow reperfusion. A radiolabeled liposome preparation (2–3 ml in HBS, up to 30 mg of total lipids and 200 to 500 μCi of ^{111}In) was injected intravenously within 30 min of reperfusion. Blood samples were taken

Table 1
Biodistribution of liposomes (5 h post-injection, % dose/g)

Size	AM	PEG	Blood		Liver		Spleen		Kidneys		Lungs		Infarct		Normal		Infarct-to-normal	
			mean	S.D.	mean	S.D.	mean	S.D.	mean	S.D.	mean	S.D.	mean	S.D.	mean	S.D.	mean	S.D.
S	–	–	0.06	0.01	0.80	0.15	0.85	0.68	0.01	0.002	0.02	0.003	0.02	0.01	0.004	0.001	5.17	2.35
S	+	–	0.16	0.10	0.37	0.16	0.55	0.15	0.02	0.01	0.03	0.01	0.14	0.05	0.007	0.002	22.70	2.38
S	–	+	0.50	0.11	0.16	0.06	0.42	0.11	0.03	0.01	0.13	0.06	0.13	0.10	0.017	0.006	8.05	5.03
S	+	+	0.35	0.11	0.13	0.03	0.26	0.14	0.05	0.04	0.15	0.12	0.25	0.14	0.02	0.01	14.10	7.15
L	–	–	0.06	0.03	0.70	0.25	0.92	0.73	0.01	0.01	0.07	0.03	0.02	0.01	0.01	0.01	4.03	1.27
L	+	–	0.10	0.05	0.39	0.09	0.47	0.28	0.03	0.01	0.06	0.04	0.09	0.04	0.003	0.001	29.67	23.76
L	–	+	0.38	0.02	0.30	0.14	0.47	0.09	0.05	0.03	0.11	0.03	0.14	0.04	0.02	0.01	7.38	1.67
L	+	+	0.41	0.08	0.47	0.38	0.60	0.32	0.04	0.02	0.16	0.04	0.15	0.02	0.02	0.004	10.43	3.30

The mean infarct-to-normal ratios were calculated from the ratios of the original values. They need not to be equal to the ratios of the means given in the table. All values were rounded off to 2 or 3 decimal places. See Section 2 for details.

serially at 1, 3, 5, 10, 20, 30, 45, 60, 90, 120, 150, 180, 240 and 300 min after injection of liposomes to measure blood radioactivity (liposomal clearance). Time within which the blood content of the liposome-associated radioactivity dropped to 50% of the injected dose was designated as $t_{1/2}$. 5 h after liposome injection, animals were killed by an overdose of pentobarbital. The heart was excised and cut into 5 mm slices, stained with 2% triphenyl tetrazolium chloride to identify necrotic areas. Each slice was further divided into smaller segments for biodistribution studies following a standard scheme [23]. Samples of normal and infarcted myocardium, and other organs of interest were dried from excessive blood by blotting on absorbant towels and then weighed and counted in a gamma-counter. The liposome accumulation in the heart was expressed as infarct-to-normal myocardium radioactivity ratio. Biodistribution of liposomes was also studied following the liposome-associated radioactivity accumulation in nontarget organs (such as liver, spleen, kidneys and lung), and expressed as %dose per g of the tissue.

Statistical treatment of the animal data. The liposomes were prepared varying three factors: size (SL and LL), PEG coating and antimyosin antibody (AM) coating. Eight subgroups of animals were studied, four for each SL and LL groups. The four subgroups for each size included plain liposomes, PEG(only)-coated liposomes, AM(only)-coated liposomes, and PEG-AM(coincorporated) liposomes. Each subgroup comprised 3–5 rabbits. The contribution of each of the three experimental factors to the infarct-to-normal myocardium radioactivity ratio and the liposome accumulation in the liver, spleen, kidney, and lung were analyzed with a three-way factorial analysis of variance. Each model was reduced to the significant terms ($P < 0.05$), and all effects were estimated. They are presented as estimate ($\pm$ standard error). Means of the observed values were used to present the biodistribution data, while means of the corresponding ratios were used to estimate infarct accumulation (Table 1).

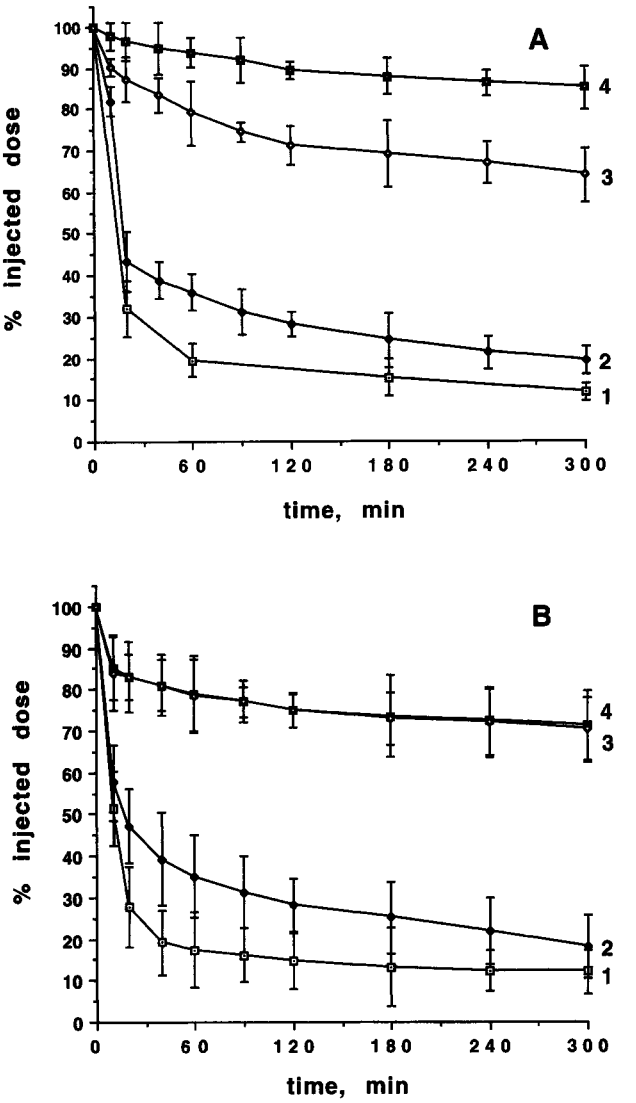

Fig. 1. Blood clearance of different liposomes in rabbits with experimental myocardial infarction. (A) Small liposomes. 1, SL; 2, AM-SL; 3, PEG-AM-SL; 4, PEG-SL. (B) Large liposomes. 1, LL; 2, AM-LL; 3, PEG-AM-LL; 4, PEG-LL.

3. Results

3.1. Immunoreactivity

The modification of antimyosin R11D10 Fab with hydrophobic substituent NGPE, and incorporation of NGPE-Fab into the liposomal membrane in this particular case decreased antibody immunoreactivity by approximately 15- to 20-times. The appropriate binding constant decreased from $5 \cdot 10^8$ for the native Fab to $3 \cdot 10^7$ for NGPE-Fab, and to $2 \cdot 10^7$ M^{-1} for an individual NGPE-Fab in liposome. However, as demonstrated earlier, such a decrease in immunoreactivity of an individual antibody is at least partially compensated by the increase in avidity due to the presence of multiple antibody molecules on the liposome surface providing specific multipoint attachment of immunoliposome to the target [26]. As a result, the apparent constant of immunoliposome binding to the antigen was in the range $(3–6) \cdot 10^8$ M^{-1} liposomes.

3.2. Blood clearance

The patterns of clearance of different liposome preparations from the circulation in infarcted rabbits are presented in Fig. 1.

3.3. Biodistribution

The biodistribution data obtained after 5 h of experiment were expressed as % of injected dose per gram of

tissue. The statistical analysis of the data related to individual organs revealed the following (see also Table 1):

Blood. The blood radioactivity at 5 h depended significantly upon the interaction (combination) of all three factors ($P = 0.006$). The strongest effect was observed for the PEG coating, which significantly ($P = 0.0001$) increased the mean level under all circumstances. The estimated effect was 0.44 (± 0.06) for SL without AM coating, 0.19 (± 0.06) for AM-SL, 0.32 (± 0.06) for LL without AM, and 0.31 (± 0.06) for AM-LL.

Infarcted area. In the infarcted myocardium, accumulation depended significantly upon the presence or absence of both the PEG coating ($P = 0.0013$) and the antimyosin antibody ($P = 0.005$). Both coatings increased the mean infarct accumulation: PEG - 0.10 (± 0.03), and AM - 0.09 (± 0.03); for all PEG-containing vs. corresponding PEG-free, and AM-containing vs. corresponding AM-free samples, respectively. Minimum accumulation was observed for both SL and LL - 0.02 ± 0.01; maximum accumulation was found for PEG-AM-SL - 0.25 ± 0.14.

Normal myocardium. Normal myocardium was demonstrably affected only by the PEG coating ($P = 0.0001$). The coating was estimated to increase the mean value by 0.013 (± 0.002).

Infarct-to-normal ratio. The infarct-to-normal ratio was demonstrably affected by the presence or absence of antimyosin antibody on the liposome ($P = 0.0002$). However, the extent of the effect depended significantly upon the presence or absence of the PEG coating together with AM ($P = 0.01$). In liposomes without any PEG coating, the AM coating increased the mean ratio by 21 (± 4). In liposomes with the PEG coating, the AM coating is estimated to increase the mean ratio by only 5 (± 4). This effect is not statistically significant ($P = 0.22$). Maximum value of the infarct-to-normal ratio was achieved for AM-SL - 22.70 ± 2.38. The value for AM-LL (29.67) can not be considered as reproducible because of large standard deviation (23.76).

Liver. Liver radioactivity accumulation was affected by both the PEG and AM coatings. Liposomes with PEG coating alone, the AM coating alone, or both had significantly ($P = 0.007$, 0.0003, and 0.0005, respectively) lower mean liver activity values than liposomes with neither coating. The differences can also be noticed between the mean values of accumulation of liposomes with either or both coatings (see Table 1).

Spleen. Spleen accumulation was not demonstrably affected by any of the three factors (size, PEG, AM).

Kidney. Radioactivity accumulation in the kidney was shown to be affected only by the PEG coating ($P = 0.002$) which was estimated to increase the mean value by 0.028 (± 0.008).

Lung. Radioactivity accumulation in the lung was shown to be affected only by the PEG coating ($P = 0.0003$) which was estimated to increase the mean value by 0.09 (± 0.02).

4. Discussion

Numerous in vitro and in vivo studies already have dealt with the interaction of long-circulating liposomes with cells and organs [27–29]. These studies have clearly documented the dependence of liposome clearance rate and the biodistribution pattern on liposome size and the presence of the protective substances on the liposome surface. However, attempts to prepare long-circulating *immuno*liposomes have added one more variable to the system, the targeting moiety (such as a monoclonal antibody). At a given phospholipid composition, the properties of the long-circulating immunoliposomes would depend on the liposome size, and the presence of both, the protective substance and antibody on the liposome. Therefore, permutations of these variables should enable the control of liposomal characteristics, such as clearance time, biodistribution, and target accumulation. This should permit preparation of liposomes specifically designed for targeted delivery of diagnostic agents (where maximum liposome accumulation in the target area and minimum accumulation in normal tissues are required to provide maximum target-to-nontarget ratio) and of therapeutic agents (where maximum absolute accumulation of drug-loaded liposomes in the target is required).

4.1. Blood clearance characteristics

Small liposomes. In case of SL, plain liposomes demonstrate the fastest clearance. Addition of AM slightly increases the circulation time probably by reducing the unaltered surface of liposomes which determines the liposome accessibility to opsonons [19,30]. Since we used Fab fragment of the antibody, immunoliposome capture via Fc fragments did not occur as in case of the whole IgG. Grafting of PEG to the liposome surface, as expected [21,23], sharply increases liposomal circulation time. Simultaneous incorporation of AM and PEG also significantly increases the circulation time, the blood clearance was marginally faster compared to the PEG liposomes. This difference in the clearance can be partly explained by a more pronounced interaction between AM and plasma proteins than between PEG and the same proteins. However, significantly longer circulation of PEG-AM-SL as compared to AM-SL allows for effective targeting as described by us previously [23].

Large liposomes. The clearance characteristics of LL are similar to SL. The only exclusion is that AM Fab incorporation practically does not influence circulation time of PEG-LL. This can be explained by size difference between SL and LL; the surface area of LL is approximately 6 times larger than that of SL, and possible irregularities in PEG location (such as the hypothetical formation of more dense PEG clusters because of polymer-to-polymer interactions which can result in the appearance of polymer-free areas on the liposome surface exposed for the

Table 2
Liposome circulation time

Liposome	Variable	$t_{1/2}$ (min)	Comments
Small	plain	10–15	Very short $t_{1/2}$
	+ AM (Fab)	15–20	Slight increase (due to partial surface protection?)
	+ PEG	> 1000	Significant increase (full surface protection)
	+ AM/ + PEG	ca. 600	Intermediate increase (interaction with plasma proteins is stronger for Fab than for PEG)
Large	plain	ca. 10	Very short $t_{1/2}$
	+ AM (Fab)	ca. 15	Slight increase; same as for SL
	+ PEG	> 600	Significant increase in circulation time, but still less than for PEG-SL.
	+ AM/ + PEG		No difference between PEG- and PEG-AM-LL: possible irregularities in surface PEG distribution (and consecutive opsonization) are more probable for LL.

opsonization) are more probable for PEG-LL than for PEG-SL. This is why the circulation time for PEG-LL is less than that for PEG-SL, and additional incorporation of AM Fab onto the surface of LL does not significantly change the clearance. Some alternative explanations are, probably, also possible.

The circulation time is therefore strongly influenced by all three factors studied – liposome size and the presence of AM, PEG or both on the liposome surface (see Table 2). However, size difference seems to be less important than surface modification with PEG or AM.

4.2. Targeting of the infarcted myocardium

Small liposomes. There is a marginally higher localization of plain SL in the infarcted myocardium as compared with remote normal myocardium. Such non-specific localization of liposomes in the injured myocardium has been shown previously [1–5]. Interestingly, both PEG-SL and AM-SL accumulate in the necrotic area almost identically if the total is expressed in absolute quantities, 0.13 and 0.14% dose/g, respectively. This indicates two different mechanisms for liposomal accumulation. Firstly, the specific one, which requires the presence of antibodies on the surface of short-circulating liposomes and permits selectively intense targeting of necrotic myocardium even after few passages over the area of interest [15]. Second mechanism is likely to be a non-specific, which slowly proceeds via impaired filtration mechanism in affected tissues with leaky vascular endothelium and requires repeated passages of liposomes through the target, i.e., prolonged circulation. The phenomenon of the accumulation of long-circulating liposomes has been reported previously in tumors with highly permeable endothelial layer [31,32], where the necrotic zone (if any) may also suffer from impaired drainage still further facilitating liposome accumulation.

Although absolute accumulation of AM-SL and PEG-SL is similar, the infarct-to-normal ratio (or relative targeting) is much higher for AM-SL than for PEG-SL; 22.70 ± 2.38 versus 8.05 ± 5.03, respectively. The reason for this interesting phenomenon is that the non-specific accumulation of AM-SL in normal tissues is very low (the time of AM-SL residence in the blood is too short). On the other hand, long-circulating PEG-SL slowly and non-specifically accumulate in both infarcted and normal tissues but the accumulation in infarcted tissued is comparatively higher due to exaggerated vascular permeability.

The combination of AM and PEG (PEG-AM-SL) on the liposome surface adversely affects the target-to-normal ratio, which is lower than that of AM-SL due to higher non-specific capture of PEG-AM-SL in normal tissue. However, in absolute terms (% dose/g) this combination provides excellent accumulation (0.25 ± 0.14) which is 2-fold higher than for short-circulating AM-SL. The intense PEG-AM-SL accumulation in the infarcted tissue can be explained by both specific and non-specific mechanisms of accumulation acting synergistically.

Large liposomes. Although, PEG and AM predominantly affected the target accumulation of liposomes, the liposome size also influences the targeting to some extent. The increase in liposome size reduces their ability for non-specific accumulation in the necrotic tissues. It can simultaneously affect the efficacy of AM-LL interaction with the target; for example, in the case when due to a short contact time with the target, part of fast-clearing AM-LL fail to form a sufficient number of Fab-antigen bonds to firmly anchor a large liposome to the target.

Similar to plain SL, no noticeable accumulation of plain LL occurs in the infarction due to brief residence time. The accumulation of both AM-LL and PEG-LL is higher. In case of LL, absolute accumulation of PEG-LL is even slightly better than that of AM-LL (0.14 ± 0.04 and 0.09 ± 0.04% dose/g, respectively). Evidently, for LL prolonged circulation can yield greater absolute accumulation through gradual accumulation than short-term specific interaction, part of which can be non-productive because of the large liposome size. Comparing these data with that of SL, one can hypothesize that to overcome the disadvantage of liposome size, at least a minimum critical residence time may be required for immunoliposome targeting.

Table 3
Liposome targeting to infarcted myocardium

Liposome	Variable	% dose/g	Comments
Small	plain	0.02	Very low accumulation
	+AM (Fab) +PEG	0.14 0.13	Good and similar. *Two mechanisms work: (1) specific binding and (2) non-specific accumulation due to impaired filtration.* Relative targeting (target-to-normal ratio) is much better for AM-SL (22.7) than for PEG-SL (8.05), because of low accumulation of AM-SL in normal tissues (fast clearance) and high accumulation of PEG-SL (slow clearance)
	+AM; +PEG	0.25	*Absolute accumulation is maximal; 0.25% dose / g: both mechanisms work!* Relative targeting is intermediate (14) because of high non-specific accumulation in normal tissues.
Large	plain	0.02	Very low accumulation.
	+AM (Fab) +PEG	0.09 0.14	Good and relatively close accumulation. Prolonged circulation might be even more effective than short-term specific interaction. Relative accumulation for AM-LL is again higher than for PEG-LL because of non-specific accumulation of PEG-LL in normal tissue.
	+Ab; +PEG	0.15	No improvement compared to PEG-LL: limiting step is non-specific accumulation in normal tissues; no room for AM additive effect.

In spite of relatively low AM-LL accumulation infarct-to-normal ratio remains higher for AM-LL than for PEG-LL due to essentially no non-specific uptake of AM-LL in normal myocardium (infarct-to-normal ratios, AM-LL 29.67 ± 23.76 vs PEG-LL 7.38 ± 1.67).

Co-incorporation of AM and PEG into the same liposome does not improve absolute accumulation of large liposomes. This can be explained by predominant effect of non-specific accumulation via impaired filtration mechanism which may offset the advantage of specific targeting in the case of LL. Infarct-to-normal ratio for PEG-AM-LL is between that for AM-LL and PEG-LL. Maximum accumulation of PEG-AM-LL is also less than that of PEG-AM-SL.

The maximum infarct-to-normal ratios could therefore be achieved for AM-SL, which makes them attractive for the targeted delivery of the agents where the maximum difference between the area of interest and normal tissues is desired (such as imaging agents). At the same time, for the requirement of maximum absolute delivery within the target tissue, liposomes combining on the surface both a protective polymer and an antibody appear to be the carriers of choice (PEG-AM-SL in our particular case) (Table 3).

4.3. Liposome biodistribution in nontarget organs

The biodistribution of both SL and LL and their surface-modified derivatives reconfirms previously known characteristics (Table 1).

Small liposomes. Plain SL and AM-SL are sequestered predominantly in the liver and spleen followed by a modest capture in kidneys and lungs. PEG modification of liposomes with or without AM modification, significantly decreases liposome accumulation in reticuloendothelial organs, while noticeably increasing it in kidneys and lungs. Pulmonary accumulation can be increased 8-fold.

Large liposomes. The common features of biodistribution pattern are preserved for different types of LL. Similar to the biodistribution of SL, sequestration of plain LL and AM-LL is observed in the liver and spleen. The protective effect of PEG modification is less pronounced than for small liposomes. LL with both AM and PEG on the surface accumulate in the spleen twice as much as SL counterpart. Lung accumulation is 3.5-times higher for plain LL than for plain SL, and increases further for PEG-coated LL. Nonspecific LL distribution in renal tissue was same as that for SL.

From the statistical analysis of the experimental data in the present study some important conclusions can be drawn with reference to the mechanisms of liposome accumulation in the target tissues, and optimization of liposome properties is suggested for their use as vehicles for various pharmaceuticals tailored to clinical demands.

References

[1] Caride, V.J. and Zaret, B.L. (1977) Science 198, 735–738.
[2] Mueller, T.M., Marcus, M.L., Mayer, H.E., Williams, J.K. and Hermsmeyer, K. (1981) Circ. Res. 49, 405–415.
[3] Palmer, T.N., Caride, V.J., Fernandez, L.A. and Twickler, J. (1981) Biosci. Rep. 1, 337–344.
[4] Caride, V.J., Twickler, J. and Zaret, B.L. (1984) J. Cardiovasc. Pharmacol. 6, 996–1005.
[5] Palmer, T.N., Caride, V.J., Caldecourt, M.A., Twicjler, J. and Abdullah, V. (1984) Biochim. Biophys. Acta 797, 363–368.

[6] Palmer, T.N., Caldercourt, M.A. and Kingaby, R.O. (1984) Biochem. Soc. Trans. 12, 344–345.
[7] Baldeschweiler, J.D. (1990) PCT Patent 9 012 595.
[8] Nguen, P.D., Orear, E.A., Johnson, A.E., Patterson, E., Whitsett, T.L. and Bhakta, R. (1990) Circ. Res. 66, 875–878.
[9] Jadot, G. and Michelson, A.M. (1987) Free Radical Res. Commun. 3, 389–394.
[10] Phelan, A.M. and Lange, D.G. (1991) Biochim. Biophys. Acta 1067, 97–102.
[11] Bkaily, G., Sperelakis, N., Elishalom, Y. and Barenholz, Y. (1983) Am. J. Physiol. 245 (Heart Circ. Physiol. 14), H756–H761.
[12] Laham, A., Claperon, N., Durussel, J.J., Fattal, E., Delattre, J., Puisieux, F., Couvreur, P. and Rossignol, P. (1988) J. Chromatogr. 440, 455–458.
[13] Khaw, B.A., Fallon, J.T., Beller, G.A. and Haber, E. (1979) Circulation 60, 1527–1531.
[14] Khaw, B.A., Beller, G.A. and Haber, E. (1978) Circulation 57, 743–750.
[15] Torchilin, V.P., Khaw, B.A., Smirnov, V.N. and Haber, E. (1979) Biophys. Res. Commun. 89, 1114–1119.
[16] Khaw, B.A., Beller, G.A., Haber, E. and Smith, T.W. (1976) J. Clin. Invest. 58, 439–446.
[17] Khaw, B.A. (1994) in Monoclonal Antibodies in Cardiovascular Diseases (Khaw, B.A., Narula, J. and Strauss, H.W., eds.), pp. 15–29, Lea and Febiger, Malvern.
[18] Allen, T. (1994) Adv. Drug Deliv. Rev. 13, 285–309.
[19] Torchilin, V.P., Omelyanenko, V.G., Papisov, M.I., Bogdanov, A.A., Jr., Trubetskoy, V.S., Herron, J.N. and Gentry, C.A. (1994) Biochim. Biophys. Acta 1195, 11–20.
[20] Torchilin, V.P., Shtilman, M.I., Trubetskoy, V.S., Whiteman, K. and Milstein, A.M. (1994) Biochim. Biophys. Acta 1195, 181–184.
[21] Klibanov, A.L., Maruyama, K., Torchilin, V.P. and Huang, L. (1990) FEBS Lett. 268, 235–237.
[22] Mori, A., Klibanov, A.L., Torchilin, V.P. and Huang, L. (1991) FEBS Lett. 284, 263–266.
[23] Torchilin, V.P., Klibanov, A.L., Huang, L., O'Donnell, S., Nossiff, N.D. and Khaw, B.A. (1992) FASEB J. 6, 2716–2719.
[24] Weissig,V., Lasch, J., Klibanov, A.L. and Torchilin, V.P. (1986) FEBS Lett. 202, 86–90.
[25] Kabalka, G.W., Buonocore, E., Hubner, K., Moss, T., Norley, N. and Huang, L. (1987) Radiology 163, 255–258.
[26] Klibanov, A.L., Muzykantov, V.R., Ivanov, N.N. and Torchilin, V.P. (1985) Anal. Bioch. 150, 251–257.
[27] Allen, T.M., Austin, G.A., Chonn, A. and Lee, K.C. (1991) Biochim. Biophys. Acta 1061, 56–63.
[28] Liu, D., Mori, A. and Huang, L. (1992) Biochim. Biophys. Acta 1104, 95–101.
[29] Litzinger, D.C., Buiting, A.M.J., van Rooijen, N. and Huang, L. (1994) Biochim. Biophys. Acta 1190, 99–107.
[30] Senior, J.H. (1987) CRC Crit. Rev. Ther. Drug Carrier Syst. 3, 123–189.
[31] Gabizon, A., Shiota, R. and Papahadjopoulos, D. (1990) J. Natl. Cancer Inst. 81, 1484–1488.
[32] Maruyama, K., Unezaki, S., Takahashi, N. and Iwatsuru, M. (1993) Biochim. Biophys. Acta 1149, 209–216.