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Extended in vivo blood circulation time of fluorinated liposomes

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The clearance from blood circulation of fluorinated liposomes made with perfluoroalkylated phosphatidylcholines was investigated in mice using liposome-entrapped 5(6)-carboxyfluorescein. The presence of a fluorinated core inside the membrane strongly retards their blood clearance. The fluorinated vesicles showed circulation half-lives of up to 8.6 h, which are 6–13 and 3–6 times larger than those of similarly sized conventional distearoylphosphatidylcholine and distearoylphosphatidylcholine/cholesterol liposomes, respectively. Their blood clearance was similar to that of some polyethylene glycol (PEG)-labelled 'stealth' liposomes and was dose-independent in a 3.3–330 μmol/kg body weight dose range.

Fluorinated liposome; Fluorinated membrane; Blood clearance; Circulation half-life; Lipophobicity; Intravascular persistence

1. INTRODUCTION

The use of liposomes as drug carrier and delivery systems is currently a topic of intensive investigation [1]. In the last few years much effort has focussed on the development of vesicles which evade the mononuclear phagocytic system (MPS) and, consequently, exhibit prolonged circulation times in the blood stream. Significant advances were achieved in this respect by including some specific natural glycolipids (such as the monosialylganglioside, G_{M1}) in order to mimick the outer composition of the red blood cell membrane [2,3] and more recently by using synthetic phospholipids with hydrophilic, bulky and mobile polyethylene glycol (PEG) groups [4–6]. Such sterically stabilized PEG-liposomes show prolonged circulating times in blood, irrespective of the dose administered, surface charge density, bilayer composition and fluidity [5,7,8]. However, the modifications made in view of extending the intravascular persistence have as yet mainly been concerned with the composition and aspect of the surface of the liposomes.

Liposome clearance from the blood stream occurs as a result of interactions with blood proteins and other plasma components, resulting in liposomes opsonization followed by macrophage uptake, and/or to liposome disintegration by lipid exchange and depletion [1,9]. In order to reduce the MPS uptake and to enhance the circulation times of liposomes after i.v. administration, we have examined the possibility of reducing these interactions by endowing the liposomal membrane with some of the characteristic properties of fluorine, i.e. its low affinity for lipidic phases. The highly stable fluorinated liposomes investigated here are made from synthetic phospholipids having perfluoroalkylated tails in their hydrophobic acyl chains (see Fig. 1) [10,11]. We have already shown that the presence of a fluorinated core inside the membrane enhances the retention of a water-soluble drug model, including human serum [12], and strongly decreases the membrane's affinity for a lipophilic probe [13].

In this paper we report the impact of the use of fluorinated phosphatidylcholines on the intravascular persistence of liposomes in mice. We have also examined the effect of the fluorinated liposomes' membrane composition and size, and the dose dependence on their clearance from circulation. Our results show that fluorinated liposomes have circulating times in the blood stream higher than those of conventional distearoylphosphatidylcholine (DSPC) or even distearoylphosphatidylcholine/cholesterol (DSPC/CH) liposomes of similar sizes. Their intravascular persistence was comparable to that of some PEG-derivatized liposomes. Most interestingly, we found also that the blood clearance of the fluorinated liposomes was dose-independent over a dose range of 3.3–330 μmol/kg mouse body weight.

2. MATERIALS AND METHODS

2.1. *Materials*

The fluorinated F6C11PC and F8C5PC phospholipids (see Fig. 1) were synthesized according to [11]. Their purity was assessed by ^1H and ^{31}P NMR, and TLC. 5(6)-Carboxyfluorescein (CF, HPLC Grade 99%), was purchased from Sigma. Phosphate-buffered saline (PBS, injection grade) came from Biomerieux. All containers and fluids used in these experiments were sterile and pyrogen-free.

2.2. *Preparation of the liposomes*

The phospholipids (50–70 mM) were hydrated above their respective phase transition (T_c = 51°C for F6C11PC, 69°C for F8C5PC) in

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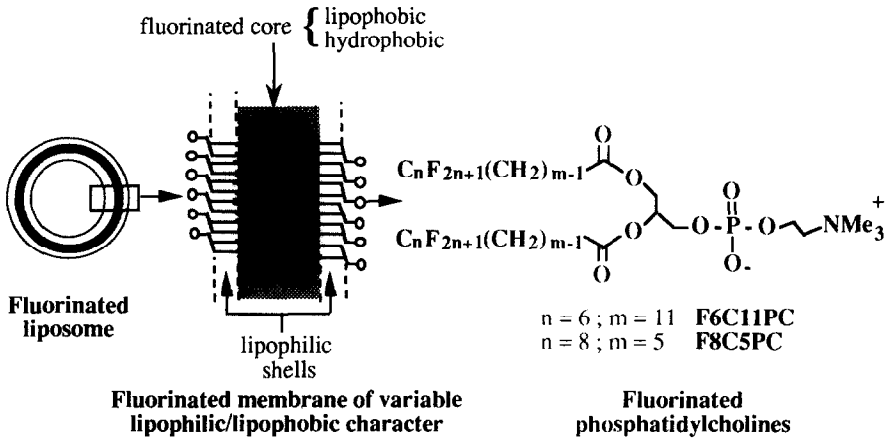

Fig. 1. Structure of the fluorinated phospholipids, membranes and liposomes.

a 100 mM CF solution (pH 7.4), followed by several cooling–heating cycles through the T_c. The crude dispersion was then sonicated using a tip sonicator to produce small unilamellar vesicles of 50–70 nm. To avoid membrane defects arising from sonication, the vesicles were annealed for 1 h above their respective T_c and then aged for 12–48 h at room temperature until they reached the desired mean diameter. Free CF was then separated from the liposome-entrapped dye by gel filtration on a Sephadex G-50 column equilibrated in PBS. Liposome sizes, which are listed in Table I, were measured by dynamic light scattering using a Coulter model N4MD sub-micron particle analyzer. The samples gave good fits with a unimodal population (polydispersity 0.1–0.18 for F6C11PC, 0.29 for F8C5PC).

Phospholipid concentration was determined by a phosphate assay [14] or by ^{19}F NMR, using CF_3CH_2OH as an internal standard. Liposomal CF was measured in appropriately diluted samples before and after addition of 10% sodium deoxycholate (DOC) on a Perkin Elmer LS50B spectrofluorimeter, using excitation and emission wavelengths of 480 nm and 520 nm, respectively. Latent CF, which is the fraction of encapsulated CF, was calculated as $100 \times (F_T - F_f/F_T$ where F_T and F_f denote the total fluorescence (after the liposomes had been destroyed by DOC) and free (released) dye, respectively.

2.3. *In vivo experiments*

OF1 Dawley male mice (3–5 per group, two independent experiments on 3 animals, $n = 6$, or a single one on 5 animals, $n = 5$) in the weight range 25–33 g were injected into the tail vein with the liposomal preparations at a dose of 0.1–10 μmol of phospholipid per mouse (ca. 3.3–330 μmol/kg mice body weight). At selected times post injection, 30 μl of blood was collected from the tail vein in heparinized micropipettes and rapidly mixed with 2.5 ml of PBS. The samples were spun out at 3,000 rpm for 10 min. The supernatant was analyzed for CF before and after addition of DOC. Latent CF was determined and expressed as % of CF latency in the respective liposomal preparation before the injection. The data were analyzed using the appropriate Student's t-test and found statistically significant ($P \leq 0.05$).

2.4. *In vitro experiments*

CF-loaded liposomes prepared from F6C11PC or F8C5PC were incubated at 37°C in a fresh mouse blood/heparine mixture or in human serum. The volume ratio of blood/liposomes was similar to that expected after i.v. injection (assuming blood volume to be 1/15 of body weight in mice) for doses in the 3.3–330 μmol/kg animal body weight range. When incubated with blood, the samples were shaken at 120 strokes/min. At selected times, 30 μl samples were collected and assayed for CF latency as for the in vivo experiments. For the experiments run in human serum, 30–50 μl of the liposomal preparations were mixed with 2 ml of serum. At selected times 20 μl were collected, diluted in 0.15 M NaCl, 20 mM HEPES buffer and assayed for CF latency. Each experiment was performed at least in triplicate.

Table I

Effect of lipid composition and liposome size on circulating half-times ($t_{1/2}$) and % of injected dose present in circulation after various times post-injection, for fluorinated, conventional or polyethyleneglycol (PEG)-derivatized liposomes after intravenous injection in mice

Lipid composition	Size ± S.D. (nm)	Injected dose (μmol/kg body weight)	Circulating half-time (h)	% injected dose	Reference
F6C11PC	110 ± 30	40	8.6	77[a]	[d]
F6C11PC	170 ± 50	40	6	66[a]	[d]
F8C5PC	210 ± 80	40	4.2	58[a]	[d]
DSPC	30–60	45	1.5	–	[18]
DSPC	150	55	0.47	~10[a]	[6]
DSPC/CH 1/1	50	33	7.5	~80[a]	[18]
DSPC/CH 1/1	200	33	1.5	~20[a]	[18]
DSPC/PEG110 9/1	90	55	9.52	~85[a]	[7,19]
DSPC/PEG5000 9/1	90	55	8.4	~80[a]	[20]
DSPC/PEG1900 1/0.1	112–136	20	–	~44[b]	[20]
DSPC/CH/PEG2000 1/1/0.6	180–200	30	–	~50[c]	[21]

[a] 2 h post-injection, extrapolated from the curves. [b] After 24 h. [c] 3h post-injection. [d] This work.

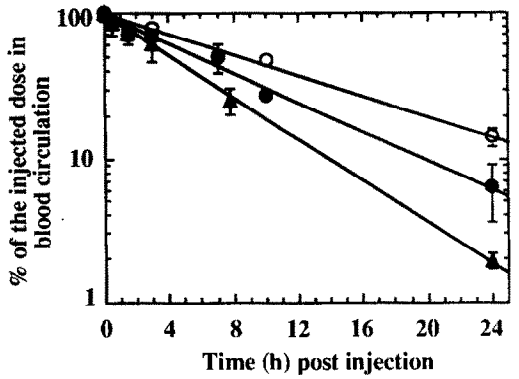

Fig. 2. Blood clearance of fluorinated liposomes after i.v. injection in mice at a dose of 40 μmol/kg body weight. Data are expressed as mean ± S.D. (n = 5 or 6). When no errors bars are seen, standard deviation is smaller than the data points. (○) 110 nm-sized F6C11PC vesicles; (●) 170 nm-sized F6C11PC vesicles; (▲) 210 nm-sized F8C5PC vesicles.

3. RESULTS

To examine the clearance of the fluorinated liposome from blood circulation, we have used 5(6)-carboxyfluorescein (CF) as a marker of their internal aqueous space. This technique has proved a convenient and accurate means of determining the half-life of the vesicles in circulation [15]. In order to measure vesicle clearance we have first assessed the extent of leakage of the fluorinated liposomes in vitro when incubated in human serum and in mouse blood in conditions close to those encountered in vivo. It was found that the fluorinated liposomes display in human serum and in mouse blood a high stability with respect to CF leakage. The kinetics of CF release were indeed very slow: less than 5–6% of CF release over a period of 24 h. It also appears that passive adsorption of the fluorinated vesicles onto erythrocytes does not contribute significantly to their in vivo clearance. CF latencies for fluorinated liposomes incubated at 37°C in mouse blood were indeed found comparable to those determined for vesicles consequently diluted in a 0.15 M NaCl, 20 mM HEPES buffer.

Fig. 2 shows the clearance of the fluorinated vesicles from the blood stream as a function of time after an i.v. administration in mice of differently sized vesicles made from F6C11PC (110 and 170 nm) or F8C5PC (210 nm) at a dose of 1.2 μmol/30 g mouse. The kinetics fitted monoexponential decays. The circulating half-lives, $t_{1/2}$, calculated from these curves are presented in Table I together with data selected from the literature showing the effects of liposome composition and size on $t_{1/2}$ and % of injected doses present 2 h after injection for conventional and PEG derivatized liposomes after i.v. injection in mice.

Fig. 3 illustrates the blood level of the 110 nm-sized F6C11PC SUVs measured at 15 min and 2 h after i.v. administration for injected doses ranging from 3.3 to 330 μmol/kg mouse body weight. It indicates that the clearance from the blood stream of these fluorinated liposomes is apparently dose-independent in the dose range investigated.

4. DISCUSSION

The main objectives of this study were to evaluate the impact on blood clearance of the presence of a fluorinated core within the membrane of liposomes made from fluorinated phosphatidylcholines (Fig. 1). We have also examined the effects of the fluorinated vesicles' size and dose on their clearance. These parameters are known, among others, to influence the intravascular persistence and biodistribution of conventional liposomes made from synthetic or natural phosphatidylcholines [16]. The impact of the fluorinated compartment was retrieved from a comparison of the fluorinated vesicles' clearance to that of liposomes made from the structurally closely related hydrogenated analog, DSPC (among the hydrogenated phosphatidylcholines, DSPC-based liposomes possess one of the highest intravascular persistences).

The most important finding of this study is that the presence of a fluorinated core inside the liposomal membrane strongly increases the blood circulation times of the vesicles, including large-sized ones: the $t_{1/2}$ measured for the F6C11PC and F8C5PC liposomes (Table I) are almost 6–13 times larger than those reported for similarly sized conventional DSPC liposomes. Liposome stability and persistence in blood circulation may be improved by the inclusion in the membrane of the latter of high levels of cholesterol [16,17]. We found that the presence of a fluorinated core within the membrane slowed down the liposomes' blood clearance even more effectively than a high content of cholesterol. When compared to DSPC/CH 1/1 vesicles of similar size, the vesicles made from fluorinated phospholipids alone exhibited 3–6 times longer blood circulation times. Most interestingly, we found that the

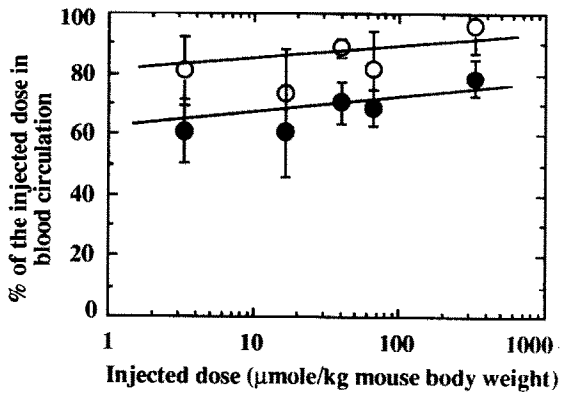

Fig. 3. Blood clearance in mice of 110 nm-sized F6C11PC vesicles as a function of injected dose. Each point represents an average ± S.D. (n = 3–5). The results are plotted as the % of the injected dose present in blood: (○) 15 min post-injection; (●) 2 h post-injection.

F6C11PC liposomes display a blood clearance similar to that of some of the long-circulating PEG-labelled liposomes, the so-called 'stealth' liposomes (Table I).

It is also noteworthy that the liposomes made of F6C11PC exhibit an almost dose-independent clearance over the 3.3–330 μmol/kg body weight range (Fig. 3), while saturation is generally observed for doses between 50–100 μmol/kg in the case of conventional liposomes [18].

The behaviour, in terms of high circulation half-times and dose-independence, of the fluorinated liposomes is comparable with that of PEG-derivatized vesicles which also show extended blood circulation times irrespective of dose for sizes below 200 nm. In the latter case, steric stabilization and surface mobility of the long polymeric PEG chains are thought to decrease opsonization and, consequently, the recognition and uptake of such liposomes by the MPS [8]. In the case of the fluorinated liposomes, it is most likely the fluorinated core inside their membrane, with its lipophobic character, which is able to reduce the adsorption and anchoring of lipophilic plasma proteins either onto the surface and/or more deeply into their membrane, thus inhibiting their recognition and uptake by the MPS. The high in vitro stability observed for the fluorinated liposomes both in serum [12] and in whole blood, as a result of an enhanced hydrophobic character of the fluorinated membrane, may also account for a decrease in their disintegration and depletion through lipid exchange induced by plasma proteins and consequently for their extended blood circulation.

Our results show further that not only direct modifications of the liposomes' surface are of prime importance for evading in vivo recognition and MPS uptake, but also more subtle and a priori 'invisible' changes introduced inside the membrane, such as here with the presence of a fluorinated core within the membrane.

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