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Synthesis of lipids for development of multifunctional lipid-based drug-carriers

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ABSTRACT

A simple approach to synthesize phospholipids to modulate drug release and track lipid-based particulate drug-carriers is described. We synthesized two ether lipids, **1** 1-*O*-hexadecyl-2-pentadenoyl-*sn*-glycerol-3-phosphocholine (C_{31}PC) and **2** 1-*O*-hexadecyl-2-pentadenoyl-*sn*-glycerol-3-phosphomethanol (C_{31}PM), and examined their ability to alter enzymatically triggered release of 6-carboxyfluorescein from liposomes incubated in TRIS buffer or fetal bovine serum solutions. Further, we demonstrated that odd-chain lipids, for example, C_{31}PC, could be identified in rat plasma without interference of endogenous lipids. This approach can be adapted to synthesize a variety of lipids for use in developing and optimizing multifunctional drug-carriers.

Lipid based drug-carriers, such as liposomes, can alter the pharmacokinetics and improve the efficacy of a variety of therapeutic agents.[1] However, mechanisms to 'control' or 'tune' their drug release kinetics and track drug-carrier disposition in vivo are limited. A variety of physical and physiological approaches have been examined to control drug release from drug-carriers, including exposure to light,[2–4] heat,[5–7] and use of ultrasound.[8,9] However, the clinical use of these strategies has been limited because of their ability to induce rapid 'burst' drug-release profiles and the inaccessibility of some tissue to light or heat.

Enzymatic approaches exploiting elevated expression of enzymes, such as esterases, in some disease states have the potential to modulate drug-release selectively. Secretory phospholipase A_2 ($sPLA_2$) is an esterase that preferentially hydrolyzes glycerophospholipids, such as phosphatidylcholine, at the *sn*-2 ester bond, releasing a fatty acid (FA) and a lysophospholipid (LP).[10] Recent studies demonstrated that the expression and catalytic activity of $sPLA_2$ is increased in prostate,[11–14] breast[15–17] and pancreatic[18–20] cancers.

We, and others, have developed $sPLA_2$ responsive liposomes (SPRL) with enhanced drug release.[21–24] $SPLA_2$-mediated degradation of phospholipids results in the formation of FA and LP; these increase membrane fluidity and result in a transition of the lipid bilayer from a gel-like to a liquid-crystalline phase. Alterations in membrane fluidity are believed responsible for the enhanced diffusion and release of contents entrapped within the aqueous-core of liposomes; however, the complete mechanism is not fully known. Our recent studies used electrospray ionization mass spectrometry to examine the selectivity of $sPLA_2$ for various lipids and quantify $sPLA_2$-mediated degradation of prototype liposomes formulations.[24] Lipid degradation was correlated to enhanced release of 6-CF, a fluorescent probe. We, and others, have hypothesized that slight modifications of existing lipids or synthesis of novel lipids and lipid-prodrugs could be used to further tune drug release and improve $sPLA_2$-activity.[21]

The specificity and activity of $sPLA_2$ is greatest for phospholipids with anionic head groups and shorter FA acyl chains.[25–27] However, optimal formulations need to balance drug retention properties with their circulation half-life, tissue distribution, and drug release kinetics. The use of high-phase transition, saturated phospholipids with neutral head groups have been shown, in combination with cholesterol (CHOL) and hydrophilic coatings (e.g., polyethylene glycol), to produce long-circulating, sterically-stabilized liposomes (SSL). Unfortunately, the clinical utility of these formulations is limited, in part, because some drugs, such as doxorubicin or vincristine, are entrapped stably and the rate of drug release is slow, whereas for other drugs, such as topotecan or paclitaxel, the rate of release is fast.[28] The synthesis of novel lipids, based on existing lipids, may allow for the modulation, or tuning, of drug release.

Another challenge associated with evaluating $sPLA_2$-mediated liposome degradation and drug release is the ability to quantify

Abbreviations: $sPLA_2$, secretory phospholipase A_2; C_{31}PC, 1-*O*-hexadecyl-2-pentadenoyl-*sn*-glycerol-3-phosphocholine; C_{31}PM, 1-*O*-hexadecyl-2-pentadenoyl-*sn*-glycerol-3-phosphomethanol; ESI-MS, electrospray ionization-mass spectrometry; PC, phosphatidylcholine; PM, phosphatidylmethanol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine, DPPC, 1,2-dipalmityl-*sn*-glycero-3-phosphatidylcholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine; DSPG, 1,2-distearoyl-*sn*-glycerol-3-phosphatidylglycerol; DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine; SSL, sterically-stabilized liposome; 6-CF, 6-carboxyfluorescein; LP, lysophospholipid; FA, fatty acid; DSPE-PEG, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethyleneglycol)$_{2000}$]; CSI, chlorosulfonyl isocyanate; BBr_3, boron tribromide; DCM, dichloride methylene; THF, tetrahydrofuran; DMF, dimethylformamide; HPLC, high performance liquid chromatography; SPRL, $sPLA_2$ responsive liposome; satd, saturated.

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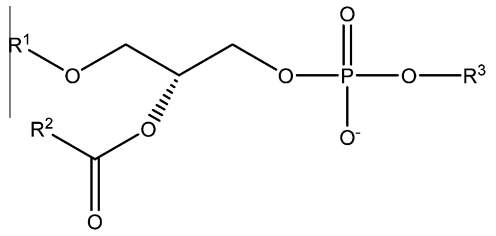


Figure 1. Structure of ether lipids.

Table 1
Structures of existing and novel $sPLA_2$-targeted ether lipids

Compound	R^1 (linkage)	R^2 (linkage)	R^3	T_m[a] (°C)
DSPC	18:0 (Ester)	18:0 (Ester)	Choline	49.6
DPPC	16:0 (Ester)	16:0 (Ester)	Choline	38.9
DSPE	18:0 (Ester)	18:0 (Ester)	Ethanolamine	74.3
DSPG	18:0 (Ester)	18:0 (Ester)	Glycerol	54.8
1	16:0 (Ether)	15:0 (Ester)	Choline	34.4
2	16:0 (Ether)	15:0 (Ester)	Methanol	52.2

(**1**) 1-*O*-C_{31}PC; (**2**) 1-*O*-C_{31}PM.
[a] T_m was determined using individual lipids hydrated in dd-water at 40 mM.

the degradation of lipids from in vivo samples. The majority of lipids used to prepare drug-carriers cannot be separated from similar lipids found endogenously. Therefore, we sought to synthesize and use lipids containing odd FA acyl chain lengths, not commonly found in vivo, thus, permitting direct assessment of liposome tissue distribution and their degradation.

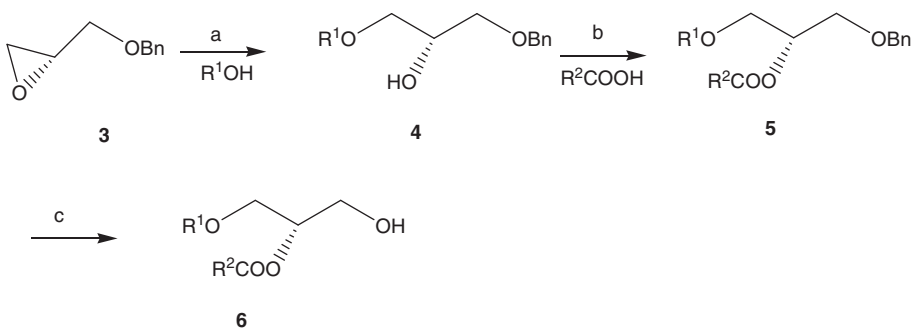
The aim of this study was to develop a simple and rapid approach to synthesize glycerophospholipids with *sn*-1 ether and *sn*-2 ester linked FA (Fig. 1) that could be used to tune drug-release and be identified in biological samples without interference from endogenous lipids. Specifically we synthesized two ether lipids, **1** 1-*O*-hexadecyl-2-pentadenoyl-*sn*-glycerol-3-phosphocholine (C_{31}PC) and **2** 1-*O*-hexadecyl-2-pentadenoyl-*sn*-glycerol-3-phosphomethanol (C_{31}PM), and examined their ability to alter enzymatically triggered release of 6-carboxyfluorescein (6-CF) from liposomes incubated in TRIS buffer or fetal bovine serum (FBS) solutions, and evaluated their ability to be identified from lipids found endogenously in plasma.

Using *R*-(*O*)-benzyl glycidol as a starting material, intermediate **6** (Scheme 1) was synthesized[29] by a three-step reaction, (i) regioselective opening of epoxide ring with ether[30], (ii) Steglich esterification to its *sn*-2 position with fatty acid[31] and (iii) deprotection reaction of benzyl group using Lewis acid boron tribromide.[25,32]

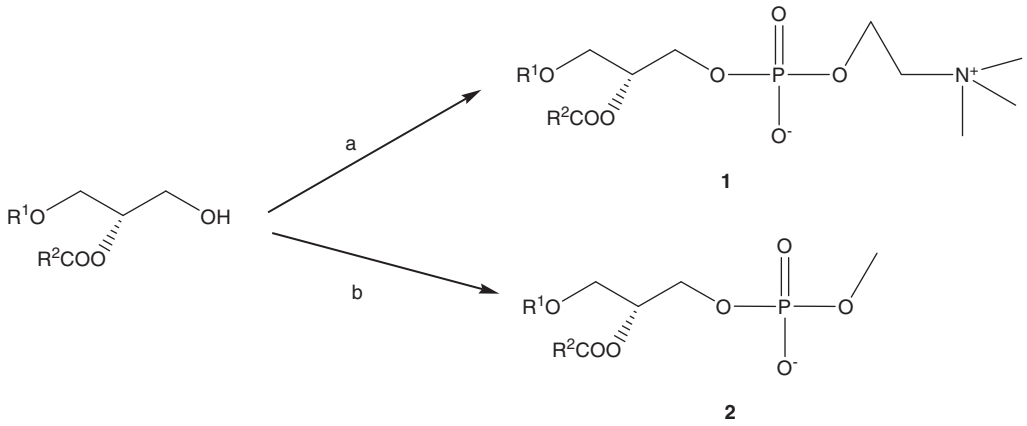
Intermediate **6** was then used as the starting material for the synthesis of both 1-*O* lipids **1** and **2** (Table 1) by conjugating either phosphocholine or phosphomethanol (Scheme 2). H2/Pd-C has been used successfully to deprotect the benzyl group, resulting in high product yield. However, this method is limited in that it can only be applied for saturated lipid synthesis. Therefore, we used alternative methods to remove the benzyl group. First chlorosulfonyl isocyanate (CSI) was tried,[33] but the result was not desirable. The failure of this reaction may have resulted from the low selectivity of CSI for ether bonds or the instability of intermediate **6** in the resulting harsh chemical environment, that is, use of sodium hydroxide and the long reaction time (up to 21 h). The instability of intermediate **6** is most likely the result of acyl migration that is accelerated at elevated temperatures and the non-neutral pH environment of this reaction. With this in mind, we adapted an existing method using boron tribromide (BBr_3).[33] The reaction was performed at −78 °C and was completed in 5 min.[32] BBr_3 displayed high selectivity over the benzyl ether group, resulting in an almost complete (~100%) formation of **6** from **5**. Furthermore, the short reaction time has also been shown to reduce acyl migration.[34] The primary drawback of this reaction is the use of large quantities of DCM solvent, that is, 0.1 g of **5** needs 100 mL of DCM to dissolve at −78 °C.

Synthesis of 1-*O*-hexadecyl-2-pentadenoyl-*sn*-glycerol-3-phosphocholine (**1**)[35] and 1-*O*-hexadecyl-2-pentadenoyl-*sn*-glycerol-3-phosphomethanol (**2**)[36] were completed using the conditions developed by Hirth, G.[37] Initial purification by chromatography using a TMD-8 resin column and a silica gel column according to the literature did not initially produce a pure product. However, purity was achieved using a gradient method in flash chromatography. It should be mentioned in the preparation of 1-*O*-hexadecyl-2-pentadenoyl-*sn*-glycerol-3-phosphomethanol (**2**) satd $NaHCO_3$ solution was used in place of water because of the resulting formation of the sodium salt form of **2** facilitated purification, compared to its free acid form.

The effect of incorporation of **1** or **2** on the release of 6-CF from prototypical SSL samples (0.05 μmol/mL) was determined in the presence and absence of $sPLA_2$, as previously described by us[24]; SSL formulations contained 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC), cholesterol and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)$_{2000}$] (DSPC/CHOL/PEG) in a 9:5:1 mole ratio. Incorporation of 10 or 30 lipid mol % of C_{31}PM resulted in enhanced $sPLA_2$-mediate release of 6-CF compared to SSL formulations (Fig. 2A). Preparations using 90% or 30 lipid mol % C_{31}PC had greater and equal release to those incorporating 10 lipid mol % DSPG (Fig. 2B). DSPG is an anionic lipid that is degraded rapidly by $sPLA_2$, but results in liposome formulations that display poor drug retention.[24] Together, these data suggest that shortening of FA acyl chains from C18:0/18:0 to C15:0/16:0, using an ester/ether linked acyl-chains and controlling the percentage of C_{31}PM or C_{31}PC can be used to tune the release from SSL-like formulations. This represents an advancement over the use of other anionic lipids, such as DSPG, that are very sensitive to $sPLA_2$, but have bulky head groups that decrease their stability



Scheme 1. Synthesis of intermediate **6**. (a) R^1OH, NaH, THF, DMF, 16 h, 80 °C (b) R^2COOH, DCC, DMAP, DCM, 24 h, room temperature (c) BBr_3, DCM, 5 min, −78 °C.



Scheme 2. Synthesis of products **1** and **2**. (a) (i) $POCl_3$, Et_3N, DCM, 1 h, room temperature; (ii) pyridine, choline tosylate, 16 h, room temperature; (iii) H_2O,1 h (b) (i) $MeOPOCl_2$, TMP, 24 h, room temperature; (ii) satd $NaHCO_3$, 2 h.

and an anionic charge that can reduce the circulation half-life of liposomes.

Serum can alter release from SSL and SPLR formulations.[24] The effect of 10% (v/v) fetal bovine serum (FBS) on $sPLA_2$-mediated 6-CF release was determined (Fig. 3). The addition of 10 or 30 mol % of $C_{31}PM$ to SSL formulations reduced the overall rate of 6-CF release compared to SSL formulations in the presence and absence of 10 mol % DSPE (Fig. 3A). This is not unexpected given the higher phase transition (T_m 52.2 °C, Table 1) of hydrated $C_{31}PM$ compared to DSPC (49.6 °C). Inclusion of DSPE (10 mol %) was

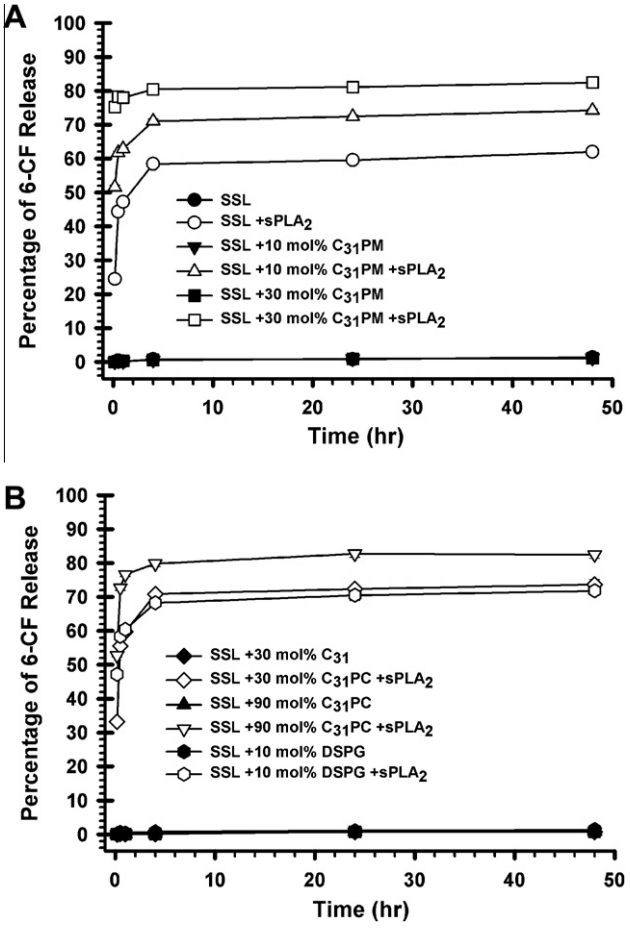


Figure 2. $sPLA_2$-mediated release of 6-CF from **A**. prototype SSL and $C_{31}PM$ (10 and 30 mol %) modified SSL and **B**. $C_{31}PC$ (90 or 30 mol %) and DSPG (10 mol %) modified SSL formulations. Liposome samples were treated with 0 (control) or 2.5 μg/mL $sPLA_2$ for 0–48 h at 25 °C in TRIS buffer (pH 7.4) and 6-CF release was determined by quantifying fluorescence at an excitation wavelength of 480 nm and an emission intensity at 510 nm. Data are represented as the mean ± SEM of a least three separate experiments, (n = 5/study).

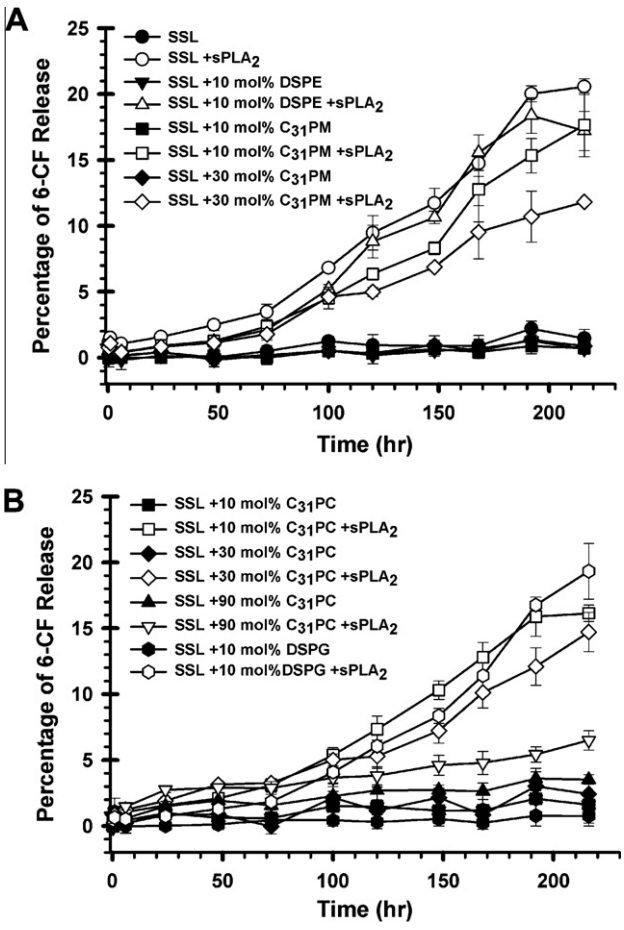


Figure 3. Effect of 10% fetal bovine serum on $sPLA_2$-mediated 6-CF release from **A**. SSL, and DSPE (10 mol %) and $C_{31}PM$ (10 and 30 mol %) modified SSL formulations and **B**. $C_{31}PC$ (10, 30, and 90 mol %) and DSPG (10 mol %) modified SSL. The effect of serum on $sPLA_2$-mediated release of 6-CF from formulations was determined over 216 h at 37 °C by quantifying fluorescence at an excitation wavelength of 480 nm and an emission intensity at 510 nm. Data are represented as the mean ± SEM of a least three separate experiments.

similar to the C_{31}PM (10 mol %). DSPE had the greatest phase transition temperature (74 °C, Table 1), that would be expected to improve membrane stability, but also has a bulkier head group relative to the phosphomethanol head group on C_{31}PM. Further, C_{31}PM is anionic, its relatively small head group may be protected from $sPLA_2$-mediated degradation, relative to other bulkier anionic lipids such as DSPE and DSPG. SSL formulations containing 10 mol % DSPG or 10 mol % C_{31}PC had similar release profiles (Fig 3B). Whereas, incorporation of C_{31}PC at 30 and 90 mol % resulted in a stepwise decrease in 6-CF release, relative to 10 mol % DSPG (Fig 3B). Although the mechanisms underlying these effects are not fully known, DSPG is an anionic lipid with a phase transition

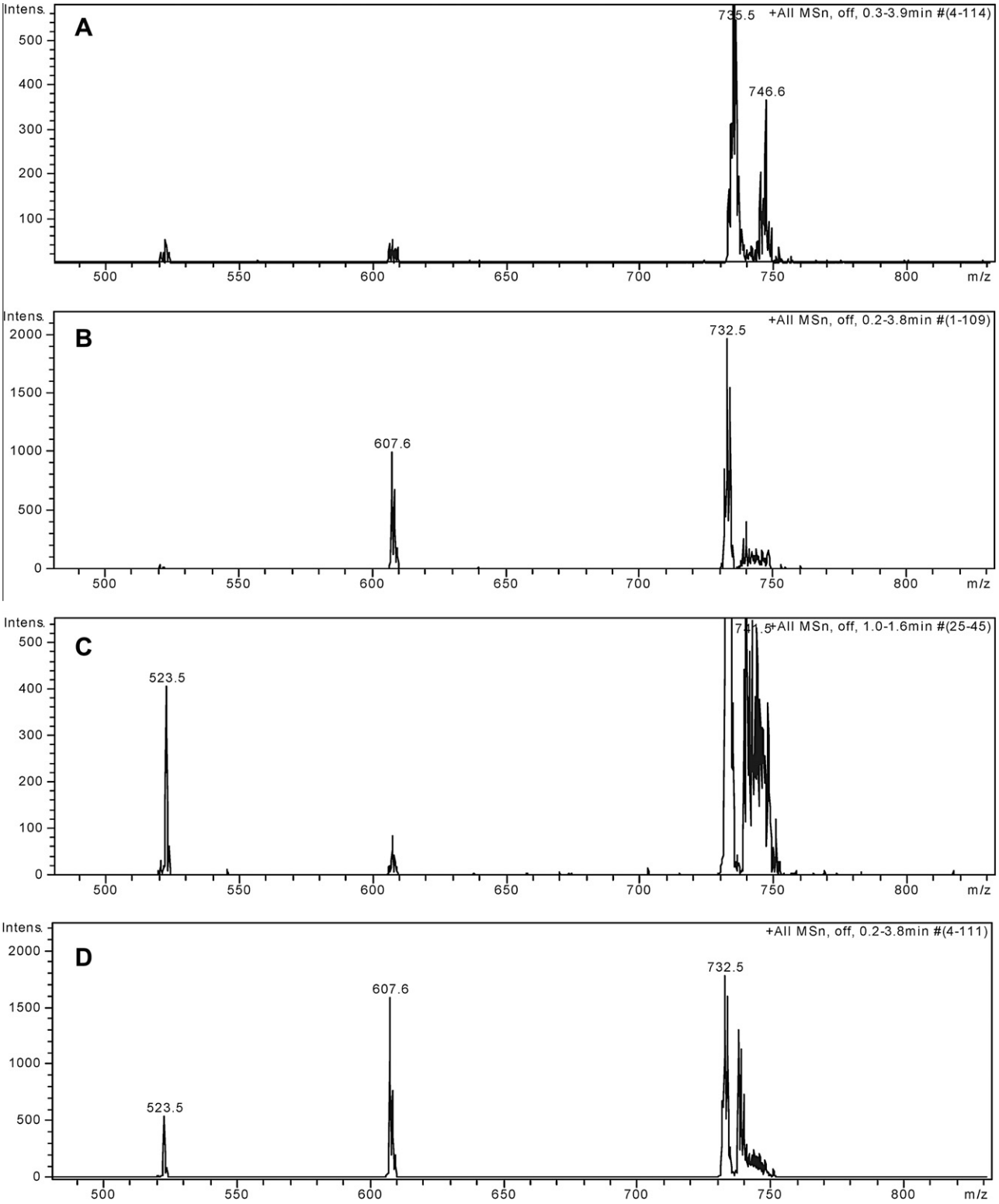


Figure 4. Mass spectra of blank rat plasma **A**. or plasma spiked DSPC **B**., P_{31}PC **C**. or SSL formulation containing DSPC and C_{31}PC in a 9:1 mole ratio **D**. DSPC (791→608 *m*/*z*) and C_{31}PC (707→523 *m*/*z*) were identified based on their ion-pair as determined using MRM.

of 54.8 °C, Table 1), whereas C_{31}PC is zwitterionic in nature. Most importantly is that the shorter acyl-chain FA used in **1** and **2** showed concentration dependent alterations in 6-CF release, suggesting that the rate and extent of release may be modulated.

These studies suggest that this approach can be used to synthesize lipids and modulate the release of intra-luminal constituents from liposomes. Although each formulation will have to be tailored to the physicochemical properties of a target drug(s), this approach provides a platform for making lipid modifications based on drug-carrier release kinetics.

Another challenge optimizing lipid based drug-carriers is our ability to track their disposition in vivo due to the presence of endogenous lipids. To overcome this challenge we synthesized odd chain lipids, not normally found in nature. Although a variety of fluorescent and radio-labeled probes exist, their effect on membrane fluidity and particulate disposition is not well known and these probes are generally not approved for human use. This is one reason why a goal of this work was to prepare lipids that were multifunctional, that is, have the ability to tune release and be identified in biological samples.

SSL formulations in the presence and absence of C_{31}PC (10 mol %) were spiked into rat plasma, extracted using a modified Bligh–Dyer assay[38], and lipids were identified via their unique ion-pairs by LC/MS/MS using mixed reaction monitoring (MRM) mode as previously described by us.[24] As expected, analysis of blank plasma demonstrated background levels of ion-pairs corresponding to DSPC (791→608 *m*/*z*) and C_{31}PC (707→523 *m*/*z*) (Fig. 4). Spiking of plasma with DSPC (Fig. 4B), C_{31}PC (Fig. 4C) and SSL formulations containing DSPC and C_{31}PC in a 9:1 mole ratio (Fig. 4D) resulted in an increased intensity for peaks corresponding to both ion-pairs. These data suggest that odd-chain lipids, for example, C_{31}PC, can be identified in rat plasma without interference of endogenous lipids. Further, the presence of a C15:0 FA on C_{31}PC may facilitate the tracking of its degradation and metabolism in vivo. Currently, determining liposome degradation in vivo is difficult without the use of tracers or radiolabels due to the high endogenous levels of even-chained lipids and their fatty acid metabolites found in biological tissues.

In conclusion, a simple approach is presented that permits synthesis of a variety of ether phospholipids from a few intermediates while decreasing acyl migration. We demonstrated the synthesis of two odd-chain ether lipids with a PC or PM head-group that could be used to alter drug release. We also demonstrated that the odd chain lipid was able to be identified after extraction from complex biological samples, for example, plasma. These data suggest that this approach may be used to make slight modifications to existing lipids or create novel lipids to tune the release of therapeutic agents from lipid based particulate carriers and track their lipids in vivo. Further, we believe this approach can be extended to use incorporation of other head groups with functional groups suitable for attaching targeting species (e.g., antibodies or peptides) or imaging probes.

Conflicts of Interest

No conflicts of interest

Acknowledgments

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29. A syringe-septum technique was used for moisture-sensitive reactions. THF was prepared freshly by distilling with sodium and benzophenone ketyl. Other solvents like DFM, DCM_3, Et_3N, and pyridine were mixed with 3 Å molecular sieves before use. Reagents were purchased from Sigma–Aldrich Chemical Co. and used without further purification. Silica gel (35–70 μm, 200–430 mesh) was used for column chromatography. Products were visualized on TLC using either iodine vapor or Hanessian's stain method. Low resolusion MS was obtained on a HPLC-LC/MSD trap XCT Ultra Plus system (Agilent), high resolution MS was obtained on a LCT Premier Orthogonal Acceleration TOF Mass Spectrometer and 400 MHz ^{1}H NMR spectra were used for chemical identifications. A Mettler Toledo DSC 1 Star System was used to determine melting temperature of DSPC, DPPC, and products **1** and **2**. DSPC, DSPG, DSPE, and DSPE–PEG were purchased from Avanti Polar Lipids Inc. (Alabaster, USA). $sPLA_2$ was purchased from Cayman Chemical Company (Ann Arbor, MI) and Genway Biotech Inc (San Diego, CA). F-12k cell culture media and FBS were purchased from Hyclone (Rockford, Illinois). CHOL was purchased from Sigma–Aldrich (St. Louis, Missouri). Acetonitrile and methanol were of HPLC grade from Fisher Scientific (Pittsburgh, PA). All other chemicals and solvents were of analytical grade, obtained from commercial sources and used without further purification. All experiments used ultrapure water (>3 MΩ) obtained from a Millipore Milli-Q synthesis system (Billerica, MA).
30. Synthesis of **4** was initiated by washing a dispersion of NaH (0.0765 g, 3.20 mmol) in (60% mineral oil) using a 50 mL N_2 protected flask with dry petroleum ether (20 mL × 3).[28] Cetyl alcohol (0.74 g, 3.05 mmol) was added followed by dry THF (10 mL) at 0 °C. The reaction mixture was refluxed at 80 °C for 1 h. (*R*)-*O*-benzyl glycidol (**3**) (0.25 g, 1.53 mmol) was added followed by addition of DMF (25 mL) drop-wise over 5 min. The reaction mixture was

stirred overnight at 80 °C. The mixture was cooled to room temperature and the reaction stopped by addition of water (2.5 mL) and solvents removed under vacuum. The residue was dissolved with diethyl ether, washed in brine three times and dried over Na_2SO_4. The product was purified using two rounds of column chromatography using ether/DCM (1:4) then ether/hexane (1:1). 1-*O*-Hexadecyl-3-*O*-benzyl-*sn*-glycerol (**4**) has a R_f 0.31 (ether/hexane 1:4), ^{1}H NMR(400 MHz, $CDCl_3$): σ 7.35 (m, 5H, Ph), σ 4.57(s, 2H, CH_2Ph), σ 4.00 (quintet, 1H, CH), σ 3.52 (m, 6H, CH_2CHCH_2, O–CH_2), σ 1.73(s, OH), σ 1.57 (quintet, 2H, β-CH_2), σ 1.26 (br s, 26H, 13 × CH_2), σ 0.887 (t, 3H, CH_3), and ESI-MS+ of 407.1 *m*/*z* (MW = 406.3).

31. Three grams of **4** (7.4 mmol), 2.7 g pentadecanoic acid (11.1 mmol), 0.17 g DMAP (1.48 mmol), 150 mL DCM and 3.1 g DCC (14.8 mmol) were added to a flame-dried, N_2 protected 250 mL flask. The reaction was cooled to 0 °C for 15 min and allowed to stand at room temperature for 24 h. The reaction was stopped by adding 5 mL acetic acid and stirred for 0.5 h. The sample was stored at a −20 °C overnight and the resulting precipitate removed by filtering. The solvent was evaporated and the resulting residues were dissolved in EtOAC, washed with 1 M HCl (30 mL × 3), satd $NaHCO_3$ (30 mL × 3), brine (30 mL × 3) and dried over Na_2SO_4. The intermediate **5** was purified using column chromatography using petroleum ether/DCM (1:4) as a mobile phase. Product yield was 69%. 1-*O*-Hexadecyl-2-pentadenoyl-3-*O*-benzyl-glycerol (**5**) has a R_f 0.25 (petroleum ether/DCM 1:4), a 631.3 *m*/*z* (MW = 630.5) by ESI-MS and ^{1}H NMR (400 MHz, $CDCl_3$): σ 7.33 (m, 5H, Ph), σ 5.18 (quintet, 1H, CH), σ 4.54–4.56 (AB, 2H, CH_2Ph), σ 3.63 (d, 2H, CH_2CHCH_2), σ 3.58 (d, 2H, CH_2CHCH_2), σ 3.42 (m, 2H, $OCH_2C_{15}H_{31}$), σ 2.34 (t, 2H, CH_2COO), σ 1.62 (quintet, 2H, β-CH_2), σ 1.53 (quintet, 2H, β-CH_2), σ 1.26 (br s, 48H, 24 × CH_2), σ 0.887 (t, 6H, CH_3 × 2), and ESI-MS+ of 631.3 *m*/*z* (MW = 630.5).
32. Intermediate **5** (0.2 g, 0.32 mmol) was added to a flame-dried, N_2 protected 250 mL flask with 200 mL DCM. The flask was bathed into a mixture of dry ice and acetone, which produced a temperature of −78 °C. BBr_3 (1 M BBr_3 in DCM, 0.64 mmol) was injected into the flask and allowed to stand for 5 min. The reaction was quenched with 20 mL satd. $NaHCO_3$ and 20 mL diethyl ether. The solvent was then washed with satd $NaHCO_3$ (30 mL × 3), water (30 mL × 3), brine (30 mL × 3) and dried over Na_2SO_4. Product **6** was concentrated under vacuum yielding a crude white solid. 1-*O*-hexadecyl-2-pentadenoyl-*sn*-glycerol (**6**) had a R_f 0.71 (DCM/diethyl ether, 10:1), ^{1}H NMR (400 MHz, $CDCl_3$): σ 5.00 (quintet, 1H, CH), σ 3.81 (d, 2H, CH_2CHCH_2), σ 3.63 (m, 2H, CH_2CHCH_2), σ 3.45 (m, 2H, O$CH_2C_{15}H_{31}$), σ 2.36 (t, 2H, CH_2COO), σ 1.63 (quintet, 2H, β-CH_2), σ 1.56 (quintet, 2H, β-CH_2), σ 1.26 (br, s, 48H, 24 × CH_2), σ 0.886 (t, 6H, CH_3 × 2), and ESI-MS+ of 541.3 *m*/*z* (MW = 540.9).
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35. To a flame-dried, N_2 protected 50 mL flask, 18 μL $POCl_3$ (0.19 mmol) and 1.5 mL DCM were added. Then 3 mL DCM with 27 μL Et_3N (0.19 mmol) and **6** (0.16 mg, 0.14 mmol) were added to the flask drop-wise over 20 min. This mixture was stirred for 1 h at room temperature. After this, 0.2 mL pyridine (1.2 mmol) and 128 mg choline tosylate (0.223 mmol) were added. The reaction was allowed to stand for 24 h at room temperature. 0.2 ml water was added to quench the reaction. The reaction mixture was concentrated and then passed through a TMD-8 resin column using THF/H_2O (9:1) as mobile phase. The resulting crude product was purified on a silica gel column using three mobile phases in a row (100 mL DCM/MeOH = 85:15, 200 mL MeOH and then 100 mL DCM/MeOH/H_2O, 65:25:4). Product yield (from **5** to **1**, two steps) was 42%. 1-*O*-hexadecyl-2-pentadenoyl-*sn*-glycerol-3-phosphocholine (**1**) has a R_f 0.35 (DCM/MeOH/H_2O, 65:25:4), ^{1}H NMR (400 MHz, $CDCl_3$): σ 5.13 (quintet, 1H, CH), σ 4.36 (s, 2H, PO$CH_2CH_2N^+$), σ 3.98 (m, 2H, CH_2CHCH_2), σ 3.88 (s, 2H, $POCH_2CH_2N^+$), σ 3.55 (m, 4H, CH_2CHCH_2, $OCH_2C_{15}H_{31}$), σ 3.44 (s, 9H, $N^+(CH_3)_3$), σ 2.32 (t, 2H, CH_2COO), σ 1.60 (quintet, 2H,β-CH_2), σ 1.53 (quintet, 2H, β-CH_2), σ 1.26 (br, s, 48H, 24 × CH_2), σ 0.887 (t, 6H, CH_3 × 2), and HRMS via TOF ES MS+ 706.5803 *m*/*z* (MW = 706.0).
36. To a solution of MOPOCL (3.4 mmol) and TMP (1.82 mmol) in dried toluene (2 mL) under N_2 protected at −20 °C, 0.4 g of 6 (0.74 mmol) in toluene (10 mL) was added drop wise. This mixture was stirred for 24 h at room temperature. After this, 2 mL satd $NaHCO_3$ was added and stirred for 2 h. This reaction mixture was concentrated by azeotropic distillation (with ethanol and toluene). The resulting crude product was purified on a silica gel column (DCM/MeOH, 4:1). Product yield (from **5** to **2**, two steps) was 40%. 1-*O*-hexadecyl-2-pentadenoyl-*sn*-glycerol-phosphomethanol (**2**) has a R_f 0.4 (DCM/MeOH = 4:1), ^{1}H NMR (400 MHz, $CDCl_3$): σ 5.18 (quintet, 1H, CH), σ 4.00 (d, 2H, CH_2CHCH_2), σ 3.61–3.57 (m, 5H, CH_2CHCH_2, $POCH_3$), σ 3.40 (m, 2H, $OCH_2C_{15}H_{31}$), σ 2.32 (t, 2H, CH_2COO), σ 1.60 (quintet, 2H, β-CH_2), σ 1.52 (quintet, 2H, β-CH_2), σ 1.26 (br, s, 48H, 24 × CH_2), σ 0.887 (t, 6H, CH_3 × 2), and TOF MS+, 657.5034 *m*/*z*, M+Na^+ adduct (MW = 633.5).
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