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Post-modification of preformed liposomes with novel non-phospholipid poly(ethylene glycol)-conjugated hexadecylcarbamoylmethyl hexadecanoic acid for enhanced circulation persistence in vivo

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ABSTRACT

We report synthesis and characterization of a novel PEG_{2000}-conjugated hexadecylcarbamoylmethyl hexadecanoate (HDAS-PEG) as a PEG-phospholipid substitute for enhancing circulation persistence of liposomes. HDAS-PEG showed critical micelle concentration of 4.25 μM. We used post-insertion technique to introduce HDAS-PEG in outer lipid layer of the preformed liposomes. The presence of surface HDAS-PEG was confirmed by altered electrophoretic mobility, confocal microscopy and PEG estimation by ELISA. The post-inserted HDAS-PEG desorbed at approximately half the rate at which post-inserted DSPE-PEG desorbed from the liposome surface. HDAS-PEG significantly reduced liposome-induced complement activation (C4d, Bb and SC5b); HDAS-PEG was more effective than more commonly used DSPE-PEG in this capacity. For studying circulation persistence, the liposomes were labeled with ^{99m}Tc radionuclide and administered in rats. ^{99m}Tc-HDAS-PEG-liposomes showed prolonged persistence in blood as compared to that shown by ^{99m}Tc-plain liposomes. After 24 h of administration, <1% of ^{99m}Tc-plain liposomes remained in blood, whereas approximately 28% of injected ^{99m}Tc-HDAS-PEG-liposomes were present in blood. In comparison, only 4.8% of ^{99m}Tc-DSPE-PEG-liposomes were measured in blood after 24 h. As expected, the clearance route of the liposomes was through liver and spleen. These results demonstrate the potential of a novel non-phosphoryl HDAS-PEG for surface modification of preformed liposomes with a goal of prolonging their circulation persistence and more effective inhibition of complement activation.

1. Introduction

Phospholipids are major constituents of biomembranes and have been the classic ingredients of liposomes. Being present naturally, they are assumed to be not toxic. However, molecular entities containing phosphoryl moiety are reactive in biological milieu. Oxidative modification and/or fragmentation of phosphatidylcholines generate potent inflammatory mediators that mimic the biologic action of platelet-activating factor (Zimmerman et al., 1995). We have also reported that negatively charged dimyristoylphosphatidylcholine (DMPG)-liposomes have a tendency to activate platelets (Awasthi et al., 2007). The net anionic charge on the phosphate moiety of polyethylene glycol (PEG)-phospholipids has been reported to play a key role in complement activation and anaphylotoxin production (Moghimi et al., 2006). On the other hand, Gamma irradiation, a potential technique for sterilization of liposome preparations, has been shown to increase hemolytic behavior of liposomes containing phospholipids (Stensrud et al., 1999). Moreover, phospholipids are susceptible to phospholipase-mediated breakdown and their extraction and synthesis is associated with significant costs. Considering these characteristics of phospholipids, the development of non-phospholipid amphiphiles as liposome constituents has considerable merit (Bastiat et al., 2007). As compared to the phospholipids, non-phospholipid substituents offer significant advantages related to economics, safety, stability and versatility.

Previously, we reported synthesis of an anionic non-phospholipid as a replacement of DMPG in liposomes, and its use in the enhancement of hemoglobin encapsulation (Agashe et al., 2010). The resultant liposome-encapsulated hemoglobin (LEH) was structurally similar to the liposomes composed only of phospholipids, and improved cerebral energy metabolism in a rat model of hemorrhagic shock (Awasthi et al., 2010). With a long-term goal of complete replacement of phospholipids in liposomes without compromising structural characteristics of liposomes, here we report a novel PEG-lipid, PEG_{2000}-conjugated hexadecylcarbamoylmethyl hexadecanoic acid (HDAS-PEG). PEG-phospholipids have been successfully used for the preparation of

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long-acting and more stable and efficacious formulations, such as liposomal amphotericin B, doxorubicin, and daunorubicin (Cattel et al., 2003). Surface modification of liposomes with PEG delays their rapid clearance by the mononuclear phagocyte system (MPS) and increases the mean residence time in circulation (Awasthi et al., 2004). It is believed that the surface PEG forms a highly hydrated film of water which sterically hinders opsonization of liposomes and their subsequent uptake by the MPS (Ahl et al., 1997; Bradley et al., 1998; Szebeni et al., 2000; Torchilin et al., 1994). This stealth property of PEG-modified liposomes is governed by the thickness of the PEG layer, which in turn is a function of the length of PEG chain.

The reported HDAS-PEG could be synthesized from readily available and inexpensive chemicals. It prolongs circulation persistence of liposomes while remaining non-reactive toward complement proteins. We introduced HDAS-PEG into preformed liposomes by post-insertion technique. While the physicochemical mechanism of post-insertion has been explained (Sou et al., 2000), a direct evidence of the presence of PEG on the post-inserted liposome surface is lacking. We provide for the first time the verification of PEG-lipid existence on the liposome surface and its time- and dilution-dependent desorption.

2. Materials and methods

Unless otherwise mentioned, all the chemicals were obtained from Sigma–Aldrich (St. Louis, MO) and/or various suppliers through VWR Scientific (West Chester, PA) and were used without further purification. Tetradecenyl succinic anhydride was a gift from Vertellus Specialties Inc. (Indianapolis, IN). Methoxy PEG amine, HCl salt (MW 2000) was purchased from Jenkem Technology (Allen, TX). For liposome preparations, the phospholipids were purchased from Lipoid (Ludwigshafen, Germany), Avanti Polar Lipids (Alabaster, AL) or NOF Corporation (Tokyo, Japan). High purity cholesterol was obtained from Calbiochem (Gibbstown, NJ). ^{1}H NMR spectra and ^{13}C NMR spectra were recorded at 300 MHz, and 75 MHz on Mercury-VX 300 (Varian Inc., CA).

2.1. Synthesis of HDAS-PEG

A novel amphiphilic lipid (HDAS-PEG) containing hydrophilic polyethylene glycol (PEG_{2000}) on one end and hydrophobic ethylene chains on the other end was synthesized (Fig. 1a) by amidification between PEG amine and NHS-ester of 2-hexadecylcarbamoylmethyl hexadecanoic acid (HDAS). The precursor lipid HDAS was obtained by reacting tetradecenyl succinic anhydride and hexadecylamine, followed by catalytic hydrogenation. The final product was purified by dialysis and characterized by ^{1}H NMR and DSC (Fig. 1). Differential scanning calorimetry (DSC) was outsourced to Photometrics, Inc. (Huntington Beach, CA). Briefly, the DSC thermograms were obtained on a 2920 Modulated DSC machine (TA Instruments, New Castle, DA) with a refrigerated cooling system. The calibration of the equipment for temperature and for enthalpy was performed using indium. Samples (5–10 mg) were placed in aluminum pans and nitrogen gas purged at 40 mL/min. An empty aluminum pan was used as a reference. Samples were heated from −20 to 150 °C, with a rate of 2 °C/min. The data was analyzed using the "TA Universal Analysis" software.

2.1.1. 2-Hexadecylcarbamoylmethyl-hexadec-3-enoic acid (1, HDA)

Tetradecenyl succinic anhydride (8.60 g, 29.21 mmol) and hexadecylamine (5.86 g, 24.27 mmol) were allowed to react in presence of pyridine (10.0 mL) at 80 °C for 3 h. The reaction mixture was extracted into dichloromethane (DCM, 200 mL) and washed three times with 10% HCl (100 mL). The organic phase was separated, dried with sodium sulfate and concentrated to obtain white solid of unsaturated HDA (11.50 g, yield 88%). ^{1}H NMR (300 MHz, $CDCl_3$): 5.77 (br, 1H, NH, exchanged with D_2O), 5.51 (1H, CH) 5.43 (1H, CH), 3.25 (m, 2H, CH_2), 2.88 (m, 1H), 2.94–2.12(m, 4H), 2.05–1.92 (m, 2H), 1.48 (br, 4H) 1.35–1.15 (m, 42H, CH_2), 0.87 (t, 6H, CH_3).

2.1.2. 2-Hexadecylcarbamoylmethyl hexadecanoic acid (2, HDAS)

HDA (5.8 g, 10.82 mmol) was dissolved in 200 mL of toluene at 60 °C and reduced by passing hydrogen gas in the presence of catalyst palladium/charcoal (5%, 50 mg) at atmospheric pressure for 48 h. The reaction mixture was filtered on a Buchner funnel and passed through a short silica gel column to remove the catalyst. The product (5.6 g, yield 96%) was obtained as white solid by evaporating toluene under vacuum. ^{1}H NMR (300 MHz, $CDCl_3$): 5.74 (br, 1H, NH, exchanged with D_2O), 3.26 (t, 2H), 2.87–2.31 (m, 3H), 1.78–1.59 (m, 2H), 1.60–1.40 (br, 4H), 1.35–1.15 (br, 48H, CH_2), 0.87 (t, 6H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): 176.81, 171.31, 41.75, 37.21, 31.90, 31.58, 29.68, 29.64, 29.58, 29.54, 22.67, 14.09. ESI HRMS calculated for $C_{34}H_{68}NO_3$ 538.42, found 538.40 $[M+H]^+$.

2.1.3. 2-Hexadecylcarbamoylmethyl hexadecanoic acid 2,5-dioxo-pyrrolidin-1-yl ester (3, HDAS-NHS)

HDAS (5.0 g, 9.29 mmol), N-hydroxysuccinimide (1.07 g, 9.29 mmol), and N,N′-dicyclohexylcarbodiimide (1.92 g, 9.32 mmol) were allowed to react in anhydrous dimethylformamide (100 mL) with vigorous stirring for 24 h. The solid precipitate appeared after the completion of reaction was removed by filtration. The solution was concentrated by evaporating the solvent under reduced pressure. The crude product was purified with silica gel column chromatography using dichloromethane and ethyl acetate (90:10) to obtain (3.8 g, yield 64%) white powder. ^{1}H NMR (300 MHz, $CDCl_3$): 5.76 (br, 1H, NH, exchanged with D_2O), 3.49 (t, 2H), 2.88–2.30 (m, 7H), 1.96–163 (br, 4H), 1.60–1.39 (br, 4H), 1.35–1.15 (m, 46H, CH_2), 0.87 (t, 6H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): 180.16, 176.84, 172.92, 40.37, 39.85, 31.90, 31.39, 29.67, 29.60, 29.55, 29.28, 25.58, 22.66, 14.08. ESI HRMS calculated for $C_{38}H_{70}N_2O_5$ (M+) 634.53, found 635.40 $[M+H]^+$.

2.1.4. Poly(ethylene glycol)-conjugated-HDAS (4, HDAS-PEG)

HDAS-NHS (0.10 g, 0.16 mmol), methoxy PEG_{2000} amine, HCl salt (0.325 g, 0.15 mmol), triethlyamine (0.040 mL) and dimethylsulfoxide (DMSO, 7 mL) were stirred vigorously at 45 °C for 24 h. The product was purified by dialysis using benzoylated cellulose tubing (MWCO 2000) against DMSO and water for 24 and 48 h, respectively. Finally, the dry product (0.24 g, 63%) was obtained by removing water under freeze drying conditions (Triad Lyophilizer, Labconco, Kansas City, MO). ^{1}H NMR (300 MHz, $CDCl_3$): 3.87 (t, 2H, CH_3O—$(CH_2$—CH_2—$O)_{44}$—CH_2—$C\underline{H}_2$—NH—CO—), 3.63 (br, 176H, CH_3O—$(C\underline{H}_2$—$C\underline{H}_2$—$O)_{44}$—CH_2—CH_2—NH—CO—), 3.54 (m, 2H,CH_3O—$(CH_2$—CH_2—$O)_{44}$—$C\underline{H}_2$—CH_2—NH—CO—), 3.54 (m, 2H), 3.37 (s, 3H, $C\underline{H}_3O$—$(CH_2$—CH_2—$O)_{44}$—CH_2—CH_2—NH—CO—), 2.71–2.54 (br, 2H), 2.35–2.26 (m,1H) 1.91–1.56 (br, 4H), 1.55–1.38 (br, 4H), 1.35–1.19 (br, 46H, CH_2), 0.87 (t, 6H, CH_3).

2.2. Critical micellar concentration (CMC) of HDAS-PEG

In order to examine the hydrophobic domain formation of HDAS-PEG in aqueous solution, we measured fluorescence spectral changes of N-phenylnaphthylamine (NPN) as a function of [HDAS-PEG]. Briefly, the samples were prepared by adding increasing concentrations of HDAS-PEG (0.5–8 μM) into a saturated aqueous solution of NPN. The solutions were kept undisturbed overnight at room temperature before measuring fluorescence spectra. The fluorescence spectra were recorded at 90° detection angle on Shimadzu