Synthesis and characterization of novel poly(ethylene glycol)-lipid conjugates suitable for use in drug delivery

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Abstract

Liposomal formulations have been used to encapsulate and deliver a wide variety of therapeutic and diagnostic agents. Their circulation can be prolonged by the addition of neutral, hydrophilic polymers such as poly(ethylene glycol) (PEG) to the outer surface. An extended circulation lifetime allows them to take advantage of the enhanced permeability and retention effect (EPR), resulting in increased delivery to target sites. Incorporation of PEG also prevents aggregation and aids in the formation of uniform, small mono-disperse particles. This is often accomplished with the use of PEG-lipid conjugates, PEG molecules with a hydrophobic domain to anchor them into the liposomal bilayer upon formulation.

Here we present data showing that some commonly used PEG-lipids are chemically unstable due to the presence of carboxylic ester bonds. This instability limits their utility in aqueous environments common to many liposomal preparations. To address this problem, we designed and synthesized three alternative PEG-lipids. Using SPLP (PEG-stabilized liposomal vesicles encapsulating plasmid DNA) as a model system, we investigated the properties of the novel PEG-lipids. An accelerated stability study was conducted at 37 °C for 42 days to confirm chemical stability and an in vivo model was used to assess the pharmacokinetics, toxicity and activity of the SPLP. We show that the novel PEG-lipids are more stable in liposomal formulation, less toxic upon systemic administration, and accordingly, are suitable replacements for the PEG-lipids described previously.

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1. Introduction

Through liposomal encapsulation, it is possible to obtain particles with the small size, low surface charge and increased serum stability necessary to increase circulation lifetime and take advantage of the “enhanced permeation and retention” (EPR) effect [1–3]. This effect occurs where fenestrated vasculature permits the extravasation and increased accumulation of encapsulated material at target sites such as tumors, sites of infection or inflammation [4–6].

Nucleic acid based drugs acquire other benefits from liposomal encapsulation [7–11]. When administered systemically in unprotected form, nucleic acids suffer from poor pharmacokinetics due to rapid degradation by intravascular nucleases. Early liposomal strategies utilized lipoplex, electrostatic complexes formed between cationic lipids and negatively charged nucleic acids [12]. However, unshielded cationic lipoplexes themselves possess poor physico-chemical characteristics for systemic delivery. Their positive surface charge leads to nonspecific interaction with anionic species in the blood, resulting in rapid clearance by the reticulo-endothelial system (RES) [13–15].

This problem can be overcome through the use of hydrophilic polymers attached to the particle’s surface. Most commonly PEG has been used [16,17], although other polymers have been described [18,19]. However, it has been shown that the presence of PEG can affect the intracellular delivery and trafficking of non-viral vectors, resulting in lower gene expression [20]. To this end, many groups have devised strategies to ensure that the presence of PEG is transient. One strategy involves the use of exchangeable PEG-lipids that rely on slow diffusion from the particle surface at a rate determined by the size of their lipid anchors [9,21]. Examples include PEG-
phosphatidyl ethanolamines (PEG-PE) [22], PEG-Ceramides (PER-Cer) [21], SAINT-PEGs [23] and PEG-succinoyl diacylglycerols (PEG-S-DAGs) [9]. Another strategy uses PEG-disulphide lipids that possess a disulphide bond between the polymer and the lipid anchor that is cleaved by thiolytic agents in the reductive environment of the endosome [24,25]. Similarly, vinyl ether PEG-lipids [26] and orthoester PEG-lipids [27–29] have been developed using chemical linkages that are sensitive to the reduced pH of the endosomal compartment.

Recently published data shows that when administering repeated doses of liposomally encapsulated, immunostimulatory nucleic acids, a strong, long-lived antibody response can be generated against PEG, a result of the powerful adjuvant effect of the nucleic acid payload [30,31]. While this effect has in the past been attributed to the immunostimulatory CpG motifs of bacterial pDNA [32], CpG free phosphorothioate ODN [33] and siRNA [34] have also been shown to be immunostimulatory. This impacts the potential for repeat administration of PEGylated nucleic acid delivery systems, causing a loss of disease site targeting, accelerated blood clearance and acute hypersensitivity upon subsequent administration. It has been shown that the use of PEG-lipids with a smaller C14 lipid anchor, thereby increasing the rate of dissociation of the PEG from the particle, abrogates this deleterious effect [30,31]. In this regard, there may be advantages to a strategy using exchangeable PEG layers, rather than those that are permanently bound or not cleaved until entry to the endosome.

Our characterization of the immune response to PEG-lipids [31] coincided with our observation that PEG-succinoyl distearylglycerol (PEG-S-DSG)-containing SPLP in a controlled, long-term stability study exhibited a steadily decreasing concentration of PEG-lipid over time (Fig. 1), ultimately leading to particle destabilization and aggregation. This led us to believe that PEG-S-DSG was chemically unstable, an undesirable attribute for a component of a potential pharmaceutical product. An examination of commercially available alternatives and the literature yielded no acceptable substitutes for PEG-S-DSG. Therefore, we set out to design, synthesize and characterize novel replacements with the aim of preparing SPLP that are at least as efficacious as those containing the original PEG-S-DSG.

2. Materials and methods

2.1. Materials and analyses

1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). 1,2-dioleoyloxy-N,N-dimethyl-3-aminopropane (DODMA) [35], PEG-S-DAGs [9] and PEG-CerC20 [21] were prepared as previously described. 3H-labelled CHE was obtained from Perkin-Elmer (Boston, MA, USA). The Picogreen Quantitation Assay and Kit was obtained from Molecular Probes (Eugene, OR, USA). GPR grade solvents were purchased from VWR Scientific (Edmonton, AB, Canada). All other chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada). The pCMVluc plasmid, encoding the luciferase reporter gene under the control of the cytomegalovirus promoter, was manufactured as described previously [36]. 1H nuclear magnetic resonance (NMR) spectrometry was performed by Spectral Data Services, Inc. (IL, USA). Elemental analysis (CHN) was performed by Canadian Microanalytical Service Ltd. (BC, Canada).

2.2. SPLP preparation

SPLP at a total lipid concentration of approximately 10 mg/mL were prepared using the method of spontaneous vesicle formation by ethanol dilution, as described previously [37]. The lipid composition was DSPC : cholesterol : PEG-lipid : DODMA (20:55:10:15 molar ratio). Nucleic acid encapsulation was determined using a PicoGreen assay, and encapsulation efficiency calculated by comparing fluorescence in the presence and absence of Triton X-100 [37]. Picogreen fluorescence was measured using a Varian Eclipse Spectrofluorometer (Varian Inc., CA, USA). Particle size was determined using a Malvern Instruments Zetasizer 3000HSA (Malvern, UK). SPLP (40 μL) were diluted with 4 mL of phosphate buffered saline (PBS (150 mM NaCl, 10 mM Phosphate, pH 7.4 buffer)). Intensity-weighted, Gaussian distribution analysis was used to determine mean vesicle diameters and population standard deviations.

Fig. 1. (A) Accelerated stability study of SPLP lipids at 40 °C. Lipid concentrations were determined by HPLC analysis using an evaporative light scattering detector. PEG-S-DSG, possessing four carboxylic ester bonds, exhibited a pronounced tendency to degrade. DSPC was also unstable at 40 °C. (B) Stability study of PEG-S-DSG in SPLP at 25 and 5 °C. Data-points are the mean of 2 analyses. Error shown is the percent relative standard deviation of six analyses of a standard containing all four lipids.
Polydispersity was reported using ‘Contin’ mode of the instruments PCS software.

2.3. SPLP stability studies and HPLC analysis

SPLP formulations were incubated at relevant temperatures (5, 25, 37 or 40 °C) for the duration of the study in question (up to 12 months for the long-term study, 42 days for the accelerated study). Samples (100 μL) were withdrawn using a Gilson pipette and prepared for HPLC analysis by diluting 1:19 in ethanol. This dissolved the SPLP lipid bilayer and resulted in precipitation of the nucleic acids and buffer salts. Precipitates were removed by filtering through a 13 mm Acrodisc 0.45 μm syringe filter ( Pall Corp., Ann Arbor, MI). A Waters Alliance 2695 HPLC (Waters Ltd., ON, Canada) with an ACE C8, 4.6 × 250 mm, 5 μm column (Canadian Life Science, ON, Canada), was used for analysis with sample injection volumes of 20 μL. Sample and column temperatures were 20 and 40 °C, respectively. The mobile phase gradient varied linearly from 80:20 A:B to 83:17 A:C (where A = methanol, B = 10 mM ammonium bicarbonate, pH 8.0, C = tetrahydrofuran (THF)) over a time period of 18 min. The mobile phase flow rate was 1.1 mL per min. A 6-min hold at the final conditions was followed by a 21-min re-equilibration period for the column (equating to a total run time of 45 min). An Alltech Evaporative Light Scattering Detector 2000 (Alltech Associates Inc., IL, USA) was used for detection, with the drift tube temperature set at 70 °C and gas flow rate set at 1.8 L per min. A standard curve was generated for each lipid. System suitability was determined prior to each run by analysis of a standard sample containing all four lipids. When the relative standard deviation (RSD) of six fractions were combined, dried over magnesium sulfate and a Beckman LS6500 (Beckman Instruments, CA, USA). For this experiment, n = 4.

2.7. Synthesis of lipid anchors

The synthesis of the C_{18} lipid anchor is described below. The C_{14} anchor was made in an analogous fashion, substituting an equimolar amount of 1-bromotetradecane for the 1-bromooctadecane in the reaction to make the analogous compound 1.

2.7.1. Preparation of 1,2-distearyloxy-3-allyloxypropane (1)

Benzene (250 mL) was added to 95% sodium hydride (11.4 g, 450.0 mmol), and the flask was sealed and flushed with nitrogen. A solution of 3-allyloxy-1,2-propanediol (6.6 g, 50.0 mmol) in benzene (75 mL) was added to the flask. 96% 1-bromoacetadecane (41.7 g, 120.0 mmol) was added and the reaction left to reflux overnight under nitrogen. The mixture was cooled to room temperature and excess sodium hydride slowly quenched with ethanol. The solution was transferred to a separatory funnel with benzene (250 mL) and washed with distilled water (2 × 200 mL) and brine (1 × 200 mL). The organic fractions were combined, dried over magnesium sulfate and concentrated. The crude product was purified by flash column chromatography (1–5% ether in hexane) to yield compound 1 as a colourless wax (21.0 g, 66%, R_{f}=0.35 (5% ether in hexane)). ^1 H NMR (400 MHz), δ_{H} (ppm): 5.90 (m, 1H, CH_{2}=CH_{2}), 5.21 (m, 2H, CH_{3}=CH), 4.01 (m, 2H, CH_{3}=CHCH_{2}), 3.61–3.40 (m, 9H, CH_{2}CH(OCH_{2})CH_{2}OCH_{2}), 1.61–1.51 (m, 4H, OCH_{2}CH_{2}), 1.38–1.20 (m, 60H, CH_{2}(stearyl)), 0.88 (t, 6H, CH_{3}, J=6.8 Hz). CHN found; C 79.06, H 13.15 (C_{42}H_{66}O_{3}=C 79.18, H 13.29; O 7.55).

2.7.2. Preparation of 1,2,3-distearyloxypropan-3-ol (2)

Compound 1 (21.0 g, 33 mmol) was dissolved in ethanol (250 mL) and trifluoroacetic acid (20 mL) and tetrakis (triphenylphosphine) palladium(0) (5.0 g, 43.3 mmol) added. The reaction mixture was refluxed under nitrogen overnight. The solvent was removed by rotary evaporator and the crude product purified by flash column chromatography (100% dichloromethane (DCM)), to yield compound 2 as a colourless wax (18.7 g, 95%, R_{f}=0.4 (chloroform)). ^1 H NMR (400 MHz), δ_{H} (ppm): 3.75–3.67 (m, 1H, OCH), 3.67–3.47 (m, 8H, OCH_{2}), 2.26 (s, 1H, O), 1.62–1.50 (m, 4H, OCH_{2}CH_{2}), 1.38–1.20 (m, 60H, CH_{2}(stearyl)), 0.88 (t, 6H, CH_{3}, J=6.8 Hz). CHN found; C 79.06, H 13.25 (C_{39}H_{60}O_{3}=C 78.46, H; 13.51; O, 8.04).
2.7.3. Preparation of N-(2,3-distearloxypropyl)phthalimide (3)

97% methanesulphonic anhydride (113.6 g, 62.8 mmol) was dissolved in DCM (anhydrous, 100 mL) and pyridine (anhydrous, 5.0 g, 62.8 mmol) slowly added. A solution of compound 2 (18.7 g, 31.4 mmol) in DCM (anhydrous, 100 mL) was added and the reaction stirred overnight at room temperature. The reaction mixture was washed with distilled water (2 × 200 mL) and brine (1 × 200 mL), dried over magnesium sulphate, and concentrated. The mixture was washed with distilled water (2 × 200 mL) and brine (1 × 200 mL), dried over magnesium sulphate, and concentrated. The crude product was purified by flash column chromatography (0 97% methanesulphonic anhydride (8.2 g, 47.1 mmol) in DCM (anhydrous, 80 mL). A solution of Me-PEG2000-OH (80 mL) and triethylamine (TEA) (3 mL, 20 mmol) was added and the reaction was stirred for 48 h. The solution was acidified via the addition of a 1% solution of hydrochloric acid (HCl) (250 mL) with agitation. The organic layer was collected, dried over magnesium sulphate and concentrated to yield a pale yellow solid. The crude product was purified by flash column chromatography (0–7% MeOH in CHCl3), then lyophilized to yield PEG-A-DSA as a colourless solid (3.4 g, 38%, Rp=0.5 (10% MeOH in CHCl3)). 1H NMR (400 MHz), δH: 7.10–7.04 (m, 1H, NH), 4.00 (s, 2H, CH2(O)NH), 3.7–3.6 (m, ~180H, OCH2PEG), 3.60–3.39 (m, 9H, NCH2CH2), 1.54–1.37 (m, 4H, OCH2CH2), 1.38–1.06 (m, 60H, CH2(stearyl)), 0.88 (t, 6H, CH3, J=6.9 Hz). CHN found; C 77.76, H 11.69, N 1.86 (C17H31NO4 = C 77.74, H 11.52, N 1.93, O 8.81).

2.8.1. Preparation of methoxy poly(ethylene glycol)2000 acetic acid (5)

Methoxy poly(ethylene glycol)2000 (Me-PEG2000-OH) (20.0 g, 10 mmol) was added to a solution of sodium dichromate (3.0 g, 10 mmol) in 10% sulfuric acid (200 mL) and stirred at room temperature overnight. The product was extracted with chloroform (3 × 250 mL) and the organic fraction combined, washed with 1 M sodium hydroxide (250 mL) and evaporated to yield a pale blue wax. This crude material was purified by flash column chromatography (0–15% MeOH–CHCl3) to give compound 5 as a colourless solid (8.0 g, 38.7%, Rp=0.3 (13% MeOH in CHCl3)). 1H NMR (400 MHz), δH: 4.0–3.9 (s, 2H, CH2CO2H), 3.7–3.6 (m, ~180H, OCH2PEG), 3.38 (s, 3H, CH3O).

2.8.2. Preparation of N-(1,2-distearloxypropyl) methoxy poly(ethylene glycol)2000 acetamide (PEG-A-DSA) (6)

Compound 5 (6.8 g, 3.4 mmol) was dissolved in benzene (80 mL) and oxalyl chloride (3.4 mL, 20 mmol) slowly added. The solution was stirred for 2 h prior to solvent removal by rotary evaporator. Compound 4 (2.15 g, 3.6 mmol), DCM (anhydrous, 80 mL) and triethylamine (TEA) (3 mL, 20 mmol) were added and the reaction was stirred for 48 h. The solution was acidified via the addition of a 1% solution of hydrochloric acid (HCl) (250 mL) with agitation. The organic layer was collected, dried over magnesium sulphate and concentrated to yield a pale yellow solid. The crude product was purified by flash column chromatography (0–7% MeOH in CHCl3), then lyophilized to yield PEG-A-DSA as a colourless solid (3.4 g, 38%, Rp=0.5 (10% MeOH in CHCl3)). 1H NMR (400 MHz), δH: 7.10–7.04 (m, 1H, NH), 4.00 (s, 2H, CH2(O)NH), 3.7–3.6 (m, ~180H, OCH2PEG), 3.60–3.39 (m, 9H, NCH2CH2CH2OCH2), 3.38 (s, 3H, CH3O), 1.60–1.50 (m, 4H, OCH2CH2(stearyl)), 1.38–1.20 (m, 60H, CH2(stearyl)), 0.88 (t, 6H, CH3J=6.8 Hz).

2.8.3. Preparation of methoxy poly(ethylene glycol)2000 mesylate (ME-PEG2000-OMs) (7)

Pyridine (3.8 mL, 47.0 mmol) was added slowly to a solution of 97% methanesulphonic anhydride (8.2 g, 47.1 mmol) in DCM (anhydrous, 80 mL). A solution of Me-PEG2000-OH (31.5 g, 15.5 mmol) in DCM (anhydrous, 120 mL) was added and the reaction stirred overnight. The reaction mixture was washed with distilled water (2 × 200 mL) and brine (1 × 200 mL), dried over magnesium sulphate, and concentrated. The crude product was purified by flash column chromatography (0–10% MeOH–CHCl3) to give compound 7 as a colourless solid (30.1 g, 92.8%, Rp=0.4 (5% MeOH in CHCl3)). 1H NMR (400 MHz), δH: 4.38 (t, 3H, CH2OS, J=4.5 Hz), 3.79–3.74 (m, 2H, CH2CH2OS), 3.7–3.6 (m, ~180H, OCH2PEG), 3.38 (s, 3H, CH3O), 3.08 (s, 3H, OSO2CH3).

2.8.4. Preparation of 1-amino methoxy poly(ethylene glycol) (Me-PEG2000-NH2) (8)

Me-PEG2000-NH2 was prepared as previously described [39]. Briefly, compound 7 (10 g, 5 mmol) was dissolved in a concentrated solution of aqueous ammonia (400 mL), sealed and left to stir for 72 h. The product was extracted with DCM (3 × 300 mL) and the combined organic fractions dried over MgSO4 and concentrated. The product was crystallized from diethyl ether to yield compound 9 as a colourless solid (9.3 g, 93%, Rp=0.15 (10% MeOH in CHCl3)). 1H NMR (400 MHz), δH: 3.7–3.6 (m, ~180H, OCH2PEG), 3.58–3.53 (m, 2H, CH2CH2NH2), 3.38 (s, 3H, CH3O), 2.90 (t, 2H, CH2NH2, J=5.2 Hz), 2.2–1.9 (bs, 2H, NH2).

2.8.5. Preparation of methoxy poly(ethylene glycol) succinimide (Me-PEG2000-Sn) (9)

Succinic anhydride (3.8 g, 38.1 mmol) was added to a solution of compound 8 (9.0 g, 4.4 mmol) in pyridine (anhydrous, 100 mL)
and the reaction stirred overnight. The pyridine solvent was removed under reduced pressure, and the residue dissolved in distilled water (100 mL) and acidified with HCl. Crude product was extracted with DCM (3×100 mL), dried over magnesium sulphate and concentrated. Purification by flash column chromatography (0–10% MeOH–CHCl₃) yielded compound 9 as a colourless solid (5.7 g, 61%, Rf=0.65 (10% MeOH in CHCl₃)). ¹H NMR (400 MHz), δH: 6.84–6.79 (m, 1H, NHCH₂CH₃), 6.40–6.34 (m, 1H, CH₂CH₂NH), 3.7–3.6 (m, ~180H, OCH₂(PEG)), 3.60–3.39 (m, 13H, OCH₂CH₂CN, NCH₂CH(OCH₂CH₂)OCH₂), 3.38 (s, 3H, CH₃O), 2.54–2.49 (m, 2H, CH₂CH₂CO₂H), 1.60–1.51 (m, 4H, OCH₂CH₂(stearyl)), 1.40–1.20 (m, 60H, CH₂(stearyl)), 0.88 (t, 6H, CH₃CH₃, J=6.9 Hz).

2.8.6. Preparation of N-[methoxy(polyethylene glycol)₂₀₀₀(carbamyl)-1,2-distearyloxypropyl-3-amine (PEG-S-DSA) (10)

N-hydroxysuccinimide (360 mg, 3 mmol) and compound 9 (3.1 g, 1.5 mmol) were dissolved in chloroform (anhydrous, 30 mL). A solution of 1,3-dicyclohexyl-carbodiimide (DCC) (490 mg, 2.25 mmol) in chloroform (anhydrous, 10 mL) was added, and the reaction stirred for 1 h. A separate solution of compound 4 (900 mg, 1.5 mmol) and TEA (0.9 mL, 6 mmol) in chloroform (anhydrous, 10 mL) was added and the reaction for a further hour. The solution was filtered through a bed of Celite and distilled water added (50 mL). The mixture was acidified with concentrated HCl, then washed again with distilled water (2×50 mL) and brine (50 mL). Organic fractions were combined, dried over magnesium sulphate and concentrated. The product was purified by flash column chromatography (0–7% MeOH–CHCl₃) to yield PEG-S-DSA, compound 10, as a colourless solid (3.5 g, 88%, Rf=0.65 (12% MeOH in CHCl₃)). ¹H NMR (400 MHz), δH: 6.84–6.79 (m, 1H, NHCH₂CH₃), 6.40–6.34 (m, 1H, CH₂CH₂NH), 3.7–3.6 (m, ~180H, OCH₂(PEG)), 3.60–3.39 (m, 13H, OCH₂CH₂CN, NCH₂CH(OCH₂CH₂)OCH₂), 3.38 (s, 3H, CH₃O), 2.54–2.49 (m, 2H, CH₂CH₂CO₂H), 1.60–1.51 (m, 4H, OCH₂CH₂(stearyl)), 1.40–1.20 (m, 60H, CH₂(stearyl)), 0.88 (t, 6H, CH₃CH₃, J=6.9 Hz).

2.8.7. N-[methoxy(polyethylene glycol)₂₀₀₀(carbamyl)-1,2-distearyloxypropyl-3-amine (PEG-C-DSA) (11)

Diphosgene (2.0 mL, 16.7 mmol) was added to a solution of PEG₂₀₀₀ methyl ether (10.0 g, 5 mmol) in DCM (anhydrous, 100 mL) and stirred under nitrogen at room temperature for 3 h. DCM and excess diphosgene were then removed under reduced pressure and compound 4 (4.2 g, 7 mmol) added. The flask was flushed with nitrogen, then DCM (anhydrous, 150 mL) and TEA (1.4 mL) added prior to stirring overnight. The solution was diluted with DCM (100 mL), washed (1% HCl (1×200 mL), water (1×200 mL) and brine (1×200 mL)), dried over magnesium sulphate and concentrated. Purification by flash column chromatography (1.5–6.0% MeOH in CHCl₃) followed by lyophilization afforded PEG-C-DSA, compound 11, as a colourless solid (11.9 g, 90%, Rf=0.35 (8% MeOH in CHCl₃)). ¹H NMR (400 MHz), δH: 5.16–5.08 (m, 1H, NH), 4.25–4.19 (m, 2H, CH₂OC(O)), 3.7–3.6 (m, ~180H, OCH₂(PEG)), 3.60–3.39 (m, 9H, NCH₂CH(OCH₂CH₂)OCH₂), 3.38 (s, 3H, CH₂O), 1.60–1.50 (m, 4H, OCH₂CH₂(stearyl)), 1.38–1.20 (m, 60H, CH₂(stearyl)), 0.88 (t, 6H, CH₃CH₃, J=6.8 Hz).

2.8.8. N-[methoxypoly(ethylene glycol)₂₀₀₀carbamyl]-1,2-dimyristyloxypropyl-3-amine (PEG-C-DMA) (12)

PEG-C-DMA, compound 12, was synthesized in a manner similar to PEG-C-DSA, compound 11, on a 5 mmol scale to yield, after chromatography, compound 12 as a colourless solid (11.1 g, 88%, Rf=0.35 (8% MeOH in CHCl₃)). ¹H NMR (400 MHz), δH: 5.21–5.15 (m, 1H, NH), 4.26–4.19 (m, 2H, CH₂OC(O)), 3.7–3.6 (m, ~180H, OCH₂(PEG)), 3.60–3.39 (m, 9H, NCH₂CH(OCH₂CH₂)OCH₂), 3.38 (s, 3H, CH₃O), 1.60–1.50 (m, 4H, OCH₂CH₂(stearyl)), 1.38–1.20 (m, 44H, CH₂(stearyl)), 0.88 (t, 6H, CH₃CH₃, J=6.9 Hz).

3. Results

3.1. PEG-S-DSG is chemically unstable in liposomal formulations

To ascertain lipid stability, we studied lipid concentrations over time in SPLP samples stored at different temperatures. Formulations containing the lipids DSPC : cholesteral : PEG-S-DSG : DODMA (20:55:10:15 molar ratio) were examined by HPLC for degradation at 5, 25 and 40 ºC. DSPC and cholesterol exhibited negligible structural instability, even under accelerated conditions (40 ºC, Fig. 1A), while DSPC degraded to a certain extent. However, PEG-S-DSG degraded rapidly, with more than 10% of the lipid degrading in the first 2 months, even at 5 ºC (Fig. 1B). As expected the instability of PEG-S-DSG was exacerbated at higher temperatures. The PEG-S-DSG half-life in aqueous liposomal formulations was 18.3, 5.2 and 2.7 months at 5, 25 and 40 ºC, respectively. Half-life values were calculated using a first order, non-compartmental model. Log values of the concentrations were plotted against time to give straight lines, the slopes of which were used to calculate half-life.

3.2. Synthesis of novel PEG-lipids

Three novel PEG-lipids were synthesized for consideration as potential replacements for PEG-S-DSG; PEG-S-DSA, PEG-A-DSA and PEG-C-DSA. Each used a different linker to join the PEG to the lipid anchor (the previously described 1,2-distearyloxypropyl-3-amine (DSA) [38]); PEG-S-DSA, PEG-A-DSA and PEG-C-DSA utilized succinimide, amide and carbamate linkers, respectively (Fig. 2). The structure of DSA allows the linkers, respectively (Fig. 2). The structure of DSA allows the possibility of synthesizing analogues with shorter alkyl chains, necessary for giving the PEG-lipids the desired programmable, exchangeable properties. The synthetic route to the lipids was comprehensively revised to allow more efficient manufacture, such that the overall yield was improved by approximately 50% while the number of synthetic steps was decreased from six to four. The synthesis and PEG-coupling processes (Fig. 3) displayed varying efficiency. Although the 2-step strategy used to obtain PEG-A-DSA had a low overall yield of 14%, this did not present a problem as the reactions are easily performed on large scale. PEG-S-DSA predictably had a low yield also (46%), having present a problem as the reactions are easily performed on large scale. PEG-S-DSA predictably had a low yield also (46%), having
analyzed by TLC and gave single spots with expected \( R_f \) values. They were further characterized by \(^1\)H NMR and, in the case of non-polymeric compounds, elemental analysis.

### 3.3. Physical characterization of formulations

The formulation characteristics of the six PEG-lipids were evaluated with particular emphasis on particle size and encapsulation efficiency. The PEG-lipids were used to prepare SPLP by spontaneous vesicle formation \[37\]. The PEG-DSPE formulation had a mean particle size of 161 nm. All other formulations had the characteristic small particle size of SPLP (approximately 120 nm diameter, Table 1). Encapsulation efficiency was also significantly less with the use of PEG-DSPE; 61% as compared to 72% to 80% for the other 5 formulations \((P<0.05, t\)-test\). Final DNA encapsulation values were all somewhat similar, as the SPLP were passed through Mustang Q cartridges to remove unencapsulated DNA at the end of the formulation process. Even so, PEG-DSPE resulted in a formulation with a significantly lower percentage of encapsulated nucleic acid in the final product, as compared to the other PEG-lipids \((P<0.05, t\)-test\).

### 3.4. The presence of a succinate linker promotes instability in PEG-lipids

Assessment of PEG-lipid stability within the vesicles was of interest. Therefore, formulations containing the six PEG-lipids were analyzed for lipid degradation in an accelerated stability study, carried out over a six-week period at 37 °C. As expected from the earlier results, PEG-S-DSG was shown to degrade steadily over the course of the experiment \((t_{1/2}=58\ \text{days})\) and PEG-CerC\(_{20}\) degraded at a very similar rate \((t_{1/2}=47\ \text{days})\) (Fig. 4). The breakdown of both lipids was shown to be significant, even after 7 days \((P<0.05, t\)-test\). The remaining PEG-lipids exhibited no obvious signs of instability during the six-week time-course, with concentrations after 42 days not significantly different from initial values \((P>N0.1\ \text{in all cases}, t\)-test\). The other lipids, cholesterol, DSPC and DODMA, were also stable during this time period. It is interesting to note that PEG-DSPE, despite containing two carboxylic ester bonds in its structure, appears to be stable within the context of this particular experiment.

### 3.5. In vivo gene expression from intravenous administration of SPLP

It has previously been shown that use of PEG-S-DSG or PEG-CerC\(_{20}\) allows for the formation of long circulating particles that yield preferential gene expression in tumor tissue \[8,9\]. Ensuring a similar performance with the new PEG-lipids was of obvious importance. Therefore, gene expression was evaluated in a mouse tumor model following intravenous (i.v.) administration of SPLP containing the \(\text{pCMV}luc\) plasmid. 48 h

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**Table 1**

<table>
<thead>
<tr>
<th>PEG-lipid</th>
<th>Mean particle diameter (nm±S.D.)</th>
<th>Initial encapsulated DNA (%)</th>
<th>Final encapsulated DNA (%)</th>
<th>Charge of PEG-lipid at formulation pH (5.0)</th>
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</thead>
<tbody>
<tr>
<td>PEG-S-DSG</td>
<td>118±4</td>
<td>80±1</td>
<td>94±1</td>
<td>Neutral</td>
</tr>
<tr>
<td>PEG-DSPE</td>
<td>161±5</td>
<td>61±3</td>
<td>84±2</td>
<td>−1</td>
</tr>
<tr>
<td>PEG-CerC(_{20})</td>
<td>109±6</td>
<td>74±2</td>
<td>94±2</td>
<td>Neutral</td>
</tr>
<tr>
<td>PEG-A-DSA</td>
<td>122±5</td>
<td>72±2</td>
<td>89±2</td>
<td>Neutral</td>
</tr>
<tr>
<td>PEG-S-DSA</td>
<td>122±6</td>
<td>75±1</td>
<td>95±1</td>
<td>Neutral</td>
</tr>
<tr>
<td>PEG-C-DSA</td>
<td>118±5</td>
<td>72±2</td>
<td>89±1</td>
<td>Neutral</td>
</tr>
<tr>
<td>PEG-C-DMA</td>
<td>122±5</td>
<td>78±2</td>
<td>95±2</td>
<td>Neutral</td>
</tr>
</tbody>
</table>

Values are the mean of 3 separate experiments, the error stated is the standard deviation.
after SPLP administration, luciferase expression was evaluated in the liver, lung, spleen, heart and tumor. As shown previously with PEG-S-DSG [9] or PEG-CerC₂₀ [40] containing SPLP, an excellent differential in luciferase protein levels was observed between the tumor and other tissues (Fig. 5). All six formulations were similar in this respect, typically resulting in tumor gene expression 2 orders of magnitude greater than that observed in other, non-target tissues.

3.6. Intravenous administration of SPLP is well tolerated

The relative toxicity of the new PEG-lipids was clearly of interest, as they are ultimately intended for in vivo applications. The toxicity following SPLP administration was examined by determining levels of the transaminases ALT and AST in mouse serum following SPLP treatment (Fig. 6). ALT is primarily regarded as a hepatocytes protein and increases in ALT are thought to indicate liver damage. AST is present in most tissues but particularly in cardiac muscle, skeletal muscle and the liver. Elevations in AST are regarded as a more general indication of systemic tissue damage. None of the formulations resulted in large increases in either enzyme, and ALT and AST levels actually remained within the normal limits for the PEG-A-DSA and PEG-C-DSA SPLP. The remaining four formulations exhibited only marginally increased levels of either enzyme.

3.7. Circulation half-life of SPLP comprising PEG-lipids with shorter lipid anchors

Formulation, stability, transfection and toxicity data, combined with the ease of synthesis, lead to the selection of the carbamate linker chemistry for further characterization. The C₁₄ analogue (PEG-C-DMA) was synthesized to confirm that, similarly to PEG-S-DAGs and PEG-Ceramides, bilayer exchangeability (and thus pharmacology) could be modulated by varying the size of the lipid anchor. SPLP were prepared incorporating a ³H-CHE-lipid marker. The percentage of injected dose remaining in circulation following a single injection of SPLP in the tail vein is displayed as a function of time (Fig. 7A). SPLP containing PEG-C-DSA exhibited a circulation half-life of 16 h, similar to that of the PEG-S-DSG containing formulation. SPLP containing PEG-C-DMA cleared more rapidly with a half-life of approximately 2 h. These results are very similar to those reported previously for SPLP containing the PEG-DAGs [9] and PEG-Ceramides [7]. The initial phase of the curve (from 0 to 8 h) was used to calculate half-life, using a first order, non-compartmental model. This initial phase was felt to be the most relevant part of the curve, as it indicates how quickly the majority of the SPLP dose is cleared. It also represents 4 out of the 5 data-points. Log values of the concentrations were plotted against time to give straight lines, the slopes of which were used to calculate half-life.

3.8. Gene expression patterns from formulations containing PEG-lipids with shorter lipid anchors

We have previously determined that the use of PEG-lipids with a shorter, C₁₄ lipid anchor is necessary to avoid the generation of an antibody response when delivering encapsulated nucleic acids [30,31]. The main drawback to this strategy is that the shorter lipid anchor leads to faster clearance, meaning less time for the
Upon storage, even at 4 °C, more than 10% of the PEG-lipid was degraded within 2 months (Fig. 1). This effectively disqualifies this PEG-lipid from incorporation into any formulation to be considered for clinical development. We hypothesized that this instability was due to the presence of readily hydrolysable carboxylic ester bonds (of which there are 4 in the structure of PEG-S-DSG) [41,42]. Of the possible replacements, PEG-Ceramides [21] and PEG-DSPE possess ester bonds. PEG-DSPE also possesses an electrostatic charge, at both formulation and physiological pH, as do SAINT-PEGs [23]. This was also considered to be undesirable, for reasons of increased blood clearance [43,44], possible complement activation, and/or possible leakage of encapsulated material [21].

Another strategy for facilitating the intracellular delivery of PEG-lipid-containing particles is the use of PEG-lipids designed to cleave in the endosome. These include the disulphide-, vinyl ether- and dithioether-linked PEG-lipids mentioned previously [24–29]. However, when considering the PEGylation strategy for nucleic acid containing particles, it is important to consider recent reports of an acquired immune response to C18 PEG-lipids with a long residence time on the particle [30,31]. The environmentally sensitive PEG-lipids described to date utilize C18 lipid anchors, and therefore may illicit similar immune responses when used to deliver immunostimulatory nucleic acids. The use of PEG-lipid linker chemistries that were designed to cleave in response to environmental triggers was rejected in favor of using exchangeable PEG-lipids.

We designed three novel PEG-lipids as potential replacements for PEG-S-DSG, each containing a different linker connecting the PEG and lipid domains. The first, PEG-S-DSA, utilized a succinimide linker, a direct (amide) analogue of the labile succinate linker in PEG-S-DSG. The yield for coupling of the PEG and lipid moieties was quite low, with an overall yield of 14%. The second and third PEG-lipids, PEG-A-DSA and PEG-C-DSA, possessed simple amide and carbamate linkers. These required fewer steps to synthesize and yields were much higher, 46% and 90%, respectively. All novel PEG-lipids utilized the same type of lipid anchor. Similarly to the linker, the carboxylic esters in the lipid anchor were replaced, in this case with ether bonds. The lipid possesses a primary amine head group to facilitate coupling to PEG. The dialkylglyceryl nature of the hydrophobic anchor was retained, to facilitate straightforward adjustment of the diffusible nature of the resulting PEG-lipid conjugate. When performing PEG-lipid coupling, an excess of lipid was used in relation to PEG. While this may seem counterintuitive (since the lipid anchor requires more synthetic steps to prepare than PEG), subsequent work up and purification of the final compounds was found to be easier with this approach. The C18 analogues of all PEG-lipids were synthesized initially to allow for direct comparison with PEG-S-DSG.

All of the PEG-lipids, with the exception of PEG-DSPE, were readily incorporated in SLP and resulted in acceptable formulations. PEG-DSPE, being the only PEG-lipid to possess a negative charge, yielded larger particles (∼160 nm) than the particles to accumulate at the disease site and typically resulting in lower gene expression at distal tumor sites. It was of interest to evaluate to what degree this phenomenon affected gene expression using PEG-C-DMA SLP. SLP were prepared using the C14 (PEG-C-DA and PEG-succinoyl dimyrystylglycerol (PEG-S-DMG)) and C18 (PEG-C-DSA and PEG-S-DSG) analogues of PEG-C-DAA and PEG-S-DAGs. Gene expression was evaluated in the liver, lung, spleen heart and tumor 48 h after a single intravenous administration of SLP. Although tumor gene expression resulting from PEG-C-DMA SLP treatment was significantly lower than that obtained with PEG-C-DSA (P<0.05), there was no significant difference between the level of tumor gene expression following treatment with PEG-S-DMG, PEG-S-DSG, or PEG-C-DMA SLP (Fig. 7B). Consistent with previous results, a marked differential was seen between the gene expression in tumor tissue and the other tissues examined, typically of 2 orders of magnitude.

4. Discussion

While conducting long-term stability tests on SLP, we discovered that the PEG-lipid, PEG-S-DSG, was unstable.
five neutral compounds (~120 nm). The model for SPLP formation and nucleic acid encapsulation involves a charge interaction between nascent cationic lipid-containing bilayer fragments and the negatively charged nucleic acid. PEG-DSPE may act to partially neutralize the charge on the bilayer fragments or actively repel the DNA by charge repulsion, as encapsulation efficiency was also reduced when using PEG-DSPE.

Stability assessment of the individual PEG-lipids incorporated in SPLP yielded interesting results (Fig. 4). PEG-S-DSG was shown to degrade steadily in aqueous solution, as was the PEG-C-DMA. PEG-DSPE, however, is stable, despite possessing two carboxylic ester bonds similar to those thought to contribute to PEG-S-DSG instability. This may be explained by the location of the ester bonds. PEG-S-DSG and PEG-C-DMA both contain ester bonds in the succinate linkers, between the PEG and hydrophobic domains of the molecule. These are expected to be located at the surface of the SPLP lipid bilayer, allowing ingress of water molecules necessary for the hydrolysis of this bond. Conversely, the ester bonds of PEG-DSPE are located in the hydrophobic domain of the molecule and would be expected to be sequestered deep within the lipid bilayer. These bonds would be less accessible to water and therefore less susceptible to hydrolysis. Of note, DSPC, which also contains ester bonds in the hydrophobic domain, also appears to be relatively stable within the limited context of this experiment. The three novel PEG-lipids, containing no ester bonds, were completely stable, as were the cholesterol and DODMA components.

To assess the utility of the PEG-lipids in formulations for systemic delivery, SPLP containing a luciferase reporter plasmid were administered to Neuro-2a tumor-bearing mice via tail vein injection. Since all the PEG-lipids used in this experiment were C18 analogues, and the hydrophobic domain of the molecule is the dominant factor in determining transfection efficiency, it was not unexpected that the six formulations performed similarly. Each formulation resulted in comparable luciferase expression of approximately 700 pg/g of tumor. This compares favorably with previously reported SPLP formulations containing pCMVluc plasmids, which have yielded 30 [7] and 100 pg/g [9]. The preferential expression of luciferase in the tumor is in part due to passive disease site targeting and the EPR effect, but also because non-viral delivery systems transflect actively dividing cells more efficiently [45].

Analysis of serum transaminase levels confirmed comparable, low toxicity for all systems. AST and ALT levels were elevated only slightly in mice treated with 50 mg/kg total lipid, of which approximately 20 mg/kg was PEG-lipid, when compared to PBS controls. This was consistent with previous results in which SPLP have been shown to be less toxic than lipoplex systems [7]. The move from readily degradable ester PEG-lipids, to those with more stable, and possibly less easily metabolised bonds, had no apparent effect on acute toxicity.

The data favored the adoption of PEG-C-DSA or one of its analogues. PEG-C-DSA is stable in the bilayer at 40 °C for greater than six weeks, it is charge-neutral, formulates well, is synthesized in good yield with a minimum number of steps, and gives particles that transflect as well as PEG-S-DSG without signs of toxicity. To confirm the diffusible PEG-lipid paradigm would apply to PEG-C-DSA analogues and to address the issue of the immune response with more stably incorporated PEG-lipids, the C14 analogue (PEG-C-DMA) of PEG-C-DSA was synthesized. The synthesis and purification of PEG-C-DMA, like its C18 analogue, were found to be straightforward and of high yield (88% compared to 90%), PEG-C-DMA particles were found to possess the same small size and high encapsulation characteristics as those containing PEG-C-DSA (Table 1). The pharmacokinetics of PEG-C-DMA-containing SPLP was confirmed in PK studies utilizing 3H-labelled SPLP. As with PEG-S-DSG/PEG-S-DMG formulations, where long (t1/2=15 h) and short (t1/2=1 h) circulating formulations were prepared by switching from the C18 to a C14 lipid anchor [9], the circulation half-life of PEG-C-DSA (t1/2=16 h) and PEG-C-DMA (t1/2=2 h) supported the diffusible PEG-lipid paradigm. Of interest, although the reduced circulation time had an effect on the resulting transfection efficiency (Fig. 7B), the tumor gene expression resulting from the administration of PEG-C-DMA-containing SPLP was as great as that obtained when using PEG-S-DSG suggesting that PEG-C-DMA may be a suitable replacement for PEG-S-DSG in nucleic acid delivery systems. Subsequently, toxicity evaluation by analysis of serum transferase levels was performed, following intravenous administration of PEG-C-DMA SPLP in A/J mice (data not shown). Similarly to the PEG-C-DSA analogue, AST and ALT levels of 125 and 78 IU/L, respectively, were barely elevated, and well within normal limits as set out by the Canadian Council on Animal Care (CCAC).

5. Conclusion

Using a simple assay developed to determine lipid degradation, we have demonstrated that two of the better-known types of PEG-lipid, PEG-S-DAGs and PEG-Ceramides, are unstable when incorporated in aqueous liposomal formulations. Accordingly, we have designed and synthesized three replacement PEG-lipids. We have shown that all three of the novel PEG-lipids are readily synthesized, stable under stressed conditions and formulate well as SPLP. The resulting particles are non-toxic and capable of transflecting distal tumors with 2 orders of magnitude of specificity over other organs. Our continuing changes to the lipid components of the SPLP particle, with no obvious penalty in terms of formulability or performance, further demonstrate the robustness of the SPLP platform and formulation methodology.

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