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Liposome clearance in mice: the effect of a separate and combined presence of surface charge and polymer coating

Tatiana S. Levchenko, Ram Rammohan, Anatoly N. Lukyanov, Kathleen R. Whiteman, Vladimir P. Torchilin *

Department of Pharmaceutical Sciences, Bouve College of Health Sciences, Northeastern University, Mugar Building 312, 360 Huntington Avenue, Boston, MA 02115, USA

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Abstract

The purpose of our work was to compare the biodistribution of liposomes with different surface properties. Phosphatidylcholine (PC)/cholesterol (Chol) liposomes were prepared containing 6% mol of a charged lipid (stearylamine, SA; phosphatidic acid, PA; or phosphatidyl serine, PS) and/or polyethylene glycol (PEG)-PE of different MW (750 and 5000). ζ-Potentials and liposome clearance in mice were investigated. In vitro, the attachment of PEG in a similar fashion neutralizes the effect of any charged component. In vivo, the chemical nature of a charged lipid becomes important. Both short PEG750 and longer PEG5000 inhibit the clearance of positively charged SA-liposomes, while only longer PEG5000 inhibits the clearance of negatively charged PA-liposomes and none of the PEGs inhibit the clearance of negatively charged PS-liposomes. The opsonins with different molecular size may be involved in the clearance of liposomes containing different charged lipids.

Keywords: Liposome; Charged lipids; Polyethylene glycol; ζ-Potential; Circulation time

1. Introduction

The use of liposomes as drug carriers requires the liposomal preparations with various clearance rates and biodistribution patterns to better fit the specifics of each particular application. Liposome charge and liposome coating with different polymers, such as PEG, are among the parameters known to strongly affect biological properties of liposomes. The effect of charge and PEG on biological behavior is well investigated (Gabizon and Papahadjopoulos, 1992; Lasic and Martin, 1995). It was repeatedly demonstrated that the incorporation of charged phospholipids into liposomes accelerates their clearance (Lee et al., 1992; Liu and Liu, 1996), while grafting liposomes with PEG and similar polymers makes liposomes long-

Abbreviations: Chol, cholesterol; DCP, dicetyl phosphate; DMSO, dimethyl sulfoxide; DTPA, diethylene triamine pentaacetic acid; HBS, HEPES-buffered saline; PA, phosphatidic acid; PC, phosphatidyl choline; PE, phosphalidyl ethanolamine; PEG, polyethylene glycol; PS, phosphatidyl serine; SA, stearylamine; TEA, triethyl amine.

* Corresponding author. Tel.: +1-617-373-3206; fax: +1-617-373-8886.

E-mail address: vtorchil@lynx.neu.edu (V.P. Torchilin).

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circulating (Klibanov et al., 1990; Torchilin and Trubetskoy, 1995).

Thus, the incorporation of PS or dicetyl phosphate (DCP) into PC/Chol liposomes dramatically enhances liposome uptake by the perfused mouse liver (Liu and Liu, 1996). The fact that the negative charge strongly increases the clearance of liposomes was also shown by Gabizon and Papahadjopoulos (1992). Negatively charged PS was found to abolish the longevity of liposomes prepared of a lipid composition resembling that of erythrocyte membrane (Allen et al., 1988). The major mechanism behind the charge-facilitated liposome clearance is an interaction of charged phospholipid head-groups with certain opsonizing proteins. The components of the complement system (fragments of C3) are involved in the clearance of PS-containing liposomes in the guinea pig and rat (Liu et al., 1995a; Huong et al., 2001). Liposomes containing PA, phosphatidyl glycerol and DCP compete in different degrees for serum components, which mediate the liver uptake of PS-containing liposomes (Liu et al., 1995a). Both negative and positive phospholipids (such as phosphatidyl ethanolamine (PE)) are strongly opsonized (for review, see Moghimi and Hunter, 2001), however, certain groups, such as PS, are especially efficient binding centers for plasma proteins (Chiu et al., 2001). Generally speaking, liposomes of different composition and charge exhibit different protein-binding properties and bind a different spectrum of proteins (Chonn et al., 1992; Liu et al., 1995b). In vitro, binding and endocytosis of liposomes by cells are mediated by specific lipid head-groups and surface charge density (Lee et al., 1992). It is interesting that in mice, the liver uptake of liposomes does not involve any specific opsonins, while the liposome uptake by the rat liver strongly depend on serum opsonins (Liu et al., 1995b). In addition, within the same species different ligands can interact with different opsonins.

The mechanisms of the protective effect of PEG on liposomes include an electrostatic and steric repulsion between PEG-grafted bilayers (Needham et al., 1992; Kenworthy et al., 1995) and the formation of protecting polymeric layer by flexible polymeric chain on the surface of liposomes (Torchilin et al., 1994). Liposome-grafted PEG prevents liposome clearance by neutralizing the surface charge of liposomes and shielding various opsonins. While PEG-PE itself demonstrates slight electronegativity, with shorter PEG producing a more electronegative product, a larger PEG can effectively shield the charge of a phospholipid block as well as the charge of the whole liposome (Needham et al., 1992; Shimada et al., 1995). This means that even if the surface potential of a PEG-liposome is negative, the net ζ-potential of such a liposome is close to neutral (Moribe et al., 1997). Thus, it was shown that the increase in the quantity of liposome-attached PEG from 0 to 10% sharply decreases ζ-potential of the liposome (Arnold et al., 1990). PEG (and other similar polymers) inhibits the attraction of opsonins because opsonins cannot bind immobilized water on the surface of liposomes and the thickness of polymer layer decreases the uptake of PEG-liposomes by macrophages (Zeisig et al., 1996), Above a certain level, an attached polymer can completely eliminate the effect of the liposome-incorporated charged and opsonin-binding groups, as was shown for PS-containing liposomes with 15% PEG-PE (Chiu et al., 2001).

An interesting question arises—does PEG provide the same effect for liposomes of all compositions or does the protective effect (and liposome clearance) depend not only on the thickness of the layer of protecting polymer on the liposome surface (i.e. on PEG concentration and MW), but also on the type of a charged and opsonin-attracting site? Assuming that different 'attraction' sites (PS, PA, PE, etc.) may predominantly interact with certain specific opsonins (Cullis et al., 1998; Moghimi and Hunter, 2001), differently sized opsonins, varying from 17.5 kDa (Kelly et al., 1992) to 80 kDa (Thornqvist et al., 1994) and up through all the intermediate sizes (Yang and Yoshino, 1990; Thornqvist et al., 1994 and see Cullis et al., 1998, for review), may demonstrate different degrees of interaction with liposomes of different compositions, especially at low and moderate concentrations of a liposome-attached polymer or at low MW of this polymer. Moreover, as a result, the shielding effect of the liposome-attached PEG may be seen differently in vitro and in vivo.

Here, we compare the biodistribution of liposomes with negative or positive surface charge additionally coated with a similar molar quantity of PEG with different MW, in order to investigate the relative role of the liposome charge and the length of the liposome-attached PEG chains on the liposome circulation time and liver accumulation in mice.

2. Materials and method

2.1. Materials

PC, Chol, PE, PS, PA, PEG750-PE and PEG5000-PE were from Avanti Polar Lipids. Triethylamine (TEA), SA and diethylene triamine pentaacetic acid (DTPA) anhydride are products of Sigma-Aldrich. 111–$InCl_3$ was obtained from NEN. All solvents and components of buffer solutions were analytical grade products.

Table 1
Size and ζ-potential of various liposomal preparations

Liposome preparation	Size (nm ± S.E.)	ζ-Potential (meV ± S.E.)
Plain LS	226 ± 60	−9.64 ± 0.53
LS+PEG750-PE	183 ± 40	−16.42 ± 1.75
LS+PEG5000-PE	179 ± 44	−12.68 ± 1.20
LS+SA	188 ± 47	8.48 ± 0.79
LS+SA+PEG-750-PE	181 ± 34	−2.47 ± 1.65
LS+SA+PEG-5000-PE	177 ± 40	−6.72 ± 1.04
LS+PA	186 ± 37	−46.37 ± 1.68
LS+PA+PEG-750-PE	180 ± 47	−20.38 ± 0.75
LS+PA+PEG-5000-PE	180 ± 45	−15.51 ± 0.70
LS+PS	180 ± 34	−39.09 ± 2.0
LS+PS+PEG-750-PE	177 ± 44	−31.10 ± 1.28
LS+PS+PEG-5000-PE	175 ± 36	−12.76 ± 1.40

2.2. Methods

2.2.1. Liposome preparation by extrusion

Liposomes were composed of PC and Chol in a molar ratio of 7:3. When necessary, the initial lipid mixture was supplemented with 6% mol (% mol is the molar fraction of the compound in the mixture multiplied by 100) of a charged component (SA, PA or PS) and/or 6% mol of PEG750-PE or PEG5000-PE (see Table 1 for the types of liposomes, LS, prepared). Additionally, 0.5% mol of DTPA-PE was added to all lipid compositions for subsequent liposome labeling with 111-ln. The organic solvent from the starting lipid or lipid/polymer solution in chloroform was removed on a rotary evaporator (Labconco) and by freeze-drying with a Freezone 4.5 Freeze Dry System (Labconco). For hydration, the film obtained was supplemented with *N*-2-hydroxymethylpiperazine-*N*′-2-ethanesulfonate (HEPES) buffered saline (HBS) (pH 7.4) and vortexed at room temperature for 15 min. The crude lipid dispersion was extruded 40 times through polycarbonate filter (pore size 0.2 μm) using LiposoFast System (Avestin).

2.2.2. Characterization of liposomes

Liposome size and size distribution were determined by the Coulter N4 MD Submicron Particle Size Analyzer (Coulter Electronics). ζ-Potentials of various liposome preparations were measured at 25 °C in HEPES buffer, pH 7.4 (0.08–0.015 mg lipids per ml) using Zeta-PULS ζ-potential analyzer (Brookhaven Instruments).

2.2.3. Preparation of radioactive labels and labeling of liposomes with 111-In

For clearance experiments, liposomes were radioactively labeled with 111-In via the liposome-incorporated amphiphilic chelating agent DTPA-PE. DTPA-PE was synthesized by the interaction of PE with DTPA anhydride in organic solvent in the presence of TEA. The product was purified by dialysis against water and lyophilized. To label DTPA-PE-containing liposomes with 111-In, the liposome suspension (2 ml) was supplemented with 30 μl of 1.0 M citrate and incubated for 1 h with a required quantity of a citrate

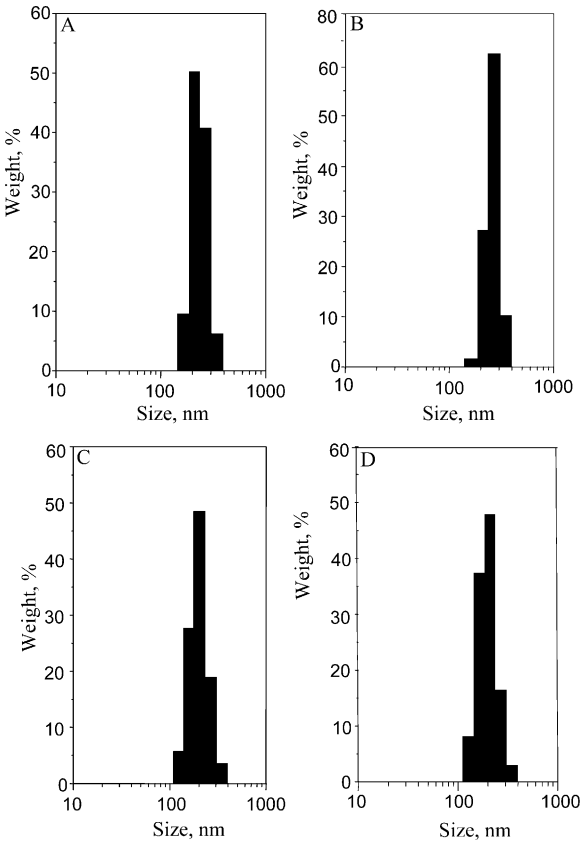

Fig. 1. Typical size distribution patterns for some liposome preparations used in our study. (A) Plain liposomes; (B) PA (6% mol)-containing liposomes; (C) PEG5000-PE (6% mol)-containing liposomes; (D) liposomes containing 6% mol of PS and 6% mol of PEG750-PE.

complex of 111-In (added as $InCl_3$) at room temperature and then dialyzed overnight against HBS at 4 °C to remove a free label.

2.2.4. Liposome clearance and biodistribution

Biodistribution studies were performed in male CD-1 mice (19–21 g). Mice were injected with 0.1 ml of radiolabeled liposome suspension (3–5 mg lipid/ml) via the tail vein (1 μCi per mouse). Each group of six mice was sacrificed at 15, 60, 180 and 300 min post injection. Blood and livers were collected and placed in the test tubes. Gamma-radioactivity of samples was determined without additional treatment or tissue solubilization in a Beckman 5500 gamma counter. Results were expressed as the percent of injected dose per gram of sample ± S.E.

3. Results

3.1. Characterization of liposomes

Liposomes of different phospholipid/polymer composition were prepared having the size of ≈200 nm and rather narrow size distribution. Several typical patterns of liposome size distribution are presented in Fig. 1; see also the corresponding data in Table 1. It is evident that under similar preparative conditions, such parameters as phospholipid composition, charge and presence of protecting polymer only minimally influence liposome size. On the other hand, as one could expect, ζ-potential of liposomes (see Table 1) was strongly influenced by the presence of charged phospholipids in the liposomal membrane. If plain and PEGylated PC:Chol liposomes are negatively charged (ζ-potential is around −10 to −15 meV (Moribe et al., 1997), the addition of 6% mol of positively charged SA increases ζ-potential to approximately +8.5 meV, while the addition of negatively charged PA and PS sharply decreases it from −40 to −45 meV (Table 1).

The attachment of PEG to charged liposomes brings their ζ-potential back close to that of plain PEGylated liposomes. As it follows from Table 1, at the same molar fraction of PEG-PE in liposomes (6% mol), PEG with higher MW (5000 against 750 kDa) provides more efficient charge neutralization. Still, even the presence of 6% mol PEG5000 does not completely compensate the positivity of SA-containing liposomes and ζ-potential of SA-liposomes with 6% mol of PEG5000-PE is approximately −7 meV compared to −12 to −15 meV for PA- and PS-liposomes with 6% mol of PEG5000-PE.

3.2. The effect of separate charge and PEG presence on liposome clearance

Fig. 2 presents the data on the clearance and liver accumulation of various liposomal preparations containing a certain individual charged lipid

or an individual PEG-PE with a certain MW after their intravenous administration in mice. As can be seen from the data presented in Fig. 2, charged lipids, especially the negatively charged PA and PS, strongly accelerate the liposome clearance, while both PEG750-PE and PEG5000-PE at the same 6% mol concentration provide practically identical prolongation of liposome circulation time (Fig. 2A) and decrease in their liver accumulation (Fig. 2B).

3.3. The effect of presence of both charged lipid and PEG on the liposome clearance

A much more complex pattern can be seen when both a charged lipid and a certain PEG are simultaneously present on the liposome surface. Fig. 3 presents the data on the clearance and liver accumulation of positively charged SA-containing liposomes. The addition of 6% mol of the positively charged SA to the liposomal lipids somewhat accelerates the clearance of liposomes. However, the attachment of PEG750-PE or PEG5000-PE to SA-liposomes brings the liposome circulation time, as well as liver accumulation, to the figures similar to that for non-charged PEG-liposomes (compare Figs. 2 and 3).

Figs. 4 and 5 present the data for PEG-coated and PEG-free negatively charged PA- and PS-containing liposomes. The net data differ not only from the data for the positively-charged SA-liposomes, but also between two negatively-charged PA- (Fig. 4) and PS-liposomes (Fig. 5). In the case of PA-liposomes, the attachment of PEG750-PE practically does not increase the circulation time: PEG750-PE-containing PA-liposomes clear as fast as PEG-free PA-liposomes, i.e. even faster than plain neutral liposomes. At the same time, the attachment of PEG5000-PE to PA-liposomes returns their circulation time to almost the level of

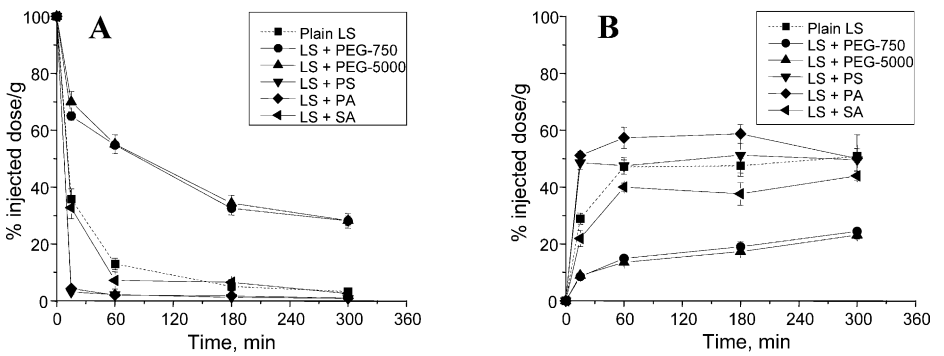

Fig. 2. The effects of charge and PEG on liposome circulation time (A) and liver accumulation (B).

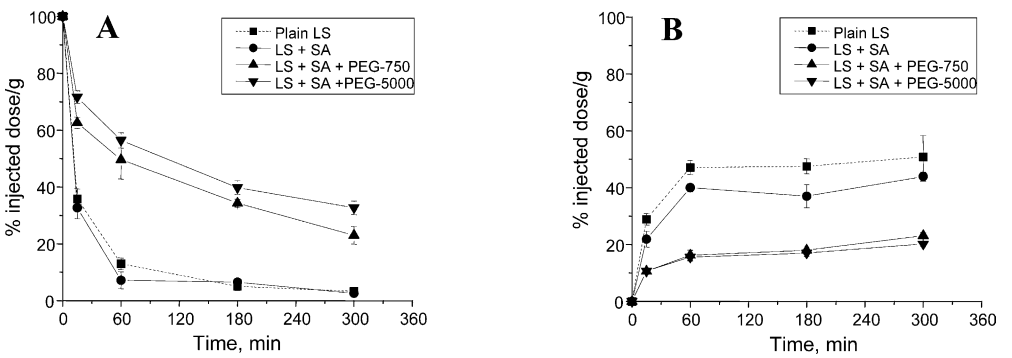

Fig. 3. The effects of PEG on the circulation limes (A) and liver accumulation (B) of SA-containing liposomes.

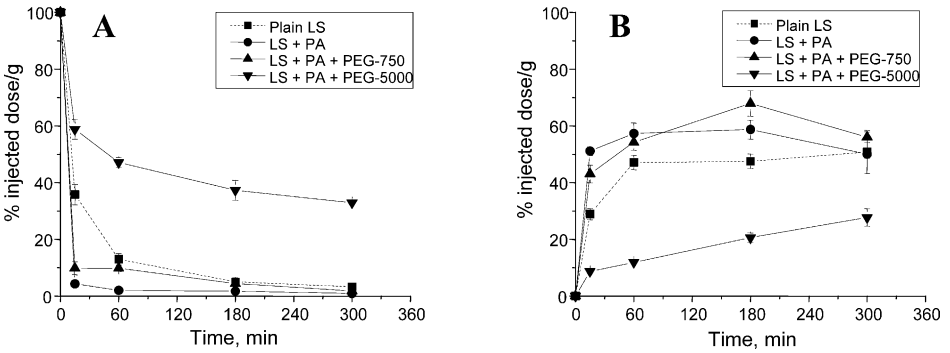

Fig. 4. The effects of PEG on the circulation times (A) and liver accumulation (B) of PA-containing liposomes.

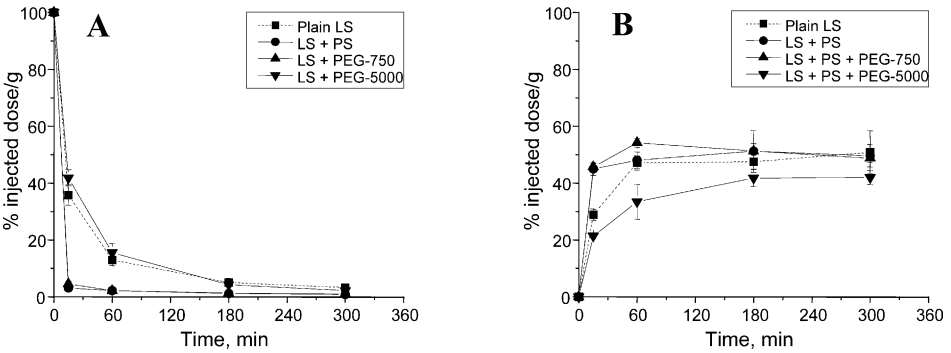

Fig. 5. The effects of PEG on the circulation times (A) and liver accumulation (B) of PS-containing liposomes.

non-charged PEG-liposomes or PEG-coated SA-liposomes.

The effect of PEG attachment in the case of the negatively charged PS-liposomes is even less expressed than in the case of PA-liposomes. PEG750-PE does not influence very fast clearance of PEG-free PS-containing liposomes at all and even the attachment of PEG5000-PE to PS-liposomes brings their circulation time back to only the level of plain non-charged PEG-free liposomes. Liver accumulation corresponds well to the clearance data.

4. Discussion

In vitro, in good agreement with some earlier reports (Arnold et al., 1990; Shimada et al., 1995; Zeisig et al., 1996), we have clearly observed the shielding effect of the liposome-attached PEG on the net charge of liposome containing any type of positively or negatively charged phospholipid. Electric properties of PEGylated liposomes look rather similar almost independently on the nature of the charged component of liposome (compare the data for PA-and PS-liposomes from Table 1). Does it, however, mean that the in vivo behavior of various liposomal preparations having the same size and ζ-potential should also be the same or, at least, similar?

If liposomes are modified with only PEG or with only a charged phospholipid, we again, quite predictably and in good agreement with the known data, observe that the liposome PEGylation increases their circulation time and decreases their liver accumulation, while the incorporation of charged components (SA, PA, or PS) facilitates clearance and liver accumulation even compared to plain liposomes.

However, if liposomes simultaneously contain both PEG and a charged phospholipid, the in vivo pattern becomes far more complex. If in vitro PEG similarly 'normalizes' properties of different charged component-containing liposomes, in vivo, liposomes of different composition still behave very differently even after the attachment of PEG. Our results show that, unlike in vitro situations, liposome properties in vivo are determined not only by the charge presence on the liposome surface, and by shielding this charge with an attached PEG. In vivo, the exact chemical nature of the charge-bearing component begins to play an important role. Thus, the fast clearance of positively charged SA-liposomes can be completely reversed by the attachment of 6% mol of PEG-chains independently on PEG MW (750 and 5000 kDa). In other words, PEG of both small and larger MW neutralizes the effect of the positive charge imparted to liposomes by SA to a similar extent. However, the fast clearance of negatively-charged PA-liposomes can be reversed by only PEG5000-PE, but not by shorter PEG750-PE, while the fast clearance of negatively-charged PS-liposomes cannot be reversed by either PEG750-PE or PEG5000-PE.

One can hypothesize that opsonins of different molecular size bind to different charged lipid components and mediate the clearance of corresponding liposomal preparations by the liver. In this case, at low or intermediate concentrations of the liposome-attached PEG, small-size opsonins may penetrate through the protective layer of both PEG750 and PEG5000 on the liposome surface. Larger opsonins may penetrate through the relatively loose protective layer created by PEG750, but not by longer PEG5000. The opsonins of the largest size can be effectively shielded by both PEG750 and PEG5000. This hypothesis is, in part, supported by the data of Chiu et al. (2001), that very high concentration of the attached PEG on the liposome surface completely eliminates the effect of charged groups on the liposome clearance, i.e. no opsonins of any size can penetrate through the protecting layer of PEG after a certain density of PEG on the liposome surface is achieved. These simple considerations may be useful for constructing liposomal drug carriers with maximized therapeutic efficacy and predictable properties in vivo.

The elucidation of the exact nature of opsonizing proteins in each particular case is of evident interest and constitutes the subject of our current research.

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