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Secretory Phospholipase A₂ Responsive Liposomes

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Abstract

Secretory phospholipase A₂ (sPLA₂) expression is increased in several cancers and has been shown to trigger release from some lipid carriers. This study used electrospray ionization mass spectrometry (ESI-MS) and release of 6-carboxyfluorescein (6-CF) to determine the effects of sPLA₂ on various liposome formulations. Different combinations of zwitterionic [1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine, and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine (DSPE)] and anionic [1,2-distearoyl-*sn*-glycero-3-phosphatidic acid, 1,2-distearoyl-*sn*-glycero-3-phosphatidylglycerol (DSPG), 1,2-distearoyl-*sn*-glycero-3-phosphatidylserine, and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-poly(ethylene glycol) 2000 (DSPE-PEG)] phospholipids were examined. DSPG and DSPE were most susceptible to sPLA₂-mediated degradation compared with other phospholipids. Increased 6-CF release was observed after inclusion of 10 mol % DSPE and anionic lipids into different liposome formulations. Group IIa sPLA₂-mediated 6-CF release was less than Group III and relatively insensitive to cholesterol (Chol), whereas Chol reduced sPLA₂-mediated release. Inclusion of DSPE-PEG increased sPLA₂-mediated 6-CF release, whereas serum reduced lipid degradation and 6-CF release significantly. These data demonstrate that ESI-MS and 6-CF release were useful in determining the selectivity of sPLA₂ and release from liposomes, that differences in the activity of different sPLA₂ isoforms exist, and that DSPE-PEG enhanced sPLA₂-mediated release of liposomal constituents. These findings will aid in the selection of lipids and optimization of the kinetics of drug release for the treatment of cancers and diseases of inflammation in which sPLA₂ expression is increased.

Keywords

controlled release; drug-carriers; lipids; liposomes; metabolism; nanoparticles; secretory phospholipase A₂; mass spectrometry

INTRODUCTION

Lipid based nanoparticulate drug carriers, such as long-circulating sterically-stabilized liposomes (SSL), can encapsulate drugs stably, alter their pharmacokinetics radically compared with free drug, and confer new pharmacological activity.^{1–3} Differences in drug carrier circulation half-life and tissue/tumor biodistribution are believed responsible for their improved antitumor activity and reduced toxicity.^{4,5} The clinical advantage of nanoparticles, such as SSL, was established with the approval of Doxil® (Centocor Ortho Biotech,

Horsham, PA) and other liposome formulations in the USA. Following administration, SSL can accumulate passively in solid tumors due to the enhanced permeability and retention effect mediated by defects in the vasculature and lack of functional lymphatics.^{6,7} Despite increased accumulation of SSL into tumors, for many drugs the rate of drug release is not optimal and clinical utility is limited.⁸ To overcome these barriers, a variety of physical and physiological approaches are being examined to facilitate and control drug release; these include exposure to light,^{9–11} heat,^{12–14} and use of ultrasound.^{15,16}

In this study, we choose to exploit pathophysiological differences in enzyme expression in normal and malignant tissues, that is, differences in secretory phospholipase A₂ (sPLA₂) expression,^{17–19} to modulate drug release. Previous studies examined the effect of porcine pancreatic- and bee venom sPLA₂ on phosphatidylcholine vesicles with different physical attributes.²⁰ These studies suggested that sPLA₂ present in some pathologies stimulate drug release from lipid-based drug carriers, such as liposomes (Fig. 1).^{21–23} In contrast, reduced expression of sPLA₂ in nondiseased tissues would limit lipid degradation and drug release, and is hypothesized to reduce toxicity (Fig. 1).

Recent studies demonstrated that sPLA₂ expression and activity is increased in prostate,^{24–27} breast,^{28–30} and pancreatic^{31–33} cancers. In prostate cancers, Group IIa sPLA₂ is reported to be expressed at levels 22-fold greater than disease-free paired controls.^{26,27,34} Increased sPLA₂ expression in cancer tissues correlates to increased immunohistochemical staining at the plasma membrane.²⁶ This coincides with the proposed mechanism of sPLA₂ action and suggests that these increases are localized to the site of injury. Typically, there is limited sPLA₂ in the systemic circulation, except in the case of septic shock or inflammation.

Secretory phospholipase A₂ are esterases that cleave glycerophospholipids, such as phosphatidylcholine, at the *sn*-2 ester bond, releasing a fatty acid (FA) and a lysophospholipid (LPC).³⁵ Many investigators have examined the effect of sPLA₂ on cellular membranes, lipid vesicles, and lipid-based drug carriers, but a majority of these studies were limited to bee venom (Group III), snake venom (Group Ia) or porcine pancreas (Group Ib) sPLA₂.^{20,36,37} In contrast, there are few published studies that examined the effect of human sPLA₂ on the degradation of lipid-based drug carriers²³ or made comparisons between the different isoforms.

The overall goal of this research was to determine the effect of sPLA₂ on lipid profiles and on the rate and extent of drug release from lipid nanoparticulate drug carriers. This was accomplished by examining the effect of different sPLA₂ on individual and mixed lipid degradation using electrospray ionization mass spectrometry (ESI-MS). This information was then used to formulate prototype sPLA₂ responsive liposomes (SPRL). The functional activity of sPLA₂ was determined by assessing the release of 6-carboxyfluorescein (6-CF), an aqueous soluble fluorescent marker encapsulated in conventional SSL and SPRL formulations in buffer and serum. Understanding the time course of sPLA₂-mediated lipid degradation and 6-CF release will accelerate the rational development of drug carriers to achieve optimal drug exposure selectively, thus enhancing drug efficacy and minimizing nontarget tissue toxicity.

MATERIALS AND METHODS

Chemical and Reagents

Phospholipids, DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine), DSPC (1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine), DSPG (1,2-distearoyl-*sn*-glycero-3-phosphatidylglycerol), DSPS (1, 2-distearoyl-*sn*-glycero-3-phosphatidylserine), DSPE (1,2-

distearoyl-*sn*-glycero-3-phosphatidylethanolamine), DSPA (1,2-distearoyl-*sn*-glycero-3-phosphatidic acid), DSPE-PEG (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol) 2000], and D₇₀-DSPC (deuterated DSPC) were purchased from Avanti Polar Lipids, Inc (Alabaster, Alabama). A listing of their structures, acyl chain lengths, phase transition temperatures, and charges under physiological conditions are provided in Table 1. 6-CF was purchased from Acros Organics (Geel, Belgium). Group III and Group IIa sPLA₂ were purchased from Cayman Chemical Company (Ann Arbor, Michigan) and Genway Biotech Inc (San Diego, California). F-12K cell culture media and fetal bovine serum (FBS) were purchased from Hyclone (Rockford, Illinois). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reagent and cholesterol (Chol) were purchased from Sigma-Aldrich (St. Louis, Missouri). Acetonitrile and methanol were of high performance liquid chromatography (HPLC) grade from Fisher Scientific (Pittsburgh, Pennsylvania). All other chemicals and solvents were of analytical grade, obtained from commercial sources, and used without further purification. All experiments used ultrapure water (>3MΩ) obtained from a Millipore Milli-Q synthesis system (Billerica, Massachusetts).

Preparation of Liposomes

Liposomes were prepared by hydration of thin films followed by a freeze-thaw and a high-pressure extrusion process. A listing of all formulations prepared is presented in Table 2. Briefly, phospholipids, Chol, and/or DSPE-PEG in chloroform were mixed together, dried under vacuum at 55°C–65°C (water bath) for 25 min using a rotary evaporator (Buchi Labortechnik AG, Postfach, Switzerland) and the thin film was hydrated using normal saline or Tris buffer (5 mM, pH 7.4) for 15–20 min to achieve a final lipid concentration of 10 mM. The formulation then underwent seven liquid nitrogen freeze-thaw cycles above the phase transition temperature of the primary lipid prior to extrusion ($n = 5$) through double-stacked polycarbonate membranes (80 nm, GE Osmonics, Trevose, Pennsylvania) using a Lipex extruder (Northern Lipids Inc., Burnaby, BC, Canada) at 65°C. All liposome formulations had a mean particle diameter of 80–110 nm, as determined using a Nicomp™ model 370 dynamic light scattering particle size analyzer (Santa Barbara, California). Samples were stored under a nitrogen atmosphere at 4°C, protected from light, and used within 24 h of preparation. Total phospholipid concentration of each formulation was quantified using an assay for inorganic phosphate following acid hydrolysis.³⁸

sPLA₂-Mediated Degradation and Lipid Extraction

Phospholipid degradation was determined by combining each lipid sample (1 μmol lipid/mL), sPLA₂ (0–10 μg/mL), and CaCl₂ (0–10 mM) in phosphate buffered saline, followed by incubation at 37°C for 0 to 24 h. Phospholipids and metabolites were isolated using Bligh and Dyer³⁹ extraction for ESI-MS analysis. Briefly, following incubation, 100 μL of methanol was added to samples followed by the addition of 300 μL chloroform, vortexing and centrifugation at 2500 × *g* for 5 min. The organic layer was removed and transferred to a clean test tube. The above extraction was repeated twice and the final organic phase was evaporated under nitrogen and reconstituted in 1 mL chloroform and methanol (3:1, v/v). A 100 μL aliquot of the resultant organic solution was diluted in 900 μL chloroform and methanol (3:1, v/v) for ESIMS analysis.

Profiling Lipid Degradation by ESI-MS

The stability of phospholipids treated with sPLA₂ was assessed by the loss of intensity of parent ions and the appearance and increased intensity of the primary metabolites, that is, LPCs and FAs. Analysis was performed on an Agilent HPLC-mass spectrometer (LC/MSD-Trap XCT Ultra Plus) system (Santa Clara, California). The mobile phase was acetonitrile, methanol, and 0.1% ammonium formate (2μ3:1, v/v/v). The flow rate was 0.15 mL/min and

the injection volume was 5 μ L. Nitrogen was used as a nebulizing gas at 25 psi and a drying gas at 8 psi. The drying temperature was 350°C. The capillary, capillary exit, and skimmer potentials were 3500, 150.3, and 40.0 V, respectively. The m/z range for scanning was 200 to 2200. The positive ion (+MS) mode was used to measure the intensity of the parent- and LPC ions, whereas the negative ion (–MS) mode was used to measure the intensity of anionic lipids and FA ions. Differences in ionization and extraction efficiencies of phospholipids and their FA and LPC metabolites prevent a simple comparison of signal intensities between individual lipids; however, changes within a specific lipid can provide insights into its relative sensitivity to sPLA₂. Furthermore, these differences were greatest in the anionic lipids (DSPA, DSPS, and DSPG) compared with zwitterionic lipids (DPPC, DSPC, and DSPE).

Quantification of Lipid Degradation by ESI-MS

The effect of sPLA₂ on individual and multilipid formulations was determined as above with the following alterations. Calibration curves of individual lipids, for example, DSPC (0.78–100 nmol) were prepared by diluting lipids in organic solvents (chloroform and methanol; 3:1, v/v) and spiked with internal standard D₇₀-DSPC (m/z 872.1). Liposome samples of the DSPC SSL formulation (0.05 μ mol/mL) were prepared and then incubated in the presence or absence of sPLA₂ as described previously. Internal standard, D₇₀-DSPC, was added to each sample prior to Bligh and Dyer³⁹ extraction, as described above. Calibration samples were prepared fresh by serial dilution of lipid standards in mobile phase or media containing serum. Standard curves were constructed by calculating the ratio of the analyte peak area to that of the internal standard, and plotting the ratio (ordinate) versus the theoretical concentration (abscissa); data were fit using weighted least squares; the inverse of the variance ($1/x^2$) of the observed data were used as the weighting factor. The standard curve was considered acceptable if greater than 90% of the standards had calculated accuracies within 15% of their theoretical value and no systematic deviations over the linear range were observed, precision had a coefficient of variation (CV) of less than or equal to 20%. The limit of quantification was determined experimentally as minimum concentration whose response was greater than five times the baseline value, with a CV less than and equal to 20% and accuracy $\pm 20\%$. For multilipid formulations, calibration curves were prepared by mixing DSPC and DSPE or DSPC and DSPG at approximately the same molar ratio as the formulations, that is, DSPC and DSPE or DSPG at 8:1 (lipid molar ratio). This was necessary because of the ion-suppression effect of the primary lipid, DSPC.

Preparation of 6-CF-Loaded Liposomes

Aliquots containing 100 mM 6-CF solution were prepared by dissolving 37.7 mg of 6-CF in 1 mL of 5 mM Tris-HCl buffer (pH 7.4, adjusted with 1 M NaOH). The liposomes were prepared as explained above, except a solution of 100 mM 6-CF was used to hydrate the lipid thin film instead of phosphate buffered saline or Tris buffer. Free 6-CF was removed by size exclusion chromatography (Sephadex G-75; GE Healthcare, Piscataway, New Jersey). The mobile phase for these separations consisted of 5 mM Tris-HCl buffer (pH 7.4, adjusted with 1 M NaOH). Phosphate assays were performed prior to conduction of enzymatic reactions to determine the concentration of total phospholipids.³⁸

Determination of sPLA₂-Mediated 6-CF Leakage and Analysis

Release of 6-CF from liposome samples (10 μ mol/mL diluted to 0.05 μ mol/mL) was determined in the presence and absence of Group IIa or Group III sPLA₂ (0–10 μ g/mL), CaCl₂ (0–10 mM), and Tris-HCl buffer (5 mM, pH 7.4). Fluorescent intensity (FI) of 6-CF was measured using a Synergy HT spectrofluorometer (Bio-Tek Instruments Inc., Winooski, Vermont) at excitation and emission wavelengths of 480 and 510 nm, respectively. Fluorescence was detected at 10, 30, and 45 min, and 1, 2, 4, 8, 12, 24, 36, 48, 72, and 108

h. After measurement at each time point, 10% (v/v) of Triton X-100 was added to the samples to calculate total 6-CF. Percentage of 6-CF leakage was calculated using the equation:

$$\text{Percentage} = [(F_t - F_0) / (F_{\text{Triton}} - F_0)] \times 100\%;$$

where F_t represents the FI at a specific time point, F_0 represents FI at time zero, and F_{Triton} represents total FI after addition of Triton X-100.

Effect of Serum on sPLA₂-Mediated Leakage

The effect of serum on the release of 6-CF and lipid metabolism was determined following incubation with F-12K with/without 10% FBS (v/v). Samples were kept at 37°C and protected from light. At 0 to 108 h, samples were removed and 6-CF fluorescence and lipid degradation by ESI-MS were quantified as described above.

Statistics

The mass spectrometry data are presented as the mean $\pm$ standard deviation, the 6-CF release are shown as the mean $\pm$ standard error of at least three separate experiments ($n = 5/\text{study}$). Differences were determined following an analysis of variance for each data set using SAS software (SAS Institute, Cary, North Carolina) followed by a Dunnett's *t*-test or a Student's *t*-test when comparisons involved a control and single variable. Differences were considered significant if the *p* value was less than or equal to 0.05.

RESULTS

ESI-MS Profiling of Phospholipids and sPLA₂-Mediated Metabolites

To demonstrate that ESI-MS can be used to profile sPLA₂-induced lipid degradation, we exposed individual phospholipids and multilipid formulations to sPLA₂. Group III sPLA₂ was chosen for the initial studies as it is readily available, has been used widely in the literature, and is known to be over-expressed in select cancers and in arteriosclerosis.⁴⁰ The concentrations of sPLA₂ used in these studies ranged from 0.5 to 10 $\mu\text{g/mL}$. The lower range of these concentrations corresponds to the activity of sPLA₂ in tissues under normal physiological conditions (0.025 to 0.5 $\mu\text{g/mL}$). The higher range of these concentrations corresponds to the activity of sPLA₂ in cancer and inflamed tissues, in which sPLA₂ levels are increased 2- to 1000-fold.⁴¹⁻⁴⁴

The zwitterionic lipid DPPC, with two C-16 acyl chains, was exposed to solvent control and increasing concentrations of sPLA₂ for 1 h and analyzed by ESI-MS in the positive ion mode (Fig. 2). The primary peak identified in control samples corresponded to undegraded DPPC (734.5 *m/z*). A concentration-dependent decrease in the intensity of DPPC, parent peak at *m/z* 734.5, resulted in a greater than 50% decrease after exposure to 10 $\mu\text{g/mL}$ sPLA₂ for 1 h (Fig. 2) and a concentration-dependent increase in the intensity of peaks corresponding to DPPC's 16:0 lysophosphatidylcholine (LPC) at 496.3 *m/z* and the 16:0 FA at 255.5 *m/z* (Fig. 2). These data demonstrated that sPLA₂ degrades DPPC in a concentration-dependent manner and that ESI-MS can be used to track sPLA₂-mediated degradation of phospholipids.

Selectivity of sPLA₂

The selectivity of Group III sPLA₂ (2.5 $\mu\text{g/mL}$) for individual zwitterionic and anionic phospholipids using ESI-MS was determined (Table 3). Although it is established in the literature that shorter acyl chain and anionic lipids are more susceptible to sPLA₂-mediated

degradation, it was unclear if this could be determined by ESI-MS and what the relative selectivity is for each of these lipids.²⁰ Furthermore, it is unclear if these differences in lipid metabolism correlated to increased release of entrapped drug. As described above, degradation was indicated by the loss of intensity of m/z values corresponding to the parent phospholipid, parallel with an increase in the intensity of the corresponding FA.

Table 3 shows the change in signal intensity of different species of phospholipids exposed to solvent control or 2.5 $\mu\text{g/mL}$ sPLA₂ for 6 and 24 h. The presence of sPLA₂ decreased the signal intensity for parent phospholipids significantly ($p \geq 0.05$) and increased the signal intensity for each of their metabolites. This was observed for all phospholipids except DSPA.

Typically, phospholipids with a shorter chain length were more susceptible to sPLA₂-mediated degradation, for example, DPPC (C-16) versus DSPC (C-18), Table 3. Among the phospholipids with 18 carbon (C-18) acyl chain length, DSPE and DSPG were the most susceptible to sPLA₂-mediated degradation (Table 3). It should be noted that intensities of parent phospholipids between different groups (i.e., zwitterionic and anionic) and their LPCs and FAs are not directly comparable due to differences in ionization efficiency that exist using ESI-MS. These data agree with the published literature that sPLA₂ shows greater activity toward anionic than neutral phospholipids.^{45,46}

sPLA₂-Mediated Release of 6-CF

Increased degradation of phospholipids does not indicate increased release of constituents entrapped in the aqueous core of liposomes. The effect on release was assessed by encapsulating 6-CF, which typically has low levels of fluorescence when encapsulated at high concentrations, but significantly higher levels when released and diluted in aqueous solutions.^{47–49} As shown in Figure 3a, we assessed 6-CF release induced by Group III sPLA₂ (2.5 $\mu\text{g/mL}$) for 0–108 h. Release of 6-CF from control samples increased slightly over time, less than 20% after 108 h. Treatment of zwitterionic formulations (DSPC and DSPC/DSPE at 9:1 mole ratio) with Group III sPLA₂ induced a rapid and significant ($p \leq 0.05$) increase in the release of 6-CF, compared with nontreated controls. The addition of 10 mol% anionic phospholipids such as DSPA and DSPG to basic DSPC formulations accelerated sPLA₂-mediated 6-CF release compared with formulations using neutral lipids alone Figure 3b.

Effect of Ca²⁺ on sPLA₂ Activity

The effect of Ca²⁺ on the release of 6-CF from DSPC liposome formulations was determined (data not shown). In the absence of Ca²⁺, 6-CF release increased linearly over time, reaching 50% after about 30 h. In contrast, the addition of Ca²⁺ (0.1–10 mM) resulted in a rapid, and concentration-dependent, increase in 6-CF release. For example, 50% 6-CF release was observed after 6 h exposure with the addition of 1mM Ca²⁺, which is the physiological requirement for this enzyme. These data agree with previous studies demonstrating the Ca²⁺ requirement of sPLA₂.³⁵

Effect of sPLA₂ on Release of 6-CF from Conventional Liposomes and SSL Formulations

The above data suggest that Group III sPLA₂ can degrade formulations containing anionic phospholipids and increase the release of 6-CF; however, SSL formulations used clinically contain polyethylene glycol (PEG) as well as Chol. Thus, we determined the effect of Chol and PEG on Group III sPLA₂ (2.5 $\mu\text{g/mL}$)-mediated release of 6-CF from DSPC formulations (Fig. 4a) and those containing 10 lipid mol % DSPG (Fig. 4b). The concentration of Chol and PEG chosen were similar to those used in Doxil® and other formulations that exhibit long-circulating properties.^{50,51}

Control samples were stable over the time-course measured as indicated by a low percentage of 6-CF release. Addition of Chol tended to decrease 6-CF release approximately 20% after the initial release (0–5 h), compared with formulations containing phospholipid alone and consistent with the literature.^{45,46} Interestingly, addition of DSPE–PEG increased the percentage of 6-CF release for all formulations. Inclusion of Chol into PEGylated formulations did not alter the percentage of 6-CF release, but did decrease 6-CF release in the absence of PEG. These data demonstrate that Group III sPLA₂ enhances 6-CF release from PEGylated and Chol -containing formulations.

Selectivity of Group III Versus Group IIA sPLA₂

Figure 4 demonstrated the effects of Chol and PEG on Group III sPLA₂-mediated release of 6-CF. However, Group III sPLA₂ is not the only sPLA₂ isoform involved in human diseases. In fact, Group IIA sPLA₂ is increased 5- to 20-fold in human breast and prostate cancer.³⁴ Thus, we tested the ability of human Group IIA sPLA₂ to release 6-CF from these formulations.

As demonstrated previously, incubation of liposomes with Group III sPLA₂ resulted in concentration-dependent release of 6-CF (Fig. 5a). 6-CF release was reduced in formulations containing Chol only, but was enhanced by the presence of PEG. Similar to Group III sPLA₂, Group IIA sPLA₂ also induced 6-CF release (Fig. 5b). The level of 6-CF release was lower than that observed after Group III sPLA₂ treatment, but was still concentration dependent. Furthermore, the presence of PEG increased 6-CF release. In contrast, Chol did not have a large inhibitory effect compared with Group III sPLA₂. These data show that different isoforms of sPLA₂ display distinct selectivity with respect to release of 6-CF from these formulations.

Effect of Group IIA sPLA₂ on 6-CF Release

To test the ability of Group IIA sPLA₂ to mediate 6-CF release from SPRL formulations, we incubated 2.5 µg/mL Group IIA sPLA₂ with DSPC alone or in combination with Chol and PEG or 10 mol % DSPE (Fig. 6). The addition of Group IIA sPLA₂ increased 6-CF release from all formulations tested in a time-dependent manner (Figs. 6a and 6b), including those containing Chol and PEG. The greatest increase was found in SSL (DSPC/Chol/DSPE–PEG) and SPRL (DSPC/DSPE/Chol/DSPE–PEG) formulations. The inclusion of Chol did not reduce Group IIA sPLA₂-mediated release of 6-CF, as observed with Group III (Fig. 5).

Effect of Serum on sPLA₂-Mediated Lipid Degradation and 6-CF Release

The association of serum proteins with drug carriers is known to alter their disposition and release profiles. The effect of serum, that is, FBS 10% (v/v), on sPLA₂-mediated lipid degradation and 6-CF release was determined. PEGylated (SSL), zwitterionic (+10 mol % DSPE), and anionic (+10 mol % DSPG) formulations were examined. The presence of serum decreased sPLA₂-mediated degradation of all lipids after 24 and 108 h exposure (Table 4). In all formulations the majority (>78%) of the lipids were degraded after 108 h in the absence of serum. Evidence of FA and LPC formation was observed in all samples, suggesting lipid degradation, but not quantified. In the presence of serum, DSPC degradation was reduced to 55%–62%, whereas DSPE degraded 26% and DSPG 3%. Likewise, the presence of serum altered the release of 6-CF (Fig. 7) compared with release profiles in buffer alone (Figs. 3 and 4). The formulation containing DSPG had the greatest release, followed by inclusion of DSPE compared with the SSL formulation. In the absence of serum (data not shown), release profiles were similar to those conducted in sPLA₂ buffer (Figs. 3 and 4). Control samples in the absence of sPLA₂ were found to have limited (<2%) release over 108 h.

DISCUSSION

Recent studies demonstrate that spatial and temporal differences in sPLA₂ expression exist in several diseases, including breast and prostate cancer and arteriosclerosis.^{24,34,40} sPLA₂ expression appears to be localized to the diseased tissue, and not expressed systemically,^{26,52} whereas in arteriosclerosis Group III sPLA₂ was expressed primarily in the arteriosclerotic lesion.⁴⁰ In this study we used ESI-MS to profile sPLA₂-mediated degradation of various phospholipids. This information was used to formulate various liposome formulations that displayed enhanced 6-CF release kinetics compared with conventional and long-circulating liposome (SSL) formulations.

Although studies have assessed the effect of phospholipases on drug release from liposomes,^{21,45,53–55} few described approaches that rapidly determine the selectivity of different sPLA₂ isoforms for different phospholipids. The use of ESI-MS to rapidly profile phospholipid degradation directly to assist in the selection of lipids susceptible to sPLA₂-mediated degradation has not been described in the literature.

These studies focused on Group IIa and Group III sPLA₂. Although other sPLA₂ exist, and are worthy of study, both Group IIa and Group III are overexpressed in several human diseases. The source for the Group III sPLA₂ was bee venom, which shares high sequence homology and structural similarities with human Group III sPLA₂. In fact, their central active-site domains are essentially equal.⁴⁰ The use of non-human sPLA₂ for such studies is not unprecedented as other studies have used snake venom PLA₂, structurally similar to human sPLA₂, to evaluate sPLA₂-mediated effect on drug release.^{54,56}

The goal of this study was to not directly compare Group IIa and Group III sPLA₂, but rather to demonstrate that differences exist and that they could be distinguished in our assays and could be exploited for controlling drug release to optimize drug exposure. We showed marked differences in substrate preference and activity of sPLA₂ Group III and Group IIa against both SSL and SPRL liposomes. Group III sPLA₂ was more effective in inducing lipid degradation and 6-CF leakage than Group IIa under *in vitro* conditions. This finding is relevant because several studies investigating the use of sPLA₂ for nanoparticle degradation use Group III sPLA₂, which may be more specific for treating arteriosclerosis, but not optimal for treatment of cancer in which Group IIa sPLA₂ is overexpressed. Furthermore, these findings suggest that liposome degradation and drug release *in vivo* is dependent on the sPLA₂ isoform expressed.

Model formulations were based on conventional SSL drug carriers. We modified a variety of lipid-based formulations, including SSL, to enhance their degradation rates by incorporating zwitterionic and anionic phospholipids that would be responsive to specific forms of sPLA₂-mediated degradation. Concentrations of anionic lipids greater than 10 mol % were not assessed in these formulations because they have been shown to have limited circulation time.^{57,58} Furthermore, liposomes with high content of anionic phospholipids may also activate the reticular endothelial system,^{57,59} which reduces retention time in the circulation and lowers therapeutic efficacy. Other factors assessed included calcium concentration, Chol, and PEG content. The goal was to find a combination of these factors that displayed enhanced degradation in the presence of sPLA₂, compared with standard SSL.

Electrospray ionization mass spectrometry was used to profile lipid susceptibility and to quantify the degradation of multiple phospholipids. In contrast to traditional lipid analysis, such as thin-layer chromatography and phosphorus analysis, ESI-MS has increased sensitivity, specificity, and speed of analysis. In this study, we quantified and profiled individual and multiple phospholipids using a deuterated internal standard. It should be noted that it is possible to quantify FA and LPC from these formulations with the use of

deuterated standards, but it is difficult to do this in multi-phospholipid containing formulations due to differences in extraction efficiency and ion-suppression effects (data not shown). Although the quantification of each FA and LPC was beyond the scope of this paper, we used ESI-MS to identify their formation, thus supporting our conclusion that the primary lipids were being degraded by sPLA₂.

Studies using ESI-MS showed that the acyl chain length, the polar head group, and the organized structure of phospholipid species, all alter sPLA₂-mediated degradation of liposomes. For example, DPPC (C-16) degradation was greater than that of DSPC (C-18). Furthermore, phospholipids with anionic polar head groups tended to display greater amounts of degradation compared with those with neutral head groups. This agrees with the reported specificity of sPLA₂ for phospholipids^{45,46} and suggests that changes in the rates of degradation are directly related to sPLA₂. This suggests that degradation profiles obtained by ESI-MS correlated to 6-CF release following sPLA₂ exposure.

It is important to note that degradation of phospholipids as assessed by ESI-MS may not always indicate that the lipid in question is a good candidate for formulations. For example, DPPC was highly susceptible to sPLA₂-mediated degradation due to its short chain length (C-16). However, it was not used for further study because of the greater permeability of DPPC, relative to DSPC (C-18) liposome formulations, which results from both its short acyl chain and relatively low phase-transition temperature (44°C).

With the above limitation in mind, data reported in this study demonstrate that ESI-MS is suitable for profiling lipid degradation rapidly and quantifying the degradation of individual lipids within multi-lipid formulations. ESI-MS is also excellent for identifying differences in structures of chemicals containing radiolabels or isotopes. We previously used it in biological tissues to distinguish differences in degradation of phospholipids labeled with deuterated standards.⁶⁰ Addition of such labels may permit the use of ESI-MS to determine the time-course of lipid-based carrier degradation *in vivo*. This could significantly advance the study of mechanisms surrounding drug-release from liposomes.

Secretory phospholipase A₂-mediated lipid degradation and 6-CF release in serum-supplemented media was greater in formulations containing anionic phospholipids, compared with those containing neutral phospholipids. Furthermore, 6-CF release from SPRL formulations was Ca²⁺-dependent, which agrees with the Ca²⁺-dependence of sPLA₂ and supports the hypothesis that degradation of the formulations is sPLA₂-dependent. However, these data do not directly demonstrate that 6-CF release is mediated by lipid degradation alone. Table 4 and Figure 7 support this hypothesis. A significant increase in 6-CF release was observed in formulations containing DSPG, but ESI-MS suggested only 3% to 8% of this lipid was degraded after 24 and 108 h. Although it has been shown that anionic lipids are most susceptible to sPLA₂-mediated degradation, with multiple lipid formulations a greater percentage of DSPC was degraded compared with DSPG or DSPE. Interestingly, the release of 6-CF was greatest in formulations containing DSPG and DSPE, suggesting carrier-sPLA₂ interactions may also have a destabilizing effect and contribute to release kinetics. These data suggest that addition of small quantities of these “helper lipids” could be used to modulate release.

An interesting finding from this study is that the effect of Chol was dependent on the isoform of sPLA₂ involved. For Group III sPLA₂, Chol reduced 6-CF release 20% to 50% in the PEG-free DSPC formulations. In contrast, inclusion of Chol into PEG-free DSPG formulations increased 6-CF release approximately 30%, when Group IIa sPLA₂ was used. This may result from differences in interactions of these individual sPLA₂ with Chol itself, or from differences in the phospholipids used. These differences suggest that inclusion of

membrane stabilizing Chol and/or other constitutes may impact sPLA₂ function significantly.

Polyethylene glycol enhanced sPLA₂-mediated 6-CF release independently of the phospholipid or sPLA₂ isoform used. This is somewhat surprising as PEG is hypothesized to alter the interaction of liposomes with serum proteins decreasing opsonization *in vivo*, thus enhancing their circulation half-life. Other studies showed decreased release of the fluorescent model drug calcein when PEG (10 mol % lipid) was included in DPPC (C-16) formulations.⁵⁴ Data from this study suggest that PEGylation using DSPE-PEG inhibits neither sPLA₂ interaction, nor activity at liposome membranes. DSPE is zwitterionic under normal physiological conditions, but is anionic when bound covalently to PEG. We believe that the anionic nature of DSPE-PEG is responsible for this enhanced activity. It is possible that PEG alters the structure of the membrane surface permitting enhanced binding or activity of sPLA₂; however, further studies are needed to test these hypotheses. Independent of the mechanism of action, this finding suggests that formulations may be developed that incorporate the benefits of long circulation half-lives (long saturated acyl chains, Chol, and PEG) with the use of sPLA₂-sensitive lipids to control drug release.

Pathological-based control of drug release has the potential to increase drug efficacy and decrease overall systemic toxicity. Differences in the expression of different sPLA₂ isoforms may be exploited to selectively degrade and modulate the rate and extent of drug release from lipid-based nanoparticulate drug carriers. In this study we used ESI-MS to profile and quantify lipid degradation, and 6-CF release to engineer prototype SPRL formulations. Additional studies are needed to gain mechanistic insights into sPLA₂ activity to optimize their *in vivo* activity and further their clinical potential.

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Abbreviations used

6-CF	6-carboxyfluorescein
Chol	cholesterol
DPPC	1,2-dipalmitoyl- <i>sn</i> -glycero-3-phosphatidylcholine
DSPA	1,2-distearoyl- <i>sn</i> -glycero-3-phosphatidic acid
DSPC	1,2-distearoyl- <i>sn</i> -glycero-3-phosphatidylcholine
D₇₀-DSPC	1,2-distearoyl(deuterated 70)- <i>sn</i> -glycero-3-phosphatidylcholine
DSPE	1,2-distearoyl- <i>sn</i> -glycero-3-phosphatidylethanolamine
DSPE-PEG	1,2-distearoyl- <i>sn</i> -glycero-3-phosphoethanolamine- <i>N</i> -[poly(ethylene glycol) 2000]
DSPG	1,2-distearoyl- <i>sn</i> -glycero-3-phosphatidylglycerol
DSPS	1,2-distearoyl- <i>sn</i> -glycero-3-phosphatidylserine
ESI-MS	electrospray ionization mass spectrometry
FA	fatty acid

FBS	fetal bovine serum
LPC	lysophospholipid
PEG	polyethylene glycol
PLA₂	phospholipase A ₂
SPRL	secretory phospholipase A ₂ responsive liposomes
SSL	sterically-stabilized liposome
sPLA₂	secretory phospholipase A ₂

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Figure 1. sPLA₂-facilitated drug release. This illustration describes the proposed mechanism of sPLA₂-mediated degradation and drug release from liposomes. Increased expression of sPLA₂ in solid tumors or other diseased tissue would enhance the degradation of phospholipids resulting in increased membrane permeability and drug release. Increased lipid degradation would be evidenced by increases in fatty acid and lysophospholipid lipid levels. Limited expression of sPLA₂ in noncancerous or diseased tissues would result in reduced lipid degradation and drug release.

Figure 2.

Concentration-dependence of sPLA₂-mediated degradation of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC). The effect of sPLA₂ on degradation of DPPC (734.5 *m/z*) and formation of 16:0 lysophosphatidylcholine (LPC) at 496.3 *m/z* and 16:0 fatty acid (FA) at 255.5 *m/z* were determined by ESI-MS. Data were normalized to signal intensity of DPPC, LPC, and FA in control samples. A concentration-dependent decrease in signal intensity of DPPC and increase in LPC and FA were observed. Data are represented as the percent mean intensity $\pm$ standard deviation ($n = 3$). Asterisk indicates a significant ($p \leq 0.05$) difference compared with control.

Figure 3.

Effect of Group III sPLA₂-mediated 6-CF release from (a) zwitterionic (neutral) formulations and (b) anionic formulations. (a) Percentage of 6-CF release from DSPC and DSPC/DSPE (9:1 mole ratio) formulations was assessed by fluorescent intensity changes in the media. Liposome samples (total phospholipid = 0.05 µg/mL) were treated with 0 (control) or 2.5 µg/mL Group III sPLA₂ for 0–108 h at 25°C. Fluorescence intensity was obtained by measuring the fluorescence at excitation and emission wavelengths of 480 and 510 nm, respectively. (b) Percentage of 6-CF release from anionic formulations containing DSPC with anionic phospholipids DSPA, DSPS, or DSPG (at 9:1 mole ratio). Data are represented as the mean ± standard error of the mean of at least three separate experiments ($n = 5/\text{study}$). Asterisk indicates significant ($p \leq 0.05$) difference compared with control.

A. Zwitterionic

B. Anionic

Figure 4.

Effect of PEG and cholesterol on Group III sPLA₂-mediated 6-CF release from modified SSL liposome formulations. The effect of cholesterol and DSPE-PEG on sPLA₂-mediated (2.5 µg/mL Group III) release of 6-CF from DSPC liposomes (total phospholipid = 0.05 µg/mL) (a) without or (b) with incorporation of 10 mol% DSPG was determined by fluorescent intensity changes in the media for 0–72 h at 25°C. Fluorescence intensity was obtained by measuring the fluorescence at excitation and emission wavelengths of 480 and 510 nm, respectively. Data are represented as the percent mean intensity ± standard error of the mean of at least three separate experiments ($n = 5/\text{study}$). Asterisk indicates significant ($p \leq 0.05$) difference compared with control.

Figure 5.

Comparison of Group Ila and Group III sPLA₂-mediated release of 6-CF from liposomes. The effect of cholesterol and DSPE-PEG on DSPC formulations (total phospholipid = 0.05 µg/mL) on 6-CF release was determined after exposure to either (a) Group III or (b) Group Ila sPLA₂ (0 to 5 µg/mL) for 6 h at 25°C. 6-CF release was assessed by fluorescent intensity changes in the media at excitation and emission wavelengths of 480 and 510 nm, respectively. Data are represented as the mean $\pm$ standard error of the mean of at least three separate experiments ($n = 5$ /study). Asterisk indicates significant ($p \leq 0.05$) difference compared with control.

Figure 6.

Comparison of Group IIa sPLA₂-mediated release of 6-CF from standard liposome versus SPRL preparations. Formulations (total phospholipid = 0.05 µg/mL) consisting of DSPC, DSPC/DSPE alone, and DSPC or DSPC/DSPE plus Chol and DSPE-PEG were exposed to 2.5 µg/mL of Group IIa sPLA₂ for (a) 30 min or (b) 6 h at 25°C. 6-CF was assessed by fluorescent intensity changes in the media at excitation and emission wavelengths of 480 and 510 nm, respectively. Data are represented as the mean ± standard error of the mean of at least three separate experiments ($n = 5/\text{study}$). Asterisk indicates significant ($p \leq 0.05$) difference compared with control.

Figure 7.

Effect of serum on 6-CF release. The effect of FBS and sPLA₂ on the release of 6-CF from SSL (DSPC/Chol/DSPE-PEG), zwitterionic (DSPC/DSPE/Chol/ DSPE-PEG), and anionic (DSPC/DSPG/Chol/DSPE-PEG) formulations at 37°C. Fluorescence intensity was obtained by measuring the fluorescence at excitation and emission wavelengths of 480 and 510 nm, respectively. Data are represented as the mean $\pm$ standard error of the mean of at least three separate experiments ($n = 5/\text{study}$). Asterisk indicates significant ($p \leq 0.05$) difference compared with DSPC/Chol/DSPE-PEG (SSL) formulation.

Table 1

Phospholipid Characteristics

Lipid	Head Group & Acyl Chain Length	Phase Transition (°C)	Charge*	Structure
DPPC	choline C16:0	44.1	z	
DSPC	choline C18:0	55.1	z	
DSPE	ethanolamine C18:0	74.2	z	
DSPG	glycerol C18:0	54.2	A	
DSPA	acid C18:0	75.4	A	
DSPS	serine C18:0	79.0	A	
DSPE-PEG	ethanolamine-(PEG ₂₀₀₀) C18:0	12.8	A	

* Z: zwitterionic; A: anionic; PEG: polyethylene glycol.

Table 2

Liposome Formulations

Formulation	Lipid Mole Ratio
DPPC	1
DSPC	1
DSPA	1
DSPG	1
DSPE	1
DSPS	1
DSPC:DSPA	9:1
DSPC:DSPG	9:1
DSPC:DSPE	9:1
DSPC:DSPS	9:1
DPPC:Chol	10:5
DSPC:Chol	10:5
DSPC:DSPE:Chol	9:1:5
DSPC:DSPG:Chol	9:1:5
DSPC:Chol:DSPE-PEG ^a	9:5:1
DSPC:DSPE:Chol:DSPE-PEG ^b	8:1:5:1
DSPC:DSPG:Chol:DSPE-PEG ^c	8:1:5:1

^aThis formulation was used as our prototypical long-circulating, sterically-stabilized liposome (SSL) formulation.

^bThis formulation was used as a prototypical SPRL formulations containing a zwitterionic lipid (DSPE).

^cThis formulation was used as a prototypical SPRL formulations containing an anionic lipid (DSPG).

Table 3Effect of sPLA₂ on Lipid Signal Intensity^a

Lipid (<i>m/z</i>)	(h)	Control Sample Intensity ($\times 10^6$)	+ sPLA ₂ (2.5 μ g/mL) Intensity ($\times 10^6$)	Percent Change
DPPC (734.5)	6	74.8 $\pm$ 1.6	29.1 $\pm$ 8.4	38.9*
	24	73.6 $\pm$ 0.7	28.7 $\pm$ 0.5	39.0*
DSPC (790.9)	6	81.0 $\pm$ 2.8	70.6 $\pm$ 3.4	87.2*
	24	66.6 $\pm$ 7.4	51.9 $\pm$ 1.8	77.9*
DSPE (748.1)	6	13.1 $\pm$ 1.0	6.87 $\pm$ 1.67	52.4*
	24	12.1 $\pm$ 0.1	2.73 $\pm$ 0.07	22.6*
DSPG (779.1)	6	2.70 $\pm$ 0.30	1.74 $\pm$ 0.19	37.0*
	24	2.09 $\pm$ 0.04	1.11 $\pm$ 0.19	53.1*
DSPA (705.0)	6	0.792 $\pm$ 0.041	0.685 $\pm$ 0.111	86.5
	24	0.735 $\pm$ 0.077	0.624 $\pm$ 0.135	84.9
DSPS (792.1)	6	0.769 $\pm$ 0.055	0.592 $\pm$ 0.093	77.0*
	24	0.779 $\pm$ 0.015	0.645 $\pm$ 0.078	83.0*

Fatty Acid (<i>m/z</i>)	(h)	Control Intensity ($\times 10^4$)	+ sPLA ₂ (2.5 μ g/mL) Intensity ($\times 10^4$)	Percent Change
DPPC-FA (255.5)	6	1.08 $\pm$ 0.13	20.0 $\pm$ 1.9	1,850
	24	0.739 $\pm$ 0.134	19.8 $\pm$ 4.4	2,670
DSPC-FA(283.3)	6	1.26 $\pm$ 0.19	8.32 $\pm$ 3.7	659
	24	1.11 $\pm$ 0.46	18.6 $\pm$ 2.5	1,680
DSPE-FA(283.3)	6	1.42 $\pm$ 0.16	28.6 $\pm$ 2.4	2,010
	24	1.56 $\pm$ 0.07	35.3 $\pm$ 0.9	2,260
DSPG-FA (283.3)	6	1.18 $\pm$ 0.21	18.4 $\pm$ 2.0	1,560
	24	1.16 $\pm$ 0.26	41.7 $\pm$ 4.6	3,600
DSPA-FA (283.3)	6	1.47 $\pm$ 0.05	1.87 $\pm$ 0.15	127
	24	1.06 $\pm$ 0.11	1.48 $\pm$ 0.15	140
DSPS-FA (283.3)	6	0.763 $\pm$ 0.034	3.62 $\pm$ 0.17	475
	24	0.926 $\pm$ 0.238	9.61 $\pm$ 0.95	1,040

^aData are represented as the mean intensity $\pm$ STD ($n = 3$).* Indicates a Significant ($p \leq 0.05$) difference compared with control.

Table 4Effect of serum on lipid degradation of different liposome formulations^a

	0h			24 h			108 h		
	Lipid	nM ± STD	FBS	nM ± STD	% ^b		nM ± STD	% ^a	
"SSL" DSPC:Chol:PEG (9:5:1 mole ratio)	DSPC	21.1 ± 0.3	-	8.99 ± 0.63	-57% [*]		0.344 ± 0.010	-98%	
			+	14.4 ± 0.0	-32% [*]		9.54 ± 0.20	-55% [*]	
"Zwitterionic" DSPC:DSPE:Chol:PEG (8:1:5:1 mole ratio)	DSPC	19.9 ± 0.3	-	9.19 ± 0.07	-58% [*]		0.99 ± 0.05	-95%	
			+	16.9 ± 0.1	-15% [*]		8.84 ± 0.10	-56% [*]	
	DSPE	1.60 ± 0.08	-	1.02 ± 0.06	-36%		ND	ND	
			+	1.16 ± 0.05	-28% [*]		1.19 ± 0.03	-26%	
"Anionic" DSPC:DSPG:Chol:PEG (8:1:5:1 mole ratio)	DSPC	16.0 ± 1.0	-	7.08 ± 0.40	-56% [*]		0.71 ± 0.03	-96%	
			+	11.3 ± 0.5	-29% [*]		6.11 ± 0.42	-62% [*]	
	DSPG	0.91 ± 0.04	-	0.54 ± 0.02	-41% [*]		0.202 ± 0.029	-78%	
			+	0.83 ± 0.11	-8.8% [*]		0.886 ± 0.124	-3% [*]	

^a FBS, fetal bovine serum 10% (v/v); ND, not detectable.^b Represents percent change relative to initial (0 h); Data are represented as the mean concentration ± STD (*n* = 3).^{*} Indicates a significant (*p* < 0.05) difference compared to control.