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Influence of poly(ethylene glycol) grafting density and polymer length on liposomes: Relating plasma circulation lifetimes to protein binding

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Abstract

The incorporation of poly(ethylene glycol) (PEG)-conjugated lipids in lipid-based carriers substantially prolongs the circulation lifetime of liposomes. However, the mechanism(s) by which PEG-lipids achieve this have not been fully elucidated. It is believed that PEG-lipids mediate steric stabilization, ultimately reducing surface-surface interactions including the aggregation of liposomes and/or adsorption of plasma proteins. The purpose of the studies described here was to compare the effects of PEG-lipid incorporation in liposomes on protein binding, liposome-liposome aggregation and pharmacokinetics in mice. Cholesterol-free liposomes were chosen because of their increasing importance as liposomal delivery systems and their marked sensitivity to protein binding and aggregation. Specifically, liposomes containing various molecular weight PEG-lipids at a variety of molar proportions were analyzed for in vivo clearance, aggregation state (size exclusion chromatography, quasi-elastic light scattering, cryo-transmission and freeze fracture electron microscopy) as well as in vitro and in vivo protein binding. The results indicated that as little as 0.5 mol% of 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine (DSPE) modified with PEG having a mean molecular weight of 2000 (DSPE-PEG_{2000}) substantially increased plasma circulation longevity of liposomes prepared of 1,2-distearoyl-sn-glycero-3-phosphatidylcholine (DSPC). Optimal plasma circulation lifetimes could be achieved with 2 mol% DSPE-PEG_{2000}. At this proportion of DSPE-PEG_{2000}, the aggregation of DSPC-based liposomes was completely precluded. However, the total protein adsorption and the protein profile was not influenced by the level of DSPE-PEG_{2000} in the membrane. These studies suggest that PEG-lipids reduce the in vivo clearance of cholesterol-free liposomal formulations primarily by inhibition of surface interactions, particularly liposome-liposome aggregation.

Keywords: Cholesterol-free; Liposomes; PEG; Protein binding; Plasma elimination

Abbreviations: ANOVA, analysis of variance; AUC, area-under-the-curve; BCA, bicinchoninic acid; CH, cholesterol; CHE, cholesteryl hexadecyl ether; DDP, didodecylphosphate; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine; DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanol-amine; ^{3}[H], tritium radiolabel; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]; HBS, HEPES buffered saline, pH 7.4; LUV, large unilamellar vesicle; MPS, mononuclear phagocytic system; PAGE, polyacrylamide gel electrophoresis; P_B, protein binding (μmol protein/μmol lipid); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEG, poly(ethylene glycol); PK, pharmacokinetic; QELS, quasielastic light scattering; SDS, sodium dodecyl sulphate; *T*c, phase transition temperature

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1. Introduction

Two factors are of paramount importance in the development of liposomal drug delivery carriers for intravenous use: liposome circulation lifetime and the release of encapsulated contents at a rate optimized for efficacy and/or toxicity endpoints. The presence of poly(ethylene glycol) (PEG) on the liposome exterior surface, typically achieved by formulation with PEG-modified lipids, is well known to significantly enhance liposome circulation lifetime [1–5]. The significance of the addition of PEG-modified lipids led to the realization of longer circulation half-lives that could facilitate higher levels of drug accumulation within sites of tumor growth [6–8] associated with improved antitumor activity [9–11].

The mechanisms governing these PEG-lipid mediated effects remain controversial. The most widely accepted hypothesis to explain the ability of PEG to prolong circulation time of liposomes is based on "steric stabilization" [12–16] that can lead to reductions in liposome aggregation [2,17] and plasma protein adsorption [18,19]. Evidence for this hypothesis has included measurements of the repulsive pressure between lipid membranes in the presence and absence of surface grafted polymers, which demonstrated that there was a larger interbilayer spacing (4 nm) in membranes modified with polymers as compared to unmodified bilayers [20]. Kenworthy et al. analyzed electron density profiles to show the distance between apposing DSPC/DSPE-PEG lipid bilayers varied as a function of the concentration of PEG-lipid in the bilayer and size of the grafted PEG chain. The extension of the PEG chain from the bilayer surface was 1.0, 2.8, 6.0 and 10.0 nm for PEGs with average molecular weights of 350, 750, 2000 and 5000, respectively [14].

Although some evidence suggests that PEG on liposome surfaces can reduce specific protein interactions [21,22], other studies have indicated that the clearance of liposomal formulations from the circulation is not correlated with total plasma protein binding to liposomes [23]. In view of the uncertainties regarding the mechanism by which PEG-lipid confers steric stabilization to liposomes, the research described here compares surface–surface interactions, including self-association (aggregation) and plasma protein adsorption, in cholesterol-free liposomes. We have chosen to perform these studies in cholesterol-free liposomes for two reasons. First, cholesterol-free liposomes have recently been recognized as an important new class of lipid-based drug delivery vehicles. Typically, the addition of cholesterol is viewed as essential for the generation of stable formulations of hydrophilic drugs. However, the studies of Dos Santos et al. [24] demonstrated that removal of cholesterol was associated with dramatic improvements in retention of the hydrophobic anthracycline idarubicin [24]. Interestingly, the liposomal formulations prepared with gel phase lipids such as DSPC ($Tc = 55$ °C) and surface stabilizing PEG-modified lipids exhibited long plasma circulation lifetimes, essentially identical to sterically stabilized cholesterol-containing liposomes. These results place a far greater importance on the role of PEG-lipids than cholesterol in governing the fate of intravenously administered liposomal formulations. Secondly, it is generally believed that cholesterol is a liposome "stabilizing" component which causes a reduction of protein binding following intravenous injection as well as an enhanced plasma circulation lifetime. Therefore, it was believed that the absence of cholesterol from these formulations would facilitate the evaluation of PEG-modified lipids on protein adsorption to the liposome surface and on liposome–liposome aggregation.

2. Materials and methods

2.1. Materials

1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC) and (DSPE)-conjugated poly(ethylene glycol) lipids (PEG average molecular weights 350, 550, 750, 2000 and 5000) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Cholesterol (CH) was obtained from the Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). ^{3}[H]-cholesteryl hexadecyl ether (CHE) (51 Ci/mmol) and ^{14}[C]-CHE (50.6 mCi/mmol) were obtained from PerkinElmer, Inc. (Boston, MA, USA). ^{3}[H]-DSPE-PEG$_{2000}$ was custom synthesized by Northern Lipids Inc. (Vancouver, BC, Canada). HEPES (N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid]), citric acid, Sephadex G-50 (medium), Sepharose CL-4B beads and all other chemicals were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Picofluor-15 scintillation fluid was obtained from Packard Bioscience (Groningen, The Netherlands).

2.2. Liposome preparation

All liposome formulations were prepared by the extrusion technique [25,26]. Briefly, the specified lipids were dissolved in chloroform and mixed together in a test tube at the indicated molar ratios. ^{3}[H]-CHE was added as a non-exchangeable, non-metabolizable lipid marker [27,28]. The chloroform was evaporated under a stream of nitrogen gas and the sample was placed under high vacuum overnight to remove residual solvent. The lipid films were hydrated in HBS by gentle mixing and heating. When preparing cholesterol-containing formulations, these were also subjected to five cycles of freeze (liquid nitrogen) and thaw (65 °C) prior to extrusion. Multilamellar vesicles (cholesterol-free and cholesterol-containing) were passed 10 times through an extruding apparatus (Northern Lipids Inc., Vancouver, BC, Canada) containing two stacked 100 nm Nucleopore® polycarbonate filters (Northern Lipids Inc., Vancouver, BC, Canada). The mean diameter and size distribution of the liposome preparations were analyzed by a NICOMP model 270 submicron particle sizer (Pacific Scientific, Santa Barbara, CA, USA) operating at 632.8 nm, and were typically 100±30 nm in the volume-weighting mode.

2.3. Pharmacokinetic analysis

Balb/c mice (breeders) weighing 20–22 g, were purchased from Charles River Laboratories (St. Constant, QC, Canada) and then bred in-house. Mice were housed in microisolator cages and given free access to food and water. All animal studies were conducted according to procedures approved by the University of British Columbia's Animal Care Committee and in accordance with the current guidelines established by the Canadian Council of Animal Care.

The plasma elimination of liposomes containing trace quantities of ^{3}[H]-CHE radiolabel was assessed. In brief, Balb/c mice were administered intravenously, into the lateral tail vein, with liposomes at a lipid dose of 165 μmol/kg. At various time points up to 24 h after drug administration, blood was collected by tail nick (collected in microfuge tubes) or cardiac puncture (collected in liquid EDTA coated tubes) and centrifuged at 1000×*g* for 10 min to isolate the plasma fraction. Lipid concentration was subsequently measured by liquid scintillation counting. Plasma area-under-the-curve values were calculated using WinNonlin pharmacokinetic software (Version 1.5, Pharsight Corp., Mountain View, CA). Biodistribution studies of tissue samples and plasma correction factors were applied as previously published [29].