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Influence of surface hydrophilicity of liposomes on their interaction with plasma protein and clearance from the circulation: studies with poly(ethylene glycol)-coated vesicles

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Well-defined liposome systems have previously established the influence of size, surface charge lipid composition and surface ligands, on in vivo fate and behaviour of model compounds entrapped in liposomes. In the present study, preformed liposomes which quantitatively retain aqueous markers were covalenty coupled via dipalmitoylphophatidyl-ethanolamine, to the hydrophilic polymer, monomethoxypoly(ethylene glycol) (MPEG 5000). Such liposomes retain the coating in the presence of plasma, and appear to adsorb plasma components more slowly than liposomes without the polymer, shown using an aqueous two-phase partitioning technique. MPEG-coupled liposomes were cleared from the blood circulation up to 30% more slowly than liposomes without MPEG after intravenous administration to mice, despite the unmodified liposomes being of a composition and size shown previously to favour achievement of maximum half-life. It is suggested that the polymer acts as a surface barrier to plasma factors which otherwise bind to liposomes in the blood and accelerate vesicle removal.

Introduction

Ways are being sought to increase surface polarity of liposomes, which is expected to prolong vesicle half-life in the circulation [1]. Rationale for this approach is based on evidence that molecules (e.g., opsonins) in the blood responsible for enhancing the uptake of liposomes by the RES, prefer to associate with hydrophobic surfaces [2,3]. Previous work [4] in which small neutral unilamellar liposomes with a highly rigid bilayer have shown a half-life of 12–20 h in mice, has set a standard for liposomal survival times in the circulation; increased solute retention and longer half-lives favour targeting of liposomes to tissues within, and even outside the RES [1,3]. However, to increase the half-life of vesicles even further, and/or to facilitate slow-release of liposomally-entrapped agents into the bloodstream using liposomes with non-rigid bilayers, other lipid components or surface coatings may be required. Previously described procedures which can confer a net increase in hydrophilicity at the liposome surface include: incorporation of gangliosides into the lipid bilayer [5], or coating the liposomal surface with glycosides [6] or poloxamers [3]. While inclusion of ganglioside in liposomes appears to increase the blood/RES ratio of liposomally associated drug for some lipid compositions [5], it is not clear to what surface property of the vesicles this can be attributed, since any increase in hydrophilicity will be accompanied by a net surface-negative charge due to the sialic acid component of the ganglioside. Furthermore, incorporation of relatively small amounts of ganglioside destabilize the liposomal membrane [7,8] to different extents dependent on lipid composition, and this is reflected in variable whole body recoveries of liposomally entrapped agent [9]. In a new approach, we have increased surface polarity of small, neutral lipo-

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Abbreviations: MPEG, monomethoxypoly(ethylene glycol); PEG, poly(ethylene glycol); TMPEG, tresylated monomethoxypoly(ethylene glycol); SUV, small unilammelar liposomes; DPPE, dipalmitoylphosphatidylethanolamine; CF, carboxyfluorescein; DSPC, distearoylphosphatidylcholine; Res, reticulo-endothelial system.

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somes which quantitatively retain aqueous solutes, without causing inter-vesicle cross-linking, and without conferring net surface charge, by covalently linking monomethoxypoly(ethylene glycol) (MPEG) to the bilayer surface. MPEG is preferred as the starting material because one of the two terminal hydroxyl groups of PEG is blocked as the methoxyether, leaving the other hydroxyl group free for derivatization. PEG and MPEG have previously been coupled to protein, the conjugates showing an increased half-life in the blood [10,11].

Aqueous two-phase partition systems of dextran and poly(ethylene glycol) (PEG) [12–14] have been used previously to investigate the surface properties of liposomes. Depending upon the size [15,16], lipid composition [15,17,18], lipid asymmetry [19] and the presence of other components [20], liposomes will partition in favour of the PEG-rich top phase or the dextran-rich bottom phase in a selected system. By altering the composition of the biphasic system with respect to the concentration and molecular weight of polymers, and/or concentration and type of buffers salts etc. (see Refs. 12, 13), partitioning of liposomes can be made sensitive to their charge (using a charge-sensitive phase system) or non-charged surface components (using a non-charge-sensitive phase system); consequently, systems can be chosen which direct liposomes of specified composition to either of the two phases or the bulk interface. Making the liposome surface more 'PEG-like' by hydrophobic association with PEG-palmitate increases liposome partitioning into the PEG-rich top phase [17]. We have, therefore, used phase partitioning to monitor the covalent attachment of MPEG to liposome surfaces.

In this report, MPEG, activated at its single free hydroxyl group with tresyl chloride [21], was covalently coupled to small unilamellar liposomes via the free amine group of dipalmitoylphosphatidylethanolamine incorporated at 20 mol% of total phospholipid, into a rigid liposomal bilayer composed of distearoylphosphatidylcholine and equimolar cholesterol. The covalent attached of MPEG and its retention on the liposomal surface after incubation with plasma at 37 °C for up to 6 h, were investigated by phase partitioning. Binding of plasma components to liposomes with and without attached MPEG was also investigated, and phase partitioning found to have advantages compared with other methods [22]. Small unilamellar liposomes with and without coupled MPEG were injected intravenously into mice and vesicle clearance from the circulation estimated from latent CF and radiolabelled lipid in blood samples.

Materials and Methods

Sources and grades of distearoylphosphatidylcholine (DSPC), dipalmitoylphosphatidylethanolamine (DPPE), cholesterol and carboxyfluorescein (CF) have been described elsewhere [4,23]. 1,2-Dipalmitoyl-L-3-phosphatidyl[*N-methyl*-^{3}H]choline ([^{3}H]DPPC), specific activity 2.81 TBq/mmol, was obtained from Amersham International, U.K. Tresylated monomethoxypoly(ethylene glycol) (TMPEG) was obtained by reacting MPEG (M_r 5000), Union Carbide, U.S.A.) and tresyl chloride (Fluka, Switzerland) as previously reported [23]. Dextran T500 (Lot MI 02434) was from Pharmacia (Sweden), PEG 6000 (M_r 6000, Lot 9159110) was from British Drug Houses (Poole, U.K.) and PEG 8000 (M_r 8000) was from Union Carbide, U.S.A. All other reagents were of analytical grade.

Preparation of small unilamellar liposomes (SUV) and assessment of liposomal stability

SUV composed of DSPC : DPPE : Chol molar ratio 0.8 : 0.2 : 1, were prepared as described previously [4] to a final concentration of 15.25 mg phospholipid/ml aqueous phase. Tracer [^{3}H]DPPC ($6 \cdot 10^6$ dpm per 30 mg phospholipid; 1.4 μ Ci/ml) was incorporated as a marker for estimating lipid concentration during procedures. To measure liposomal retention of water-soluble molecules during the coupling reaction and subsequent procedures, CF was partially purified and entrapped at 0.15 M [4] and latency measured as reported [22]. Free CF was removed by gel filtration before MPEG was coupled to liposomes.

Coupling of MPEG to SUV

0.5 ml SUV were mixed with an equal volume of (TMPEG) made up in 0.05 M sodium phosphate buffer (pH 8.5) containing 0.0125 M sodium chloride (PBS) (125 mg/ml). After incubation for 2 h at 22 °C, free TMPEG was separated from MPEG-liposomes by gel filtration on Sepharose 4B-CL. A control sample of SUV (c-liposomes) was incubated in PBS and also subjected to the gel filtration step. The concentration of phospholipids in the preparations obtained after filtration was 2 mg/ml as measured [22] by [^{3}H]DPPC content. These preparations (MPEG-liposomes and c-liposomes) were used for subsequent studies of partitioning, plasma interactions and in vivo clearance. In one experiment, preparations obtained after filtration were subjected to dehydration within dialysis tubing placed on PEG flakes, to increase the concentration of phospholipids to 4 mg/ml. The average diameter of vesicles before and after reaction with TMPEG was determined in a separate experiment using Quasi-elastic light scattering (Nicomp Model 270 particle analyser). Liposomes composed of 20 mol% PE were pre-sized to approx. 100 nm using a freeze-thaw extrusion method [24], and reacted with TMPEG under similar conditions to those described above to give an average diameter of 115 ± 17 nm before the reaction compared with 144 ± 28 nm afterwards.

Phase partitioning: preparation of phase systems

Two-phase systems of 5% (w/w) dextran and 5% (w/w) PEG were prepared in 0.01 M sodium phosphate (pH 6.8), containing 0.15 M sodium chloride (a non charge-sensitive system) by mixing the required weights of the following stock solutions: 20% Dextran T-500, either 40% (w/w) PEG 6000 or 40% (w/w) PEG 8000, 0.44 M sodium phosphate (pH 6.8), 0.6 M sodium chloride and distilled water. The two phases were allowed to equilibrate at 25°C, separated and stored at 4°C until required.

Phase partitioning of SUV

Partitioning of SUV was carried out as described previously [15]. In brief, liposomes (50 μl) were applied to a mixture of upper and lower phases (1 ml of each phase) equilibrated at 25°C in a 50 × 10 mm tube. The phases were mixed for 1 min by repeated inversion, total radioactivity samples (50 μl), and after a further 20 min at 25°C, triplicate samples (50 μl) from each of the top and bottom phases removed for counting (tracer radioactive lipid) or for measurement of CF in Triton X-100 (1% final concentration). Distribution of liposomes between phases was expressed as a percentage of total material added to the phase system; radioactivity or CF present at the interface was estimated as the difference between the total, and the sum of the material in the top and the bottom phases.

Phase partitioning of plasma proteins

Mouse plasma (50 μl) was combined with 1 ml of each phase (top and bottom) equilibrated at 25°C and mixed for 1 min as above. After complete separation of phases (within 30 min) samples of top and bottom phases were removed for analysis of plasma protein concentration either colorimetrically [25] or by radioactivity measurements (^{125}I-radiolabelled plasma) when liposomes were present. Distribution of the proteins between the phases is expressed as the partition coefficient, defined as the ratio of protein concentration in top and bottom phases.

Incubation of liposomes with plasma

Liposomes (0.1 ml) with or without attached MPEG, were incubated at 37°C with 0.5 ml fresh plasma (mouse) or buffer. Samples of incubation mixtures were removed at time intervals, and liposome partitioning measured. In some experiments, fresh plasma was radiolabelled with ^{125}I by the method of Fraker and Speck [26] to a specific activity of 0.14 MBq/μg protein; $7 \cdot 10^5$ cpm (2 μl) are added to 0.3 ml unlabelled plasma for use. For a non-plasma treated control, liposomes were incubated with PBS.

In-vivo behaviour of SUV coupled with MPEG

Liposomes (0.2 ml containing 0.4 mg phospholipid in one experiment, 0.8 mg in another) were injected intravenously into the tail vein of male TO mice (five each group). Liposome clearance was assessed from latent (entrapped) CF and ^{3}H-radiolabelled phospholipid measured in blood (plasma) samples (25 μl) withdrawn from the tail vein at time intervals [22]. In one experiment (0.8 mg dose), the liposome suspension was subjected to ultracentrifugation at 100000 × g for 1 h, and supernatant containing small vesicles 20–100 nm (av. 50 nm) in diameter [4], injected. Liposomes were dialysed overnight against PBS (4°C) and the c-liposomes were diluted to the same lipid concentration on the basis of ^{3}H-lipid measurements, as the MPEG-liposomes.

TABLE I

The effect of coupling MPEG to liposomes: partitioning in a PEG-Dextran two-phase system

SUV composed of 10 mol% DPPE, 40 mol% DSPC and 50 mol% cholesterol, were incubated with 25 mg/ml TMPEG at pH 8–8.5 for 1–2 at room temperature (PEG-treated), or with buffer in the absence of MPEG (control). Partition of ^{3}H-phospholipid-labelled liposomes was determined using a biphasic system composed of 5% (w/w) PEG 8000, 5% (w/w) Dextran T500 and sodium phosphate buffer (0.01 M) (pH 6.8) in 0.15 M NaCl.

Liposomes	Partition (% of total ± S.D. *)		
	top	interface	bottom
Control	1.4 ± 0.2	36.0 ± 5.0	62.5 ± 5.1
MPEG-coupled	96.5 ± 1.0	1.4 ± 1.1	2.1 ± 0.4

* $n = 6$.

Results and Discussion

Partitioning behaviour of liposomes: effect of coupling with MPEG

Table I shows that a non charge-sensitive phase system formed by 5% dextran and 5% PEG 8000 can distinguish between SUV incubated in presence or the absence of TMPEG. Liposomes incubated in PBS distribute to the interface and the bottom phase (62.5 ± 5.1% and 36.0 ± 5.0, respectively) with little partitioning into the top phase, whereas liposomes incubated with TMPEG distribute almost exclusively (96.5 ± 1.0%) into the top phase (Table I). This major change in partitioning reflects the presence of MPEG on the liposomal surface. Adsorption of MPEG to the surface rather than a covalent linkage to the amino group of the DPPE is considered unlikely, because the partition coefficient of liposomes incubated with untresylated MPEG is similar to that of liposomes incubated in buffer (data not shown). Partitioning behaviour of liposomes was re-examined after preparative procedures (gel filtration, dehydration/rehydration, centrifugation) and was unchanged prior to in vivo administration of liposome preparations (data not shown), suggesting that MPEG remains coupled to the vesicle surface throughout these procedures. The aqueous marker CF, was quantitatively

TABLE II

Partition of plasma proteins in the presence and absence of MPEG-liposomes

Partition of plasma proteins as measured by Coomassie assay and ^{125}I-radioactivity in a phase system consisting of 5% (w/w) PEG 6000, 5% (w/w) Dextran-T500, 0.01 M sodium phosphate (pH 6.8) and 0.15 M sodium chloride. TMPEG-treated liposomes and control liposomes were mixed with plasma and partition of plasma proteins measured at time intervals. Unless otherwise indicated each value is the mean of two independent experiments each measured in triplicate. The partition coefficient (K) is the ratio of protein concentration in the top and bottom phases.

Incubation time	Partition coefficient (K) of plasma proteins		
	without liposomes	with liposomes	
		control	+MPEG
1 min	0.47 ± 0.02 ($n = 4$)	0.44	0.46
1 h		0.47	0.48
6 h		0.46	0.47

retained in liposomes during procedures, indicating that structural integrity of the vesicles was maintained during and after the coupling reaction, as well as during phase partitioning (data not shown).

Effect of plasma proteins on the phase-partitioning of liposomes

Preliminary experiments showed that plasma increased SUV partition to the bottom phase. Therefore, a non charge-sensitive phase system of 5% Dextran T500 and 5% PEG 6000 was selected (in contrast to PEG 8000 used above) to provide substantial top phase partitioning of control liposomes so that the change in partition on serum treatment could be followed more sensitively.

Fig. 1 shows that c-liposomes incubated with PBS at 37°C for up to 6 h showed no alteration in partition (ca. 35% top; 50% interface; 15% bottom), whereas contact with plasma for 1 min caused a marked change in partition in favour of the bottom phase (5% top; 35% interface; 60% bottom). This effect of plasma did not alter on longer incubation (up to 6 h). Plasma proteins have a low partition coefficient (Table II) i.e., they distribute mainly (68%) to the bottom phase. Thus, c-liposomes incubated only briefly in plasma, mostly partition to the same place as plasma alone, thus indicating surface properties similar to that of plasma itself. This suggests an association between the plasma proteins and the liposomal surface which probably reflects a change in the liposomal surface that takes place instantaneously on contact with plasma.

By way of contrast to c-liposomes, liposomes coupled to MPEG partition exclusively into the top phase of the system in the absence of plasma (Fig. 1), but on contact with plasma, also partition to some extent into the interface (11%) and bottom phase (12%), the remaining 77% being in the top phase. As contact with plasma continues, top phase partitioning is reduced further to 65% (1 h), and to 33% by 6 h, with liposomes being displaced to the bottom phase and interface (Fig. 1). As with control liposomes, MPEG-liposomes are displaced from the top phase towards the bottom phase during incubation with plasma, but this process takes longer for the MPEGylated vesicles. It is unlikely that changes in partitioning of MPEG-liposomes represent loss of MPEG during the incubation because on removal of plasma, MPEG-liposomes re-partition to the same phase as before exposure to plasma (Fig. 1, 6 h*). Thus, small changes in partitioning of MPEG-liposomes as the incubation with plasma proceeds, may reflect gradual plasma protein adsorption onto the surface of liposomes, so that the whole surface is not instantaneously coated with plasma proteins as appeared to occur with control liposomes. Furthermore, Table II shows that partitioning of the plasma proteins themselves is not altered by the presence of liposomes despite the observed changes in liposome-partitioning (Fig. 1). That plasma proteins continue to partition in the same ratio (partition coefficient) in the presence or absence of liposomes suggests that the amount of plasma protein interacting with liposomes must be below the detection limit of the methods used to determine protein concentrations in each phase.

Attempts to detect whether any plasma protein was associated with liposomes after removal of the bulk of plasma proteins using gel filtration, were carried out using liposomes with and without coupled MPEG and incubated in plasma as before; liposome fractions recovered after gel filtration were pooled, and samples (50 μl) applied to the phase partitioning system. Surprisingly, the partitioning behaviour of liposomes after gel filtration whether coupled with MPEG or not, was the same as that obtained in the absence of plasma (Fig. 1, 6 h*). This suggests that any plasma components associating with liposomes can alter the partitioning behaviour, but are effectively removed by gel filtration since the original, preincubation partitioning behaviour is restored. Although it is known that some higher-molecular-weight plasma components may co-elute with liposomes under the conditions used in this experiment [27], the majority of plasma proteins will be removed [22]. Furthermore, any plasma component eluting with the liposomes has no detectable effect on the partitioning behaviour of MPEG- or control liposomes. It therefore appears that gel filtration removes the plasma component(s) causing phase-partitioning changes (Fig. 1). Thus, phase partitioning techniques may allow identification of plasma factors which can become associated with liposomes yet are removed by filtration. If this is indeed the case, phase partitioning is confirmed as a useful tool for studying liposome surface interac-

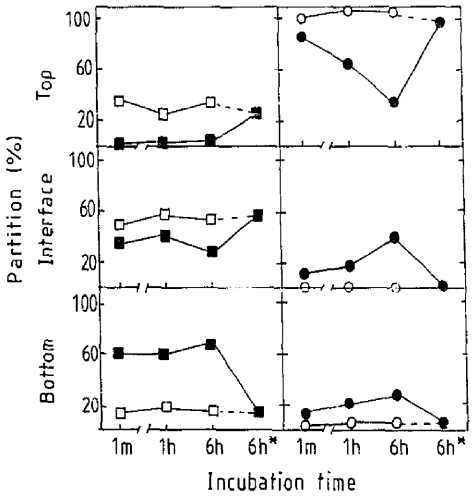

Fig. 1. The effect of plasma on the partitioning of MPEG-modified SUV. Control (left) (■, □) and MPEG-modified (right) (●, ○) SUV composed of DSPC, DPPE and cholesterol (0.8:0.2:1 molar ratio) were incubated with plasma (●, ■) or buffer (○, □). At time intervals the partitioning of the vesicles was measured in a two phase system of 5%/5% PEG 6000 and Dextran T500 in 0.15 M NaCl buffered with 0.01 M Na phosphate (pH 6.8). After 6 h (6 h*) the vesicles that had been exposed to plasma were re-isolated by gel chromatography and re-partitioned.

tions; such interactions may involve weak binding, possibly of a transitory nature.

In-vivo clearance of SUV; effect of coupling MPEG

Clearance of control and MPEG-coupled liposomes after intravenous administration to mice, is shown in Fig. 2 for a sonicated, uncentrifuged preparation. These preparations presumably contain some larger vesicles which are cleared rapidly within 1 h after injection. However, the slower clearance phase corresponding to 50–60% of the lipid dose (0.2–0.3 mg) shows a marked difference in half-life for MPEG-liposomes and control liposomes (10 h and 7 h, respectively). The effect of MPEG on SUV half-life was also determined using SUV devoid of larger vesicles and at higher concentration of phospholipid, 0.8 mg, similar to that used in previous studies [4]. Liposomes were centrifuged at 100000 × g, and their clearance shown in Fig. 3. Again, there is a longer half-life (14 h) for MPEG-liposomes, than for the control (12 h). The extent to which DPPE in the liposomal bilayer is saturated with MPEG was not determined in these studies, but it is conceivable that altered binding conditions could result in more MPEG being attached, and vesicle half-life increased further. Recent work has produced similar increases in half-life for SUV composed of DSPC: Chol: GM_1 molar ratio 1:1:0.1 (Senior, J. and Gregoriadis, G., unpublished data).

In conclusion, grafting of polar ligands onto the surface of liposomes has been investigated with a view to increasing their circulation half-lives. Using small unilamellar liposomes of a composition that has been previously shown to produce the longest half-lives in the absence of further surface modification, a modest increase in half-life (ca. 30%) was obtained. This increase appears to demonstrate the validity of the concept that increasing hydrophilicity of the liposome surface will lead to longer half-lives in the blood, presumably reflecting a reduction in the uptake by liver, spleen and bone-marrow. This may have applications in slow-release of drugs, or in conjunction with other ligands, to increase the amount of ligand-bearing vesicles in the circulation available for interaction with the target. Increased retention of particles (polystyrene microspheres) coated with polyoxyethylene compounds (poloxamers) in the circulation, has been reported [28], and the effectiveness of the coating in reducing the rate of removal of particles from the circulation related to an increase in the hydrophilicity of surface coating [30]. Previous attempts (unpublished) to coat liposomes with poloxamer did not appear to prolong the clearance of SUV composed of PC and equimolar cholesterol, but it was not possible to measure how much poloxamer had bound to the vesicles and/or remained on them in circulation. MPEG has been coupled to proteins to

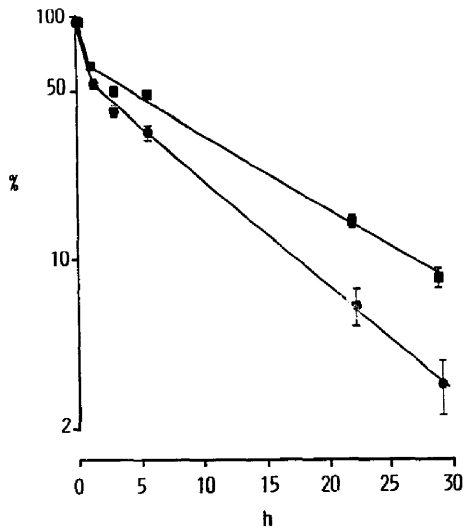

Fig. 2. Comparison of the clearance of MPEG-modified and unmodified SUV from the circulation of mice (i). SUV of composition DSPC: DPPE: Chol (molar ratio 0.4:0.1:0.5), either MPEG-modified (■) or unmodified (●), were injected intravenously into mice (0.4 mg/25 g mouse). Total blood levels of CF (% dose ± S.E. five animals) are shown; ^{3}H-phospholipid clearance was similar (not shown). Total blood levels based on an assumed blood volume of 75 ml/kg body weight [22].

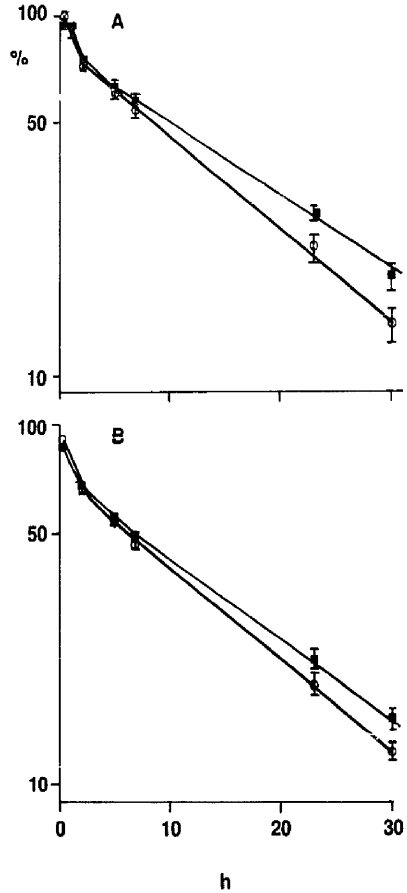

Fig. 3. Comparison of the clearance of MPEG-modified and unmodified SUV from the circulation of mice (ii). Conditions were identical to Fig. 2, except that the SUV preparation was centrifuged at 100000 × g for 1 h to remove larger vesicles and the injected doses were 0.8 mg phospholipid/25 g mouse. Both CF clearance (A) and ^{3}H-phospholipid clearance (B) (% of injected dose in total blood) are shown for MPEG-treated (●) and untreated (○) vesicles. 23 and 30 h time points are significant at $P = 0.06$ and 0.05, respectively (Unpaired, 2-tail Student's t-test).

prolong their circulation times [11], but this is the first attempt to attach the polymer to liposomes for this purpose. Preliminary studies with MPEG-bearing liposomes of a slightly larger size (approx. 100 nm) prepared by a different method (see above) suggest that particle size is insignificantly increased by the attachment of the polymer, although any increase in particle diameter would be expected to reduce the half-life of the vesicles [4]. It is possible that PEG acts to slow down liposome clearance from the circulation by acting as a steric barrier for attachment of plasma proteins including those involved in accelerating particle clearance.

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