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Long circulating, cationic liposomes containing amino-PEG-phosphatidylethanolamine

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Abstract Ligand attachment to polyethylene glycol (PEG) grafted, long circulating liposomes at the polymer terminus is of interest for targeting but the effect of positively charged groups is unknown. Amino-polyethylene glycol-phosphatidylethanolamine (AminoPEG-PE), prepared in four steps from α-amino-ω-hydroxy-PEG, was tested for influence on liposome interactions in vivo: blood circulation and biodistribution. Despite surface amines on each liposome conferring cationic behavior, in vivo properties are comparable to those obtained with methoxy-PEG-PE. The consequences are profound for targeting and possibly systemic delivery of cationic lipidic-polynucleotide complexes.

Key words: Liposome; Polyethylene glycol; Targeting; DNA

1. Introduction

Nonspecific interactions and uptake in vivo of liposomes can be minimized when methoxypolyethylene glycol (mPEG) is anchored to their surfaces. This is typically achieved by incorporation of mPEG- phosphatidylethanolamine (mPEG-PE) conjugate into, so called, sterically stabilized liposomes [1]. The reduced biological recognition leads to prolonged circulation in blood and selective localization into many different sites of pathology: tumors, infections, and inflammations. The use of these surface-modified liposomes with therapeutic agents have been shown to have considerable advantages over their conventional counterparts for a number of therapeutic applications, not only for release of encapsulated drugs to the blood [2] but also for preferential delivery of encapsulated agents into pathological tissues [3].

In addition to mPEG-DSPE, which is the derivative of choice for preparation of sterically stabilized liposomes, a few end-group functionalized analogs were recently reported (for review see [4]). In one study testing the mechanism whereby surface modified liposomes avoid recognition and uptake in vivo, anionic carboxyl groups were positioned at the ends of PEG chains. Interestingly, decreased circulation time of carboxyl-PEG-containing liposomes was reported, perhaps due to the negative charge introduced to the periphery of the graphed polymer, and certainly contrary to the expectations of a mechanism relying exclusively on formation of hydrophilic coatings [5]. In contrast, hydrazido- PEG-containing liposomes, developed for conjugation of antibodies, showed identical behaviour to the original sterically stabilized vesicles [6,7]. Hydrazido groups ($pK_a \approx 3$) are neutral at physiological conditions. More recently, some deleterious effects on retention of sterically stabilized liposomes in the blood by protein ligands, antibodies, have been observed [7,8]. Furthermore, positively charged ligands, in particular, may increase interactions with the predominantly negatively charged blood components [9,10]. An approach to this problem might be to attach small ligands to the lipid surface underneath the polymer. Studies with antibodies, though, indicate that a loss of binding would be expected with such an approach [11].

Surprisingly, little is known of the fate of cationic liposomes in vivo as only a few reports exist with ambiguous or conflicting results [12]. Generally they have been viewed as incompatible in vivo giving accelerated clearance from blood [10] with substantial toxicity, despite an early study indicating only a small influence of the cationic surface charge on circulation [13]. Recent in vitro studies with plasma revealed the expected tendency for interactions with negatively charged components leading to large aggregates and even hemolysis, although the specific details are somewhat dependent on the cationic lipid and other liposome components [10]. Despite such a pessimistic perspective, in vivo gene expression studies were performed using intravenous administration of cationic liposome complexes with DNA, e.g. see [14], but without examination of the in vivo fate of the actual complex. Consequently, the ability to utilize cationic ligands or cationic DNA-lipid complexes for in vivo applications has been unclear.

In this report, an amino functionalized PEG conjugate of PE was prepared and substituted for mPEG-PE in sterically stabilized liposomes. The terminal amino group is protonated and thus is positively charged at physiological pH, which results in an overall neutral, albeit zwitterionic, molecule given the negatively charged phosphate group of the PE residue. Nevertheless, the relatively large separation between the positive amino group and the negative phosphate group by the PEG effectively results in a positive surface as determined by direction of migration in electrophoresis. Blood circulation kinetics and tissue distribution has been determined for these cationic sterically stabilized liposomes using an aqueous compartment label, ^{67}Ga- desferal [15] showing results comparable to mPEG conjugates. These findings indicate that small positively charged ligands may be amenable with sterically stabilized liposomes when attached to the end of the polymer coating.

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Abbreviations: PEG, polyethylene glycol; mPEG, methoxy-PEG; PE, phosphatidylethanolamine; mPEG-PE, conjugate of mPEG and PE; aminoPEG-PE, conjugate of aminoPEG and PE; TEA, triethylamine; SC, succinimidyl carbonate; TLC, thin layer chromatography; Boc, *tert*-butyloxycarbonyl; PC, phosphatidylcholine; DF, desferoxamine mesylate; DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine.

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2. Materials and methods

2.1. General

Partially hydrogenated egg PC (iodine value 40, Asahi Chemical, Japan), Cholesterol (USP grade, Croda), DSPE (CalBiochem), DSC and Boc_2O (Fluka). Partial conversion of hydroxyl end groups of PEG-2000 (Fluka) into primary amines [16] followed by ion-exchange purification according to [17] was employed to prepare α-amino-ω-hydroxy-PEG. TLC on silica gel G (Analtech) was visualized with iodine vapor, Dragendorff- [18] and ninhydrin spray reagents. Liposome particle size distribution was determined by dynamic light scattering (Coulter N4SD). Phospholipid concentrations were measured by phosphorus determination.

2.2. Preparation of Boc-HN-PEG-OH

A solution of α-amino-ω- hydroxy-PEG (2.0 g, 1.0 mmol) in dioxane (8 ml) was treated with Boc_2O (0.436 g, 2.0 mmol) overnight. The solution was concentrated under reduced pressure and then ethyl ether (40 ml) was added. The precipitated product was filtered and dried in vacuo over P_2O_5. Yield: 1.89 g (90%).

TLC ($CHCl_3$-CH_3OH 8:2) $R_f = 0.70$, ninhydrin positive after exposure to HCl vapor. H-NMR (D6 DMSO): d 1.37 (s, ^{t}Bu, 9H), 3.06 (m, CH_2NH, 2H), 3.51 (s, PEG, 180H), 4.52 (t, HO-PEG, 1H).

2.3. Preparation of Boc-HN-PEG-SC [19]

Boc-NH-PEG-OH (1.9 g, 0.9 mmol) was dissolved in acetonitrile (2 ml) and treated with DSC (0.46 g, 1.8 mmol) and pyridine (0.36 ml, 4.55 mmol) overnight. The product was precipitated with ether and then recrystallized from isopropanol, filtered and dried over P_2O_5 in vacuo. Yield: 1.8 g (85%).

TLC ($CHCl_3$-CH_3OH 9:1) $R_f = 0.38$. H-NMR (D6 DMSO): d 1.37 (s, ^{t}Bu, 9H), 2.81 (s, SC, 4H), 3.05 (m, CH_2NH, 2H), 3.51 (s, PEG, ≈180H), 4.44 (t, CH_2-SC, 2H), 6.7 (br s, NH, 1H) ppm. H-NMR ($CDCl_3$): d 1.44 (s, ^{t}Bu, 9H), 2.83 (s, SC-group, 4H), 3.30 (br m, CH_2NH, 2H), 3.64 (s, PEG, ≈180H), 4.46 (t, CH_2-SC, 2H), 5.0 (br s, NH, 1H) ppm.

2.4. Preparation of Boc-HN-PEG-DSPE

DSPE (0.33 g, 0.48 mml) was added to a solution of Boc-HN-PEG-SC (1.0 g, 0.47 mmol) in chloroform (7 ml), followed by TEA (0.24 ml, 1.6 mmol). The resulting suspension was vigorously mixed and maintained at 40°C for ≈10 min, while the reaction mixture became completely clear. The solvent was rotary evaporated and the residue taken up in acetonitrile (12 ml). After overnight storage at 4°C the solution was centrifuged to separate traces of insoluble unreacted DSPE, evaporated, and the product dried over P_2O_5 in vacuo. Yield: 1.1 g (89%). TLC ($CHCl_3$-CH_3OH 8:2) $R_f = 0.52$, ninhydrin positive after exposure to HCl vapor. H-NMR ($CDCl_3$): δ 0.88 (t, $J = 6.8$ Hz, CH_3, 6H), 1.26 (s, CH_2, 56H), 1.44 (s, ^{t}Bu, 9H), 1.58 (br, CH_2CH_2C = O, 4H), 2.31 (m, CH_2C = O, 4H), 3.31 (br m, CH_2NH, 4H), 3.64 (s, PEG, ≈ 180H), 4.0–4.3 (overlaping m, CH_2O-P & CH_2OCONH, 6H), 4.39 (dd, $J = 3.2$, 12 Hz, glycerol CH_2O- C = O, 2 H), 5.2 (m, CH, 1H) ppm. Presence of TEA was also detected: δ 1.41 (t, $J = 7$ Hz, CH_3, 3H), 3.12 (q, $J = 7$ Hz, CH_2, 2H) ppm.

2.5. Preparation of amino-PEG-DSPE

Cleavage of the amino protecting group from Boc-HN-PEG-DSPE was carried out in 4 M HCl in dioxane for 2 h. After removal of the solvent and all the volatiles by drying over P_2O_5 in vacuo, the product was quantitatively recovered in the form of a white solid. TLC ($CHCl_3$-CH_3OH-H_2O 90:18:2) $R_f = 0.29$, ninhydrin positive. Disappearance of the ^{t}Bu peak at 1.44 ppm and appearance of two separate peaks at 3.21 (br m, CH_2NH, 2H) and 3.46 (m, CH_2NH, 2H) instead of 3.31 (br m, CH_2NH, 4H), present in the starting material, indicated that the deprotection went to completion.

2.6. Preparation of liposomes and in vivo studies

Liposomes composed of amino-PEG-DSPE, partially hydrogenated egg PC, and cholesterol in a mole ratio of 0.15:1.85:1 with an average particle size distribution of 1000 Å and 30 to 100 mmol phospholipid/ml were prepared by extrusion of multilamellar vesicles (MLV) using defined pore filters (Nuclepore) according to Woodle et al. [20]. Loading of ^{67}Ga-DF as a liposomal radiolabel was achieved as described previously [15,20]. In vivo studies of blood circulation and tissue distribution following intravenous administration were performed with male and female adult Sprague–Dawley rats (220–400 g) following standard procedures described elsewhere [20]. Briefly, liposome samples prepared at 10 mM phospholipid in 10 mM desferoxamine mesylate in isotonic saline and radiolabeled with ^{67}Ga-oxine [15] were administered intravenously at a dose of about 10–20 mmol phospholipid/kg body weight. Blood levels at various times were determined by retro-orbital bleeding while tissues were obtained surgically after 24 h. Tissue and blood levels of ^{67}Ga radioactivity were determined by gamma counter.

3. Results and discussion

Sterically stabilized liposomes appear to have many applications for retention in blood and passive localization of therapeutic agents to sites of pathology. However, their ability to be actively targeted with ligands at the end of the polymer coating has yet to be fully utilized. Most studies to date have explored large proteins, e.g. antibodies [6–8], although small ligands may prove more amenable to minimize nonspecific biological interactions. For these types of ligands, a cationic nature may be often encountered. As part of an ongoing effort to prepare functionalized PEG-lipids for covalent attachment of potential ligands, we synthesized amino- PEG-DSPE. The conjugate was prepared as schematically depicted in Fig. 1. Note from the conjugate structure that two groups are ionized at near neutral pH: the phospho diester of PE residue and the terminal amino group. Thus, apart from the presence of long polymeric spacer separating the two charged groups the molecule is actually similar to PE.

The synthesis starts with heterobifunctional polymer, α-amino-ω-hydroxy-PEG. First the amino group was selectively protected by Boc-group [16]. The remaining three steps of the synthesis were performed as in a previously published method for preparation of hydrazido- PEG-DSPE [19]. Briefly, a suc-

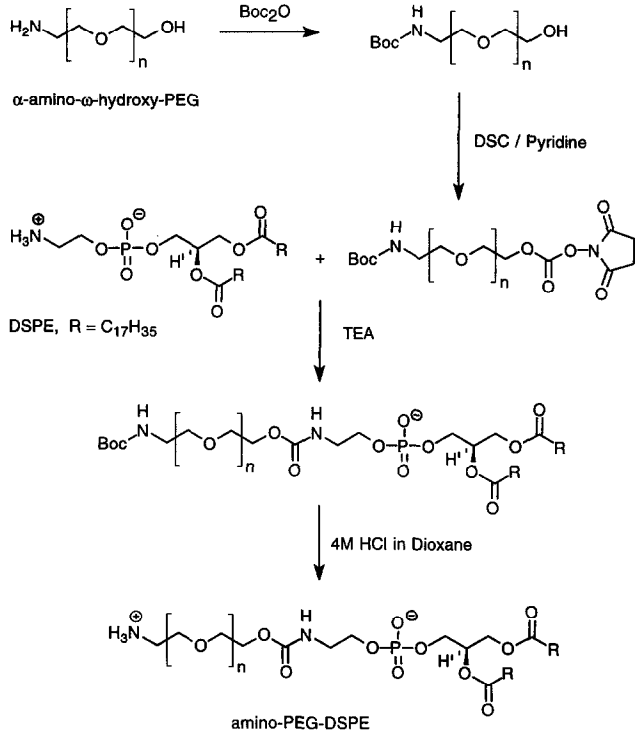

Fig. 1. Synthesis of amino-PEG-DSPE ($n \approx 45$).

Table 1
Tissue distribution in rats 24 hours after I.V. injection

Sample[1]	% Injected dose (SD) in selected tissues						
	Blood	Liver	Spleen	Kidney	Lung	Heart	L + S/Blood
mPEG-PE	32.3 (0.4)	8.4 (1.5)	2.7 (2.3)	0.9 (0.3)	0.3 (0.3)	0.7 (0.1)	0.34
aminoPEG-PE	28.0 (1.8)	7.7 (0.5)	3.7 (0.6)	0.9 (0.2)	0.8 (0.2)	0.5 (0.1)	0.41

[1]Liposome samples as described in section 2 and used in Fig. 2.

cinimidyl carbonate group, introduced at the hydroxy-end of the polymer, was used to form a urethane linkage to the amino group of PE and the primary amine functionality of the polymer regenerated by acidolytic removal of the Boc- group. The final product and all the intermediates were characterized by both TLC and H-NMR.

Substitution of amino-PEG-DSPE for the mPEG-PE traditionally used for sterically stabilized liposomes did not give rise to any noticeable differences in liposome formation nor to radiolabel incorporation using the remote loading method forming ^{67}Ga-DF [15]. The blood circulation over the first 24 hours for liposomes containing either amino-PEG-DSPE or mPEG-PE at 5 mol% of phospholipid is shown in Fig. 2 and selected tissue distribution after 24 hours given in Table 1. These results demonstrate comparable blood circulation and tissue distribution regardless of the terminal group of the polymer coating while all other properties of the polymer conjugate and liposome composition are the same.

Liposomes prepared from neutral, yet zwiterionic, amino-PEG-DSPE, PC and cholesterol might be expected to carry a net neutral charge. Nonetheless, observations of migration direction in an electrical field were made to determine the effective particle surface charge polarity with a microelectrophoresis apparatus (Rank Brothers Mark II) using MLV dispersions (large particles) in low ionic strength buffer (10 mM NaCl). The results obtained were not quantitative but indicated that these liposomes move toward the negative electrode and thus the amino group must effectively convey a net positive surface charge to the liposomes. How this may occur is as follows. Since a lipid vesicle of 1000 Å contains approximately 80,000 phospholipid molecules, incorporation of 5 mol percent of amino-PEG-PE results in positioning of ≈ 2000 of positively charged ammonium ions on the exterior of each vesicle. Their exact location relative to the liposomal surface will depend on the conformation and dynamics of the polymer. Previous estimates of polymer coating thickness with mPEG- PE of the same molecular weight, is on the order of 50 Å [21,22]. The same thickness is expected for the amino- PEG-PE. Finally, PEG is known to shield charges when it is attached to proteins [23] or liposomes [21]. This is expected to nullify, at least partially, the polar head group charges underneath the polymer. The combination of these effects gives rise to the effectively cationic behavior of the amino-PEG-PE liposomes.

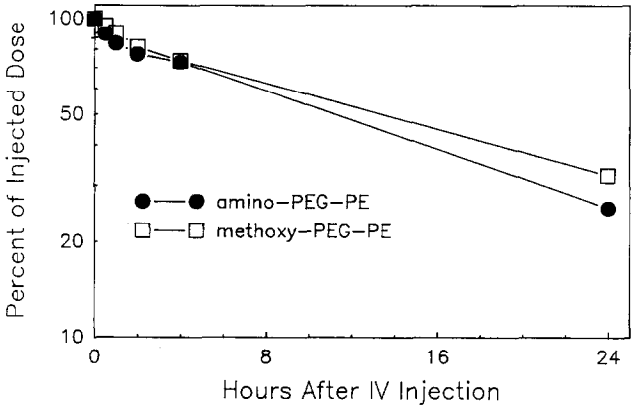

Fig. 2. Blood levels in rats of radiolabeled liposomes expressed as a percentage of the initial time point reading (1–2 min). Circles, amino-PEG-DSPE containing liposomes; squares, mPEG-DSPE containing liposomes.

In conclusion, the system described here containing cationic PEG periphery of liposomes is well tolerated in vivo without loss of prolonged circulation and without noticeable deviations in biodistribution as compared to the neutral polymer-containing vesicles. It is pertinent to note that cationic liposomes frequently used to complex with polynucleotides for transfection are typically unstable in physiological saline. The cationic liposomes described here do not exhibit such problems. Whether this difference depends on surface charge density or on the charge being located at the end of a flexible polymer, remains to be determined. Regardless, the results have important potential implications for utilization of cationic ligands for targeting and perhaps, for overall positively charged polynucleotide complexes.

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