

Specific targeting with poly(ethylene glycol)-modified liposomes: coupling of homing devices to the ends of the polymeric chains combines effective target binding with long circulation times

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One possibility for bringing drugs to their specific targets is to use the drug-laden liposomes that have been made target-specific by the attachment of appropriate proteins. Such 'directed' proteoliposomes and most other particles are rapidly removed from the bloodstream, however, by the mononuclear phagocytes in the liver and spleen. This causes suboptimal drug accumulation at the target site. Coating the liposome surface with poly(ethylene glycol) (PEG) may prolong the circulation time of liposomes. Using plasminogen as a homing device we have shown that the PEG-modified liposomes with such a homing device coupled to the ends of the long PEG chains may combine long vesicle circulation times in the blood with high target binding capability. The PEG-coated proteoliposomes with homing devices attached at the very bilayer surface, on the contrary, are longlived but have only little or no capability to bind to their targets.

Various methods have been devised for attaching the homing devices, such as antibodies, to the liposome surfaces (for a review, see Ref. 1). Even with such techniques available, the achievable proteoliposome site-specific targeting capability has remained low, however. Perhaps the most important for this is the very rapid elimination of the targetable proteoliposomes from the blood stream by the cells of mononuclear phagocyte system (MPS). This prevents most of the administered liposomes from reaching their designed target site. The recent development of long-circulating proteoliposomes could represent a major breakthrough in this field.

Liposomes that remain in the blood for a long periods of time have initially been made from the glycolipids such as monosialoganglioside (GM1) or hydrogenated phosphatidylinositol (HPI). These glycolipids were typically combined with the phospholipids with a high chain-melting phase transition temperature

[2,3]. More recently derivatives of the synthetic phosphatidylethanolamines (PE) with the hydrophilic poly(ethylene glycol) chains (PEG) attached to the polar lipid heads [4–9] have been introduced. Compared with previous formulations containing GM1 or HPI, incorporation of the PEG-PE conjugates into the lipid bilayers has allowed a far greater versatility of lipid compositions and doses without that the ultimate vesicle lifetime in blood and the tissue distribution were altered substantially [6,10].

Inhibition of the specific and/or nonspecific interactions between the PEG-modified liposomes and MPS has been invoked to explain these favorable characteristics; it has also already been confirmed by the first successful experimental tests [4,6,11,12].

The surface attached PEG-groups inhibit more than just the uptake of liposomes by the MPS, however. PE molecules with the long PEG 5000 heads also hamper the efficient binding of the proteoliposomes with such modified surfaces to their targets [13–17]. Some authors have therefore suggested using shorter, PEG 1900, heads for this purpose [13,28]. In spite of the fact that some binding between such liposomes and their cellular targets is observed [28], even such relatively

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short heads, when present at the liposome surfaces at the required high concentration, tend to suppress the interactions between the target-receptors and the homing devices [13]. The use of longer spacers with the terminally attached homing devices has therefore also been discussed to achieve the desired goal [15].

In this study we describe an even simpler and more elegant solution: we propose that the target-recognizing proteins should be attached to the ends of the polymeric chains that render liposomes long-lived. Using Glu-plasminogen as a model target-recognizing molecule we show that the corresponding proteoliposomes are both longlived *in vivo* and capable to bind to their natural target, fibrin layers.

Distearylphosphatidylethanolamine (DSPE) was obtained from Boehringer (Mannheim, Germany), soybean phosphatidylcholine (SPC) from Nattermann Phospholipids (Köln, Germany) or Lipoid KG (Ludwigshafen, Germany). Phospholipids purity was assayed by thin-layer chromatography (TLC); it was higher than > 99% for the synthetic and > 98% for the biological lipids. [³H]Cholesteryl hexadecyl ether was from Amersham Buchler (Buckinghamshire, UK). Methoxypoly(ethylene glycol) with 110 repetitive units, activated with cyanuric chloride, polyoxyethylene bis(acetic acid) containing 77 repetitive units, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (ECDI) and cholesterol were all purchased from Sigma (Munich, Germany). Glu-plasminogen was a gift of Kabi Pharmacia (Stockholm, Sweden).

DSPE-triazine-PEG 110 (DSPE-PEG 110) and DSPE-PEG 77-COOH, with an amide bond, were synthesized as described previously [4,6]; the first by the method of Abuchowski et al. [18] and the latter according to Kung and Redemann [19].

Standard liposomes with a short protein anchor contained SPC/Chol/DSPE-*N*-glutaryl (67.6:27:5.4, all values in molar terms); the surface modified, long-circulating vesicles were made of SPC/Chol/DSPE-*N*-glutaryl/DSPE-PEG (60.8:27:5.4:6.8) and the liposomes with terminally attached proteins ('long coupling anchor') consisted of SPC/Chol/DSPE-PEG 77-COOH (67.6:27:5.4).

Vesicle suspensions typically contained 5 weight% of lipids (as determined by the Fiske-SubbaRow assay [20]) in a 10 mM Hepes buffer (pH 7.2). After 20 extrusions through a polycarbonate filter (50 nm pore size) the resulting average vesicle diameter was maximally 80 nm, for the DSPE-*N*-glutaryl containing vesicles, and 120 nm, for the vesicles consisting of DSPE-PEG 77-COOH. These values were determined by the dynamic light scattering (Malvern, UK). (Vesicles for the *in vivo* studies contained traces of [³H]cholesteryl hexadecyl ether (0.5 μ Ci/mg phospholipid).)

Glu-plasminogen was coupled to 1 day old liposomes as described by Kung and Redemann [19]. In

brief, liposomes (50 μ moles phospholipid in 1 ml Hepes buffer at pH 7.2 and room temperature) were activated for 45 min by the addition of 200 μ l of 1 M ECDI in 0.155 M NaCl and acidification (to pH 5.5) by 1 M HCl. Subsequent pH adjustment to pH 8.8 with 1 M NaOH (200 nmoles) was followed by the addition of Glu-plasminogen in 600 μ l Hepes buffer (pH 7.2). The ionic strength of suspension was then increased to 0.12 with NaCl and Glu-plasminogen was allowed to bind to the lipid vesicles at the resulting pH 8.5 for 4 h at room temperature. Free protein was removed by the gel-chromatography on a Sephacryl S1000 superfine column. Finally, liposomes were concentrated by centrifugation in a Centriscart I vial (SM 13239E, Sartorius, Göttingen, Germany) at 3000 $\times g$ for 20 min. Protein concentration was measured spectrophotometrically using a modified Bradford [21] assay (Bio-Rad, Munich, Germany) and was shown to be nearly unaffected by the staining of amino-bonds on the PEG-PE molecules.

Plasminogen-fibrin association was determined as described previously [23,24]. In brief, the solutions of free or the suspensions of the liposome-bound Glu-plasminogen were brought into contact with a fibrin-coated surface. After an incubation for 12 h at room temperature the supernatant was collected and the surface was washed three times. The amount of bound protein was determined according to Ref. 22.

For the measurements of liposome lifetime in the blood circulation normal female Balb/c mice were used [4]. In each test, three mice (25 g) received 0.25 ml of an appropriate liposome suspension through the tail vein; this corresponds to 2–2.5 mg phospholipid per mouse.

In order to develop long-circulating proteoliposomes which bind well to their targets we have addressed two basic problems: (1) Does the presence of the superficially bound PEG affect the effectiveness of the proteoliposome-target association? (2) Does the presence of Glu-plasminogen on the outer surface of the PEG-coated liposome interfere with the liposome longevity *in vivo*?

To answer these two questions we have first studied the properties of various surface-modified liposomes which are schematically shown in the inset to Fig. 1. This representation makes clear that the PEG-layers on the liposomal surface may, but need not, mask the interactions between the homing devices and their targets. In order to quantify this phenomenon we have explored the binding properties of three different types of the plasminogen-containing proteoliposomes:

- (i) PEG-free liposomes with Glu-plasminogen covalently linked to the short lipidic anchor, DSPE-*N*-glutaryl (spacer length 1 nm, type A);
- (ii) PEG-coated proteoliposomes containing PEG 5000-DSPE, with 110 repetitive PEG units, and a headgroup length of < 20 nm [25,26] with Glu-

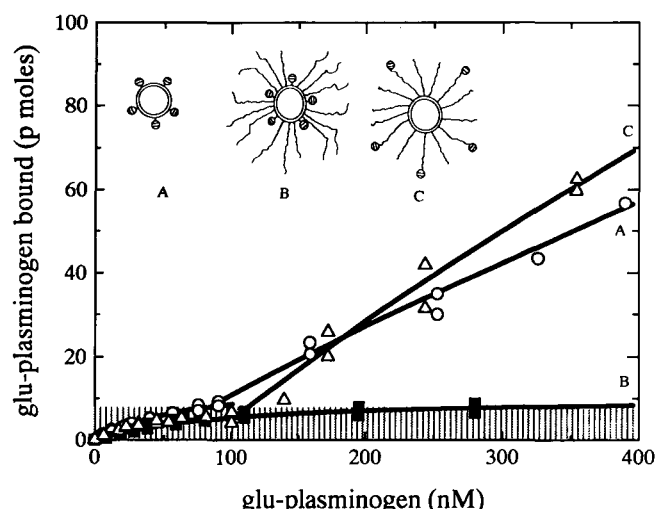


Fig. 1. Specific binding of the liposome-associated Glu-plasminogen to the fibrin-multilayers in vitro. ○, PEG-free proteoliposomes with Glu-plasminogen covalently linked to the short lipidic anchor DSPE-*N*-glutaryl (spacer length about 1 nm, type A). ■, PEG-coated proteoliposomes containing DSPE-PEG 5000, which has headgroups with 110 repetitive PEG units and a length of less than 20 nm, where Glu-plasminogen is covalently linked to the DSPE-*N*-glutaryl (type B). △, proteo-PEG-coated liposomes with Glu-plasminogen attached to the DSPE-PEG 3500-COOH-terminus, each long headgroup containing 77 repetitive PEG units having a length of 12 nm (type C). Lipid concentration increases with increasing protein concentration (cf. Table I). To allow for the nonspecific-binding, the results pertaining to free plasminogen were subtracted from the other measured data. Shaded area shows the limit of confidence for such a subtraction method.

plasminogen covalently bound to the DSPE-*N*-glutaryl (type B);

(iii) proteo-PEG-coated liposomes with Glu-plas-

minogen covalently attached to the ends of the DSPE-PEG 3500-COOH with 77 repetitive units and a headgroup length of about 12 nm (type C).

Characteristics of all three proteoliposome types are given in Table I. PEG content in the lipid bilayer was chosen to be always similar, nominally 6.8 mol% and 5.4 mol% (of the total lipid, TL) for the types B and type C, respectively, to facilitate the direct comparison with previous results of other authors.

The final protein/lipid ratios under our coupling conditions was found to be between $2 \cdot 10^{-3}$ mmol/mol TL (for the liposomes of types A and C) and $1.2 \cdot 10^{-3}$ mmol/mol TL (for the liposome of type B). This corresponds to approx. 180, 140 and 600 protein molecules per liposome, in the case of vesicles of the types A, B and C, respectively. The much higher numbers of the plasminogen molecules attached to the surfaces of the PEG-coated proteoliposomes (type-C) are due to the vesicle size effects. The relative coupling efficiencies for the PEG-free and PEG-modified liposomes are nearly identical (55%). Non-specific adsorption of plasminogen to liposomes is rather low ($< 2.5 \cdot 10^{-4}$ mmol/mol TL).

The potential problem of the PEG-dependent reduction of the liposome-target association was assessed in vitro by measuring the binding of the liposome-associated plasminogen to its natural target, fibrin, in vitro (cf. Fig. 1). The degree of fibrin binding of the proteo-PEG-coated liposomes (type C) was found to be similar to that of the PEG-free proteoliposomes (type A). This suggests that target binding of the Glu-plasminogen molecules attached to the ends of the polymeric chains is not affected by the coupling procedure. (In fact, the efficacy of the liposome-mediated protein binding to its

TABLE I

Proteoliposome characteristics

| | Type A PEG-free proteoliposomes | Type B PEG-coated proteoliposomes | Type C proteo/PEG-coated liposomes |
|--|---------------------------------------|---|--|
| PEG content (mol% of TL) ^a | 0 | 6.8 | 5.4 |
| Coupling ratio ($\mu\text{g protein}/\mu\text{mol TL}$) ^b | 182 ± 15 | 97 ± 4 | 164 ± 9 |
| Coupling efficiency (%) ^c | 56 ± 5 | 30 ± 2 | 50 ± 3 |
| Mean particle size (nm) | 65 ± 13 | 80 ± 19 | 125 ± 29 |
| Estimated number of protein molecules per liposome | 180 | 140 | 600 |
| Protein density at the liposome surface (nm^{-2}) | $3.4 \cdot 10^{-3}$ | $5.7 \cdot 10^{-4}$ | $3 \cdot 10^{-3}$ |
| Enzymatic activity (%) ^e | 150 | 110 | 190 |

^a Nominal PEG content expressed as mol% of total lipid (TL).

^b The coupling ratio ($\mu\text{g protein}/\mu\text{mol TL}$) was determined following the separation of the liposome-bound protein from the unreacted protein by gel-chromatography as described in the text.

^c Coupling efficiency (%) was calculated by dividing the coupling ratio by the nominal ratio of the protein and lipid concentrations during the coupling reaction ($\times 100$).

^d The number of protein molecules per liposome, at the coupling ratio indicated, was estimated by using the following assumptions: mol. wt. of Glu-plasminogen is 81000; surface area is $1 \cdot 10^{11} \text{ nm}^2/\text{mmol TL}$; each vesicle has 1.5 bilayers, on the average; mean particle diameter is as indicated.

^e Enzymatic activity is expressed as percentage of the activity of native, free Glu-plasminogen.

receptor is, in molar terms, higher than that of the free plasminogen under comparable conditions [23].)

In contrast to this, the efficiency of the binding of the PEG-coated proteoliposomes (type B) to fibrin layers is negligible. This illustrates the surface masking problem: proteins that are associated with the B-type proteoliposomes are buried in the PEG-layers which severely hampers target binding.

Partial suppression of the interaction between the PEG-coated immunoliposomes and their targets has been observed previously both *in vitro* [15,29] and *in vivo* [13,16,17]. Diminished target binding capability has been correctly attributed to the interference between the PEG-reach region on the liposome surface and the designed liposome binding site. The surface density of the PEG-groups as well as the molecular mass of the PEG- and homing-segments used both play some role in this.

The investigated PEG-coated proteoliposomes contain lipids with relatively heavy, 5000 Da headgroups. Cosgrove et al. [25] have measured the thickness of the PEG 5000 rich layers, created by the terminal polymer attachment to the latex surface, to be less than 20 nm. This suffices to prevent an approach of the plasminogen-residues, with an estimated size of approx. 5 nm, to their designed binding sites on the fibrin multilayers. Terminal protein attachment to a polymeric chain (for example the DSPE-PEG 3500-COOH molecule) eliminates this problem.

We have also examined whether the Glu-plasminogen molecules attached to the outer surface of the PEG-ylated liposomes (types B and C) interfere with the liposome longevity *in vivo*. Fig. 2 shows the kinetics of elimination of different proteoliposome types from

the murine blood circulation. As expected, the PEG-coated proteoliposomes (type B) remain in the circulation much longer than the PEG-free proteoliposomes (type A), their longest half-times of elimination being 11.6 h and 5 h, respectively. Corresponding PEG-liposomes without surface attached Glu-plasminogen are cleared from the bloodstream with half-times of 9.9 h and 4.8 h (results not shown).

This demonstrates that surface PEG-ylation prolongs the circulation time of liposomes *in vivo* even in the presence of surface-bound proteins.

24 h after injection, $26 \pm 0.4\%$ (mean \pm S.D.) of the dose of the PEG-coated proteoliposomes that were administered *i.v.* are typically still found in the blood. This value is much higher than the $4.0 \pm 0.4\%$ achieved by using PEG-free proteoliposomes. These results agree qualitatively with those of other authors (see, for example, Refs. 27 and 28). Proteo-PEG-coated liposomes (type C) also remain in the blood circulation much longer than the standard, PEG-free proteoliposomes (cf. Fig. 2): 24 h after the administration of the former, $18.4 \pm 0.6\%$ of the injected dose still circulate in the blood. Liposome targeting by means of the homing devices that are attached directly to the end of the PEG 3500 chains is thus perfectly compatible with the vesicle longevity *in vivo*.

In summary, we have introduced and tested a new type of the long-circulating proteoliposomes which can effectively bind to their designated target sites *in vitro*. This was achieved by the direct coupling of plasminogen, a model homing device, to the ends of the PEG chains that render liposomes long-lived in the bloodstream. Preparation of such targetable, long-circulating liposomes is relatively simple and generally applicable. We believe that such surface PEG-ylated liposomes with the terminally attached targeting moieties will provide valuable assets for the site-specific delivery of the liposomal drug carriers *in vivo*.

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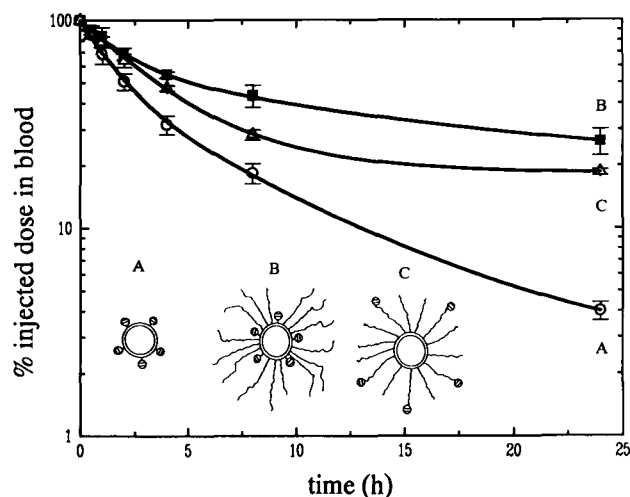


Fig. 2. Kinetics of the elimination of the Glu-plasminogen proteoliposomes from the murine blood-stream after an *i.v.* injection of 2.5 μ moles of phospholipid. \circ , SPC/Chol/DSPE-*N*-glutaryl (67.6:27:5.4, mol/mol/mol); \blacksquare , SPC/Chol/DSPE-*N*-glutaryl/DSPE-triazine-PEG 110 (60.8:27:5.4:6.8, mol/mol/mol); \triangle , SPC/Chol/DSPE-PEG 77-COOH (67.6:27:5.4, mol/mol/mol).

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