Rapid Light-Triggered Drug Release in Liposomes Containing Small Amounts of Unsaturated and Porphyrin–Phospholipids

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***P**rompt membrane permeabilization is a requisite for liposomes designed for local stimuli-induced intravascular release of therapeutic payloads. Incorporation of a small amount (i.e., 5 molar percent) of an unsaturated phospholipid, such as dioleoylphosphatidylcholine (DOPC), accelerates near infrared (NIR) light-triggered doxorubicin release in porphyrin–phospholipid (PoP) liposomes by an order of magnitude. In physiological conditions in vitro, the loaded drug can be released in a minute under NIR irradiation, while liposomes maintain serum stability otherwise. This enables rapid laser-induced drug release using remarkably low amounts of PoP (i.e., 0.3 molar percent). Light-triggered drug release occurs concomitantly with DOPC and cholesterol oxidation, as detected by mass spectrometry. In the presence of an oxygen scavenger or an antioxidant, light-triggered drug release is inhibited, suggesting that the mechanism is related to singlet oxygen mediated oxidization of unsaturated lipids. Despite the irreversible modification of lipid composition, DOPC-containing PoP liposome permeabilization is transient. Human pancreatic xenograft growth in mice is significantly delayed with a single chemophototherapy treatment following intravenous administration of 6 mg kg^{-1} doxorubicin, loaded in liposomes containing small amounts of DOPC and PoP.*

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1. Introduction

Limited accumulation of bioavailable anticancer drugs in solid tumors is a challenge for effective chemotherapy treatments.[1–4] Nanoparticles capable of site-specific drug release via intrinsic triggers such as pH,[5–7] or externally applied triggers such as heat[8–12] and light[13–22] have been designed to enhance drug deposition in tumors while sparing the other healthy organs. Release of drugs from liposomes, even in the case of passive release, is an important design characteristic.[23] For nanocarriers designed for stimuli-induced drug release inside tumor blood vessels, rapid triggered release is preferred and it has been estimated that liposomes traveling through a 1 cm diameter tumor have a residence time as small as 50–100 s.[24,25]

Porphyrin–phospholipid (PoP) can be used to generate self-assembled nanostructures with light-triggered cargo

release,[26–28] and other theranostic properties.[29–33] The inclusion of relatively small amounts (e.g., 2 mol%) of PoP to conventional sterically stabilized liposomal doxorubicin (Dox) did not interfere with the long circulating nature of conventional sterically stabilized stealth liposomes, and enhanced local phototherapeutic efficacy due to light-induced drug release and vasculature permeabilization.[27] Liposomes have been developed that contain reactive unsaturated lipids that can be directly photopolymerized under ultraviolet light to induce membrane permeabilization.[34,35] Alternatively, singlet oxygen can oxidize unsaturated phospholipids,[36–38] leading to increasing permeability of lipid bilayers.[39,40] This has been demonstrated with liposomes containing plasmalogen and membrane-bound photosensitizers, where release of entrapped calcein was achieved in 20 min with near infrared (NIR) light.[18,41] The release mechanism in these liposomes relied on the changes of membrane phase or permeability upon cleavage of plasmenylcholine to a single chain surfactant via photooxidization of the plasmalogen vinyl ether linkage. Conventional photosensitizers (but not PoP, which remains stably inserted in the membrane in biological fluids) have been used in liposomes containing large amounts of unsaturated lipids for triggered cargo release, but reports of in vivo applications have been sparse.[42] Such an approach was recently demonstrated in vivo using a palladium phthalocyanine inserted into a liposome system for on demand local release of a nerve blocking agent.[43]

PoP liposomes release cargo under NIR irradiation but until now have not been formulated with unsaturated phospholipids. The mechanisms of light-induced permeabilization of PoP liposomes have been unclear, although mechanisms related to a thermal transition were ruled out.[26] Here, we show that inclusion of a small quantity of a conventional unsaturated lipid such as dioleoylphosphatidylcholine (DOPC) greatly accelerates light-induced cargo release in PoP liposomes and this accelerated release process is related to the oxidation of unsaturated lipids.

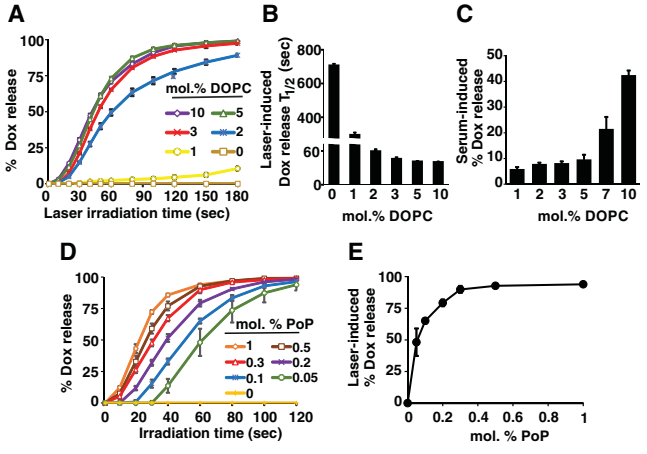

Figure 1. Rapid light-triggered release of Dox in liposomes containing small amounts of DOPC and PoP. A) Release of Dox from PoP liposomes (0.3 mol% PoP) with various amounts of DOPC upon irradiation at 310 mW cm^{-2} in 50% bovine serum at 37 °C. B) Time required to reach 50% release of Dox from PoP liposomes (0.3 mol% PoP) with various amounts of DOPC. C) Serum-induced Dox release after 4 h incubation in 50% bovine serum at 37 °C. D) Release of Dox from PoP liposomes with various amounts of PoP with laser irradiation (310 mW cm^{-2}) in 50% bovine serum at 37 °C. E) Amount of Dox released at 60 s of irradiation for PoP liposomes containing varying amounts of PoP. Data are presented as mean ± S.D., $n = 3$.

2. Results and Discussion

2.1. DOPC Accelerates Light-Triggered Release Rates of PoP Liposomes

We previously demonstrated that incorporation of lipid-anchored polyetheyleneglycol (PEG-lipid) retards light-triggered release in PoP liposomes.[27] The exact reason for this is not clear, however, it may be that PEG-lipid can sterically stabilize liposomal structures to prevent Dox leakage. Also, immunogenicity of PEG-lipid has been identified as a possible concern in patients.[44–46] Due to these factors, we elected to characterize PoP liposomes lacking PEG-lipid. PoP liposomes were made with 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), DOPC, cholesterol, and pyro-lipid (molar ratio, 59.7–*x*: *x*: 40: 0.3, *x* = mol% DOPC). DSPC was replaced with DOPC ranging from 0–10 mol% and the effects on the light-triggered release under irradiation with 665 nm NIR light were assessed (**Figure 1**A,B). Inclusion of just 2 mol% DOPC accelerated light-triggered release, resulting in an 11.6 fold decrease (713 s vs 61 s) in the time required to release 50% of Dox. Increasing amounts of DOPC (more than 3 mol%) further increased release rates, and liposomes with 5 mol% DOPC released 50% of loaded Dox in 43 s. Serum stability of Dox-loaded PoP liposomes with various amounts of DOPC revealed that PoP liposomes with DOPC content above 5 mol% were not stable when incubated in 50% bovine serum at 37 °C for 4 h, leading to 22% and 42% leakage of Dox at 7 mol% and 10 mol% DOPC, respectively (Figure 1C). Thus, 5 mol% of DOPC was selected as it enabled both rapid light-triggered release and good serum stability in the absence of NIR irradiation (just ≈10% Dox release in 50% bovine serum in 4 h). We previously demonstrated that the loading efficacy of Dox for liposomes containing 2 mol% pyro-lipid was just ≈50% without the inclusion of PEG-lipid.[27] Interestingly, incorporation of DOPC allowed for ≈95% Dox loading efficacy in PoP liposomes lacking PEG-lipid (Figure S1, Supporting Information). However, when the amount of pyro-lipid was decreased to less than 0.5 mol%, high loading efficiencies of Dox were achieved without DOPC (loading efficiency 97.3% for the formulation with 0 mol% DOPC in Figure 1A). Inclusion of 5 mol% PEG-lipid reduced the light-triggered release rate in PoP liposomes containing DOPC (336 s vs 46 s for 50% Dox release, Figure S2, Supporting Information).

By using a small amount of DOPC (5 mol%), rapid release of Dox was achieved using less than 1 mol% PoP (Figure 1D). Irradiation times required to reach 50% Dox release was less than 30 s for liposomes containing 0.5–1 mol% PoP. ≈90% of the Dox could be released in 60 s for liposomes containing 0.3 mol% or more PoP (Figure 1E). Increasing amount of PoP enhances light-triggered cargo release, however, administration of photosensitizers to

patients also increases the risk of potentially phototoxic side effects.[47–49] Thus, 0.3 mol% PoP was selected for further investigation, as it was associated with the minimal amount of PoP used while rapidly releasing contents in 60 s. Unless otherwise noted, the final formulation used for subsequent studies was [DSPC:DOPC:Cholesterol:PoP], [54.7:5:40:0.3, mol%] with a drug to lipid molar ratio of 1:8.

Dox release at lower fluence rates (25 to 250 mW cm^{-2}) was assessed in 50% bovine serum at 37 °C (Figure S3A, Supporting Information). At low fluence rates (25 mW cm^{-2}), 57% Dox release was observed in 2 min of NIR irradiation. The time required to reach 90% Dox release was not linear (Figure S3B, Supporting Information), so that the total energy required to reach 90% release was not constant. This is in contrast to our previous observation that DOPC-free liposomes release cargo with a constant amount of energy regardless of fluence rate.[26] The fluence required for 90% release increased in a linear relationship with fluence rate, with lower total energy required at lower fluence rate (Figure S3C, Supporting Information). This suggests that an alternative mechanism exists in PoP liposomes containing DOPC. As singlet oxygen generation is less efficient at higher fluence rates due to depletion of oxygen,[50–52] we hypothesized that the release mechanism could be related to singlet oxygen generation during irradiation.

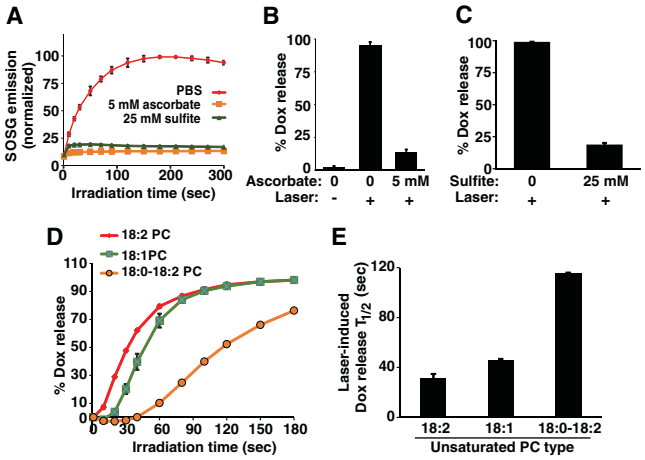

Figure 2. Enhanced light-triggered release is singlet oxygen related. A) Singlet oxygen generation during irradiation (310 mW cm^{-2}) of PoP liposomes in phosphate buffered saline (PBS), PBS containing 5 mM sodium ascorbate, or 25 mM sodium sulfite. Singlet oxygen was reported by SOSG relative fluorescence. B) Dox release upon irradiation (250 mW cm^{-2} for 3 min) was inhibited in PBS containing 5 mM sodium ascorbate at room temperature. C) Dox release upon irradiation (310 mW cm^{-2} for 3 min) was inhibited in PBS containing 25 mM sodium sulfite at room temperature. D) Dox release profiles of PoP liposomes (0.1% PoP, 5 mol% unsaturated lipids, 40 mol% cholesterol, and 54.9 mol% DSPC) containing 18:1(*cis*)PC, 18:2(*cis*) PC, or 18:0-18:2 PC upon irradiation (310 mW cm^{-2}) in 50% bovine serum at 37 °C. E) Time required for PoP liposomes (0.1% pyro-lipid, 5 mol% unsaturated lipids, 40 mol% cholesterol, and 54.9 mol% DSPC) to reach 50% Dox release. Data are presented as mean ± S.D., $n = 3$.

2.2. Enhanced Light-Triggered Release is Singlet Oxygen Related

Upon light irradiation in the presence of oxygen, photosensitizers (PoP in this case) can generate reactive single oxygen.[53–55] Cellular membranes are known to be a target of singlet oxygen in photodynamic therapy.[56–58] It was hypothesized that the rapid light-triggered release observed was related to singlet oxygen generation. To test this, the reporter fluorophore singlet oxygen sensor green (SOSG) was used to detect the presence of singlet oxygen during liposome irradiation. The antioxidant sodium ascorbate and the molecular oxygen scavenger sodium sulfite were used to inhibit singlet oxygen generation. Under NIR irradiation, singlet oxygen was generated by the PoP liposomes, but this was inhibited by ascorbate and sulfite (**Figure 2**A). Dox release from PoP liposomes was inhibited in the presence of 5 mM ascorbate (Figure 2B). No Dox release was observed in the absence of light treatment, with or without sodium ascorbate. Light treatment of Dox-loaded PoP liposomes induced 95% Dox release in 3 min, but inclusion of 5 mM sodium ascorbate led to an 81% reduction in Dox release. Similarly, light-triggered Dox release in the presence of 25 mM sodium sulfite was reduced by 80% (Figure 2C).

The light-triggered release of Dox-loaded PoP liposomes containing different unsaturated phospholipids was examined, including 18:1(*cis*) PC (DOPC), 18:2 (*cis*) PC, and 18:0–18:2 PC (Figure 2D). Other unsaturated phospholipids also enhanced Dox release from PoP liposomes upon irradiation. Lipids with greater degree of unsaturation induced faster release (Figure 2E). Under NIR light, 18:2(*cis*) PC (4 unsaturated bonds) liposomes released 50% of Dox in 31 s, while that time increased to 46 s for liposomes containing 18:1(*cis*) PC (2 unsaturated bonds). Although 18:2 (*cis*) PC resulted in faster release compared to 18:1 (*cis*) PC, PoP liposomes containing 18:2 (*cis*) PC demonstrated a lower loading efficiency (75% loading efficiency, Table S1, Supporting Information). Interestingly, 18:0–18:2 PC has the same unsaturation extent as 18:1(*cis*) PC, however, the light-triggered release rate was slower, achieving 50% Dox release in 116 s. This might be due to a lower probability of singlet oxygen accessing the unsaturated bonds of 18:0–18:2 PC that are on the same chain. Further studies demonstrated that the chemical configuration of the unsaturated lipid was critical, as 18:1(*trans*) PC did not show obvious enhancement in light-triggered release (Figure S4A, Supporting Information). Irradiation for 505 s was required to reach 50% Dox release in 18:1(*trans*) PC, compared to 31 s for 18:1(*cis*) PC (Figure S4B, Supporting Information). Local defects or destabilization may occur during this process and ultimately assist in the disruption of the lipid bilayers by 18:1 (*cis*) PC but not 18:1(*trans*) PC.

2.3. Oxidization of DOPC during Light-triggered Release

Singlet oxygen can cause oxidation of unsaturated phospholipids and cholesterol.[36,56,59,60] The DOPC content of the PoP liposomes before and after NIR irradiation (310 mW cm^{-2} for 4 min) was assessed by liquid chromatography and mass spectrometry (LC-MS). 96% of DOPC was eliminated

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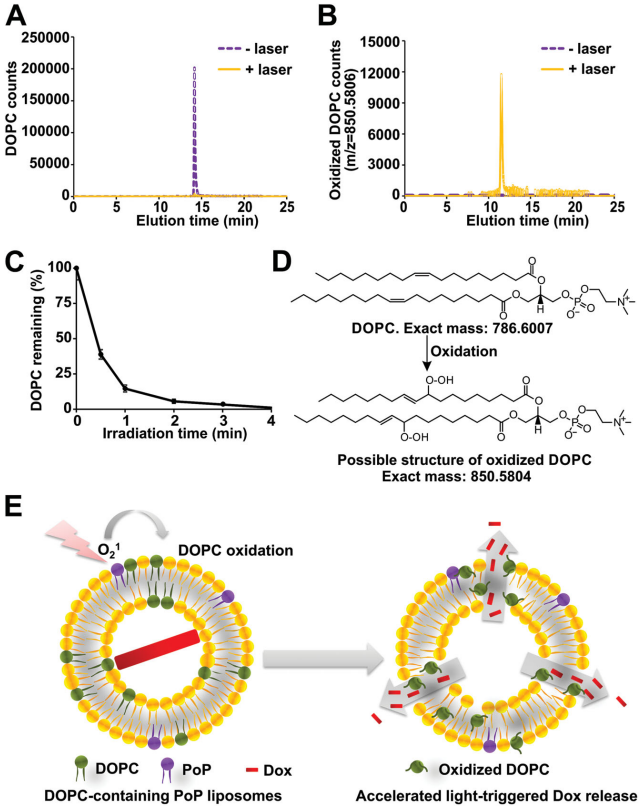

Figure 3. Light irradiation of PoP liposomes results in oxidization of DOPC. A) DOPC content in PoP liposomes before and after irradiation for 4 min at 310 mW cm^{-2}. B) New lipid species generated after irradiation (m/z: 850.5806). C) DOPC oxidation kinetics (% DOPC present compared to nonirradiated samples) while irradiated at 310 mW cm^{-2}. Data are presented as mean ± S.D., n = 3. D) Structure of DOPC and possible structure of oxidized DOPC product (Exact mass 850.5804, matching the detected oxidized species with m/z: 850.5806). E) Schematic of DOPC oxidation in PoP liposomes by singlet oxygen, leading to release of Dox.

following irradiation (**Figure 3**A) and three DOPC-related oxidized species (m/z: 832.5814, 834.5927, and 850.5806; phospholipid head groups confirmed, Figure 3B and Figure S5A, Supporting Information) were identified. In addition to these DOPC-related species, three cholesterol-related oxidized species (m/z: 367.3388, 383.3298, Figure S5B, Supporting Information) were also identified. DOPC oxidization kinetics under NIR irradiation showed that 85% of DOPC was oxidized after 1 min, a time point that at which ≈90% of loaded Dox was released (Figure 3C). DOPC was further oxidized with prolonged NIR irradiation, with 99% of the DOPC oxidized after 4 min. The amount of DSPC remained constant throughout the course of irradiation (data not shown). A possible lipid structure with 9-hydroperoxides matching the correct mass of the observed DOPC oxidized species (m/z 850.5806) is presented in Figure 3D. Singlet oxygen reacts with carbon at either end of a double bound by concerted addition (or "ene" reaction) and produces an allylic hydroperoxide in the *trans* configuration.[36–38] It is likely that both side chains of DOPC were oxidized, forming a mixture of 9- and 10-hydroperoxides. Lipid hydroperoxides are not stable species and prone to secondary oxidization. There was a relatively high variation of DOPC oxidized species detected

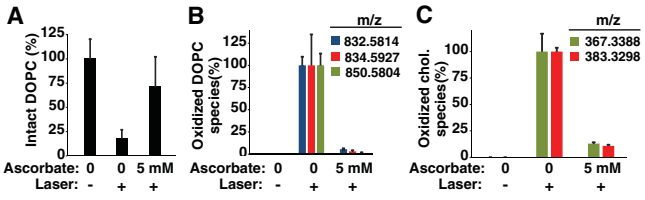

Figure 4. Ascorbate inhibits light-triggered lipid oxidation. PoP liposomes were irradiated with a 665 nm laser diode for 4 min at 310 mW cm^{-2} in the presence or absence of 5 mM sodium ascorbate. Signals in laser without sodium ascorbate were normalized as 100%. A) DOPC oxidization by light treatment was inhibited by sodium ascorbate. Sodium ascorbate inhibited generation of B) DOPC-related and C) cholesterol-related oxidized species upon NIR irradiation. Data are presented as mean ± S.D., n = 3–6.

(Figure S5A, Supporting Information), while cholesterol oxidized species (Figure S5B, Supporting Information) were relatively consistent.

The formation of allylic hydroperoxides can lead to a decrease in hydrophobic interactions that maintain liposome integrity,[61–63] and likely caused acceleration of leakage and release of Dox (Figure 3E). Further studies revealed that in the case of DOPC-free liposomes, the ability of PoP liposomes to release cargo upon NIR irradiation was dependent on oxidization of cholesterol. PoP liposomes lacking both cholesterol and DOPC could not effectively release encapsulated dyes (Figure S6A, Supporting Information), but inclusion of cholesterol enabled light-triggered release. The oxygen scavenger sodium sulfite inhibited light-induced dye release (Figure S6B, Supporting Information).

Lipid oxidization upon NIR irradiation was inhibited by sodium ascorbate (an antioxidant shown to inhibit light-triggered release in Figure 2B), as monitored by LC–MS. In the absence of the antioxidant, only 18% of the intact DOPC remained following irradiation. NIR-triggered loss of DOPC was inhibited in the presence of ascorbate, with 72% of the DOPC remaining following irradiation (**Figure 4**A). In the presence of sodium ascorbate, DOPC-related oxidized species were reduced to 5% compared to samples lacking sodium ascorbate (Figure 4B). Generation of cholesterol-related oxidized species also decreased to just ≈10% in the presence of ascorbate (Figure 4C). Taken together with the dependence of DOPC to enhance light-triggered release, these results suggest that DOPC oxidization by singlet oxygen was responsible for the enhancement of Dox release upon NIR irradiation.

2.4. Transient Permeabilization of PoP Liposomes upon NIR Irradiation

We previously reported that PoP liposome membranes are only temporarily permeabilized, based on the observation that with exposure to NIR light, external calcein can be loaded into the core of the liposomes.[26] However, for DOPC-containing PoP liposomes, the unsaturated lipid component is irreversibly oxidized, so the permanence of membrane permeabilization was of interest. Size and polydispersity index (PDI) were recorded before and after

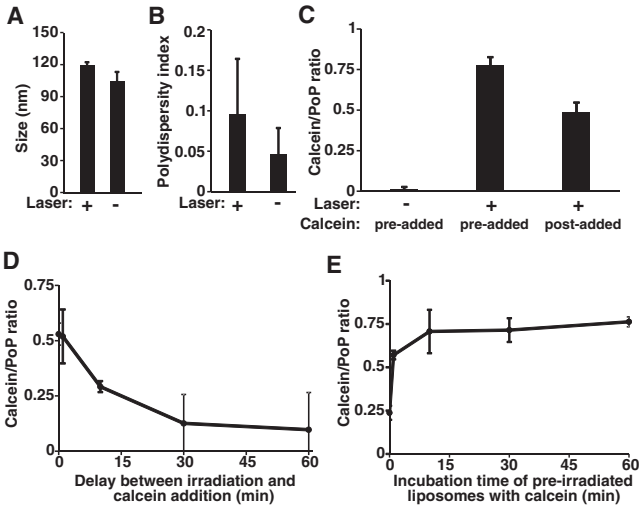

Figure 5. Transient permeabilization of PoP liposomes containing DOPC with NIR irradiation. A) Size of PoP liposomes with or without laser irradiation (250 mW cm^{-2} for 3 min), measured in PBS. B) Polydispersity index (PDI) of PoP liposomes with or without laser irradiation (250 mW cm^{-2} for 3 min), measured in PBS. PDI slightly increased but not significantly (one tailed t test). C) Passive loading of calcein (presented as calcein/PoP emission ratio) with calcein addition prior or after irradiation (250 mW cm^{-2} for 3 min). D) Passive loading of calcein into empty preirradiated PoP liposomes (250 mW cm^{-2} for 3 min). Calcein was added at indicated times following irradiation and incubated at room temperature. E) Passive loading of calcein into preirradiated empty PoP liposomes (250 mW cm^{-2} for 3 min). Calcein was added to empty PoP liposomes immediately after irradiation and incubated for various amounts of time at room temperature. Data are presented as mean ± S.D., $n = 3$.

irradiation (**Figure 5**A,B). Liposome size increased with statistical significance (105 vs 119 nm, $P < 0.05$) following light treatment, although this can be considered a modest change in liposome diameter. PDI increased but the change was not statistically significant (0.046 vs 0.096). Thus, the physical size changes that occurred in the liposomes during irradiation were subtle. Water soluble dyes such as calcein could passively load into empty PoP liposomes under NIR irradiation. This is reflected by the calcein:PoP fluorescence ratio of 0.77 in the liposome-containing fractions following removal of the free dye by gel filtration chromatography (Figure 5C). In these conditions, nonirradiated liposomes had a calcein:PoP fluorescence ratio close to 0. Interestingly, when calcein was added to empty PoP liposomes after NIR irradiation (as opposed to prior to, which is how the assay was usually performed), calcein became encapsulated in preirradiated empty PoP liposomes (calcein/PoP ratio 0.49). This suggests that irradiated DOPC-containing PoP liposomes did not reseal themselves immediately after irradiation. To investigate whether the preirradiated liposomes resealed themselves at all, calcein was added to preirradiated empty DOPC-containing PoP liposomes at different time points following irradiation. As shown in Figure 5D, the amount of calcein encapsulated decreased over time, with the calcein:PoP fluorescence ratio decreasing from when the calcein was immediately added after NIR irradiation by 45% at 10 min postirradiation and by 82% at 60 min postirradiation. Thus, preirradiated PoP liposomes appeared to gradually reseal themselves over time, preventing calcein from being encapsulated. This was further verified in another experiment in which calcein was added to empty PoP liposomes immediately after irradiation and then incubated for 0, 1, 10, 30, and 60 min at room temperature. Prolonged incubation of preirradiated empty PoP liposomes in the presence of calcein led to enhanced calcein encapsulation (Figure 5E). Most of the light-triggered loading occurred in the earlier time points with little further increase after 30 min, suggesting that the membrane reorganization and resealing occurred in ≈10 min. Since irradiated liposomes reformed membrane structures that were sufficiently intact to retain calcein over a gel filtration column, we investigated whether preirradiated liposomes could actively load Dox. Active loading of Dox was inefficient (≈2% Dox loaded) for empty PoP liposomes preirradiated with NIR light in ammonium sulfate (Figure S7, Supporting Information), suggesting that PoP liposomes with oxidized DOPC and cholesterol were not stable enough to maintain an internal ammonium sulfate gradient that is required for active Dox loading.

2.5. In Vivo Evaluation

Dox-loaded, DOPC-containing PoP liposomes prepared for animal studies were ≈120 nm and spherical in shape (Figure S8A,B, Supporting Information). Liposomes were stable in storage at 4 °C (protected from light exposure) for at least 3 months. No discernable drug leakage, changes of sizes or polydispersity index were observed (Figure S8C–E, Supporting Information). For pharmacokinetic studies, liposomes were intravenously injected into CD-1 mice at a Dox dose of 10 mg kg^{-1} (**Figure 6**A and **Table 1**). A circulating half-life of 8.3 h was observed for this PEG free formulation ([DSPC:DOPC:Chol:PoP] molar ratio of [54.7:5:40:0.3]), which was shorter than the 21.9 h half-life of a PEGylated stealth PoP-liposome formulation ([DSPC:PEG-lipid:Chol:PoP] molar ratio of [53:5:40:2]), we recently reported.[6] With the same injection dose, non-PEGylated PoP liposomes exhibited only half the Dox peak serum concentration (119 vs 250 μg mL^{-1}) 0.5 h after injection, one third the median residence time (MRT, 9.6 vs 29.3 h), and 18% the area under the curve (AUC, 851 vs 4837 μg h mL^{-1}) compared to PEGylated stealth PoP liposomes. The clearance rate of PEG free PoP liposomes was six times faster (0.012 vs 0.002 mL h^{-1} g^{-1}) and the volume of distribution at steady state was 18.8 times larger than that of PEGylated PoP liposomes.

A dual tumor model was used to assess chemophototherapy-induced Dox accumulation in tumors, with one flank of tumor irradiated and the other used as a nonirradiated control. Tumor uptake of Dox immediately after laser treatment (250 mW cm^{-2} for 40 min) was determined (Figure 6B). A 5.6 fold increase of tumoral Dox accumulation was achieved in the irradiated tumors, compared to the nonirradiated tumors. However, the amount of Dox accumulation in both the nonirradiated and irradiated tumors (0.5 vs 1.0 μg g^{-1}, 2.6 vs 7.0 μg g^{-1}, respectively) was lower compared to the previously reported PEGylated PoP liposomes at an injection dose 5 mg kg^{-1}. The shorter circulating times for the

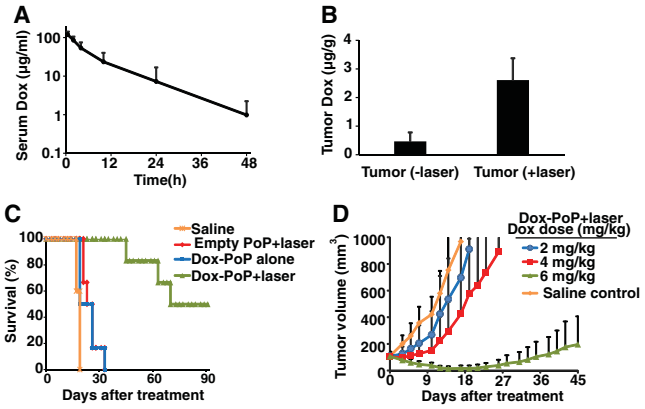

Figure 6. In vivo evaluation of Dox-loaded DOPC-containing PoP liposomes. A) Pharmacokinetics of Dox after intravenous injection of DOPC-containing Dox–PoP liposomes (10 mg kg^{-1} Dox). Data show mean+S.D., $n=4$. B) Tumoral uptake of Dox immediately after intravenous administration and phototreatment with Dox–PoP liposomes (6 mg kg^{-1} Dox) with or without laser irradiation (250 mW cm^{-2} for 40 min). A dual tumor model was used, with a tumor on one flank irradiated and the other non-irradiated as a control. The irradiated tumors had statistically significantly more Dox uptake based on the unpaired t test (***$P < 0.001$). Data are presented as mean ± S.D., $n = 4$. C) Kaplan–Merier survival curves for nude mice bearing Mia Paca-2 xenografts. Mice were intravenously administered Dox–PoP liposomes (6 mg kg^{-1} Dox, 0.25 mg kg^{-1} PoP), empty-PoP liposomes (0.25 mg kg^{-1} PoP) or saline. 10 min following injection, tumors were irradiated (250 mW cm^{-2} for 40 min, 600 J cm^{-2}) as indicated. Mice were sacrificed when tumors reached 10 times their initial volume. D) Tumor volumes of nude mice bearing Mia Paca-2 xenografts. Mice were intravenously injected with saline or Dox–PoP liposomes at 2, 4, or 6 mg kg^{-1} Dox. 10 min following injection, tumors were irradiated for 40 min at 250 mW cm^{-2} with a 665 nm laser diode. Data are presented as mean + S.D., $n = 5–6$.

non-PEGylated liposomes could be a contributing factor for the decreased deposition. Also, PoP can also induce a photodynamic mediated vascular permeabilization effect, which could contribute to the enhanced accumulation of nanoscale therapeutics.[33,64–66] Thus the lower PoP dose (0.3 vs 2 mol%) with the non-PEGyated liposomes, and diminished tumor vascular damage effects are likely reason for the relatively low Dox accumulation in the irradiated tumors. Dox distribution in key organs was determined immediately after laser treatment and revealed that most of the Dox was in kidney, spleen, and liver, with a substantial amount of Dox-loaded PoP liposomes remaining in circulation after light treatment (Figure S9A, Supporting Information).

The antitumor efficacy of Dox loaded PoP (Dox–PoP) liposomes containing 5 mol% DOPC was assessed in mice bearing MIA PaCa-2 xenografts (Figure 6C). A 6 mg kg^{-1} dose of Dox–PoP liposomes with light treatment was significantly more effective than the same dose of Dox–PoP liposomes without light treatment (median survival 80.5 vs 22.5 d, ***$P < 0.001$), or empty PoP liposomes with light treatment (median survival 80.5 vs 24.5 d, ***$P < 0.001$). The 6 mg kg^{-1} dose of Dox–PoP liposomes without light irradiation slightly delayed tumor growth compared to saline control (median survival 22.5 vs 19 d,*$P < 0.05$). The equivalent dose of empty PoP liposomes with laser treatment was also marginally effective in tumor growth inhibition compared to saline control (median survival 24.5 d vs 19 d, **$P < 0.01$). The enhanced efficacy of Dox–PoP liposomes with light treatment, compared to the other two monotherapies (chemotherapy with Dox–PoP alone or equivalent photodynamic therapy with empty PoP liposomes) could be due to the enhanced tumoral drug accumulation due to drug release and synergistic effects of chemotherapy and photodynamic therapy.[67–69]

Given the effectiveness of the single-treatment chemophototherapy, a dose response of Dox–PoP liposomes with light was performed (Figure 6D). Dox–PoP liposomes at just a 2 mg kg^{-1} Dox dose with laser treatment inhibited tumor growth compared to the saline control (median survival 23.5 vs 19 d, *$P < 0.05$). 4 mg kg^{-1} dose of Dox–PoP liposomes was not significantly more effective than 2 mg kg^{-1} (median survival 28 vs 23.5 d). 6 mg kg^{-1} dose of Dox–PoP liposomes was significantly more effective than 4 mg kg^{-1} (median survival 80.5 vs 28 d, **$P < 0.01$), with 2 out of 6 mice permanently curved (33% cure rate). Based on the tumor volume data, on day 19 after phototreatment, 2 mg kg^{-1} dose of Dox–PoP liposomes did not statistically significantly inhibit tumor growth compared to saline, while 4 mg kg^{-1} dose of Dox–PoP liposomes was effective in tumor growth control (*$P < 0.05$). 6 mg kg^{-1} dose of Dox–PoP liposomes was significantly more potent than 4 mg kg^{-1} Dox–PoP liposomes (*$P < 0.05$). Taken together, the 4 mg kg^{-1} dose was effective in tumor growth inhibition, and 6 mg kg^{-1} Dox–PoP liposomes was more effective and a 33% cure rate could be achieved. The body mass of mice revealed no weight loss during the course of treatment (Figure S9B,C, Supporting Information). There was no significant heating during the laser treatment, as the tumor surface temperature did not exceed 40 °C based on measurements with a thermal camera (data not shown).

Table 1. Noncompartmental pharmacokinetics analysis of Dox–PoP liposomes.

$T_{1/2}$ [h]	C_{max} [μg mL^{-1}]	$AUC_{0-\infty}$ [μg h mL^{-1}]	$MRT_{0-\infty}$ [h]	Cl [mL h^{-1} g^{-1}]	V_{ss} [mL g^{-1}]
8.3	119.0	851	9.6	0.012	0.113

MRT, median residence time; AUC, the area under the product of $c \bullet t$ plotted against t from time 0 to infinity; Cl, clearance; Vss, volume of distribution at steady state.

3. Conclusion

Incorporation of unsaturated lipids, including DOPC, into PoP liposomes dramatically accelerated NIR light-triggered release. This allowed for the use of very low amounts of PoP (0.1–0.3 mol%) to trigger rapid light release while preserving serum stability in the absence of NIR irradiation. The mechanism of enhanced light release rate was related to the oxidation of DOPC by singlet oxygen. In the case of DOPC-free PoP liposomes, cholesterol oxidization led to light-triggered cargo release. Tumor inhibition of MIA PaCa-2 xenografts demonstrated the efficacy of chemophototherapy. The strategy of combining small amounts of unsaturated phospholipids together with photosensitizers stably inserted in the bilayer (such as PoP) is a promising strategy for inducing rapid light-triggered intravascular release of therapeutics.

4. Experimental Section

Materials: DSPC, Cholesterol, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (18:1 (Δ9-*cis*) PC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (MPEG-2000-DSPE, PEG-lipid) were obtained from Corden Pharma. (1, 2-dilinoleoyl-*sn*-glycero-3-phosphocholine (18:2(*cis*) PC), 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (18:0-18:2 PC), and 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine (18:1(*trans*) PC) were obtained from Avanti Polar Lipids. Other chemicals were obtained from Sigma unless noted otherwise. The PoP used was pyro-lipid and was synthesized as previously reported.[26]

Liposome Preparation: Unless noted otherwise, various formulations of liposomes were prepared by the same method as previously described.[27] Unless otherwise noted, the finalized PoP liposome formulation in this study was [DSPC:DOPC:Cholesterol:PoP], [54.7:5:40:0.3], mol%, with a drug to lipid molar ratio 1:8. To generate 5 mL of PoP liposomes (20 mg mL^{-1} total lipids) of the indicated formations, lipids were dissolved in 1 mL ethanol at 60 °C, followed by injection of 4 mL of 250 mM ammonium sulfate (pH 5.5) buffer at 60 °C. The liposome solutions were then passed 10 times at 60 °C through sequentially stacked polycarbonate membranes of 0.2, 0. 1, and 0.08 μm pore size using a high pressure nitrogen extruder (Northern Lipids). Free ammonium sulfate was removed by dialysis in an 800 mL solution composed of 10% sucrose and 10 mM histidine (pH 6.5) with at least 2 buffer exchanges. For sulforhodamine (SRB) loaded PoP liposomes, lipids of the indicated formulations were dissolved in ethanol and hydrated with 50 mM SRB, sonicated at 45 °C for 30 min. Liposomal fractions were collected with gel filtration.

Cargo Loading and Characterization of PoP Liposomes: Doxorubicin (Dox, LC Labs # D-4000) was loaded via the ammonium sulfate gradient method.[70] Dox with a drug to lipid molar ratio of 1:8 was added into the liposome solution and incubated at 60 °C for 1 h. Liposomes sizes and polydispersity index were determined by dynamic light scattering in phosphate buffered saline (PBS). Dox loading efficiency were determined by running 20 μL of liposomes (20 mg mL^{-1} lipids) diluted in 1 mL of PBS over a Sephadex G-75 column. 24 × 1 mL fractions were collected and Dox fluorescence in each fraction was measured using a TECAN Safire fluorescence microplate reader (excitation and emission wavelengths of 480 and 590 nm, respectively). Loading efficiency was determined as the percentage of drug in the liposome-containing fractions (first 3–8 fractions). Negative stained transmission electron microscopy was performed using a JEM-2010 electron microscope with 1% uranyl acetate staining. Serum stability was performed by incubating PoP liposomes (20 mg mL^{-1} lipids) diluted 200 times in 50% sterile bovine serum (Pel-Freeze) at 37 °C for the indicated times. 0.25% Triton X-100 was added to read the total Dox fluorescence. Dox release was measured by florescence using the formula: %Dox release = $(F_{final}–F_{initial})/(F_{x–100}–F_{initial})$ ×100%.

Light-Triggered Drug Release: Light-triggered release experiments were performed with a power-tunable 665 nm laser diode (RPMC Lasers) at the indicated fluence rate (310 mW cm^{-2} or 250 mW cm^{-2}, as noted). Dox release was recorded in real time in a fluorometer (PTI). Irradiation was performed with PoP liposomes (20 mg mL^{-1} lipids) diluted 600 times in 50% sterile bovine serum (Pel-Freeze) at 37 °C. Temperature was measured by inserting a K-type thermocouple probe directly into the irradiated solution. 0.25% Triton X-100 was added after laser irradiation to read the total fluorescence. Dox release was assessed by measuring Dox fluorescence before and after treatment with the formula: %Dox release = $(F_{final}–F_{initial})/(F_{x–100}–F_{initial})$ ×100%. Inhibition of Dox release by sodium ascorbate was performed in a 96 microplate with 2 μL PoP liposomes (20 mg mL^{-1} lipids) diluted 100 times in PBS containing 5 mM of sodium ascorbate. Samples were irradiated at 250 mW cm^{-2} for 3 min. Inhibition of Dox release by sodium sulfite was performed in a cuvette with 5 μL PoP liposomes (20 mg mL^{-1} lipids) diluted 600 times in PBS containing 25 mM sodium sulfite. Samples were irradiated at 310 mW cm^{-2}.

Singlet Oxygen Determination: Singlet oxygen sensor green (Life Technologies # S-36002) was employed for the detection of singlet oxygen generated by pyro-lipid during irradiation. SOSG fluorescence (exc./em. 504 nm/525 nm) was recorded during irradiation in a fluometer (PTI). Light irradiation was performed in PBS containing 500 nM SOSG and Dox-loaded PoP (420 nM PoP) liposomes. PBS containing 5 mM sodium ascorbate or 25 mM sodium sulfite were employed to inhibit singlet oxygen generation.

Liquid Chromatography–Mass Spectrometry (LC–MS): Dox-loaded PoP liposomes (20 mg mL^{-1} lipids, [DSPC:DOPC:Cholesterol:PoP], [54.7:5:40:0.3], mol%) were diluted 100 times in PBS and irradiated (310 mW cm^{-2}) for 0.5, 1, 2, and 4 min for oxidization kinetics. For oxidization inhibition, samples were irradiated for 4 min at 310 mW cm^{-2} in PBS containing 5 mM sodium ascorbate.1 mL of treated liposomes was then extracted with a methanol:chloroform 1:2 (v/v) solution. The organic layer was collected and the aqueous layer was re-extracted. The organic layers were combined and dried under vacuum and stored in –80 °C. Lipids were resuspended in chloroform for LC–MS use. LC–MS data acquisition was performed using LC-ESI-QTOF [Agilent 1260 HPLC coupled to Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight instrument (Agilent Technologies, Santa Clara, CA, USA)] in positive electrospray ionization mode. Chromatographic separation was achieved using a Luna C5 reversed phase column (5 μm, 4.6 mm × 50 mm, Phenomenex) with a C5 reversed phase guard cartridge. Mobile phase A and B were 95:5 water:methanol (v/v) and 60:35:5 isopropanol:methanol:water, respectively. Each mobile phase was supplemented with 0.1% (v/v) formic acid and 5 mM ammonium formate. The gradient started after 3 min at 0% B and then increased to 100% B over 10 min followed by 100% B for 7 min before equilibration for 8 min at 0% B. The flow rate was 0.5 mL min^{-1}. A Dual JSI fitted electrospray ionization source was used. Capillary and fragmentor voltages were set to 3500 and 175 V. Drying gas temperature was 350 °C with a flow rate of 12 L min^{-1}. Data were collected using an m/z range of 50–1700 in extended dynamic range.

For targeted analysis, the corresponding m/z for each ion (for DOPC m/z = 786.6007, $[M+H]^+$, and for DSPC m/z = 790.6320, $[M+H]^+$) was extracted in MassHunter Qualitative Analysis (version B.06.00, Agilent Technologies). Peak areas for each ion in extracted ion chromatogram were manually integrated and were presented as ion counts. DOPC and DSPC were confirmed by their MS/MS fragmentation patterns. MS/MS experiments were carried out in a similar way. Different collision energies were used to get optimal ionization. Fragmentation patterns were observed at 15, 35 and 55 V. In order to identify emerging species after irradiation, raw data obtained was imported into MassHunter Profinder (version

B.06.00, Agilent Technologies) for peak alignment. Statistical analysis and filtering of the newly identified species were carried out in Mass Profiler Professional (version 12.6.1, Agilent Technologies).

Light-Induced Calcein Encapsulation: 10 μL of empty PoP liposomes (20 mg mL^{-1} lipids) were diluted 20 times in PBS in a microplate well. Laser irradiation was performed at 665 nm and 250 mW cm^{-2} for 3 min at room temperature. Calcein (50 mM) was added before or after irradiation as indicated. Liposome samples were loaded onto a Sephadex G-75 column immediately after treatment (Figure 5C). For the kinetics of calcein encapsulation into preirradiated empty PoP liposomes, calcein was added immediately after, 1, 10, 30, or 60 min after irradiation. Samples were then added to G-75 columns after incubation at room temperature for 3 min (Figure 5D). Alternatively, calcein was added before irradiation (250 mW cm^{-2} for 3 min) and incubated at room temperature for 0, 1, 10, 30, and 60 min (Figure 5E). Calcein encapsulation efficiency was determined by gel filtration with a Sephadex G-75 column. 16 × 0.5 mL fractions were collected, and calcein (485 nm/525 nm) and PoP (420 nm/670 nm) fluorescence were read with a TECAN Safire fluorescence microplate reader. Calcein/PoP ratios in the liposomal fractions (6–9 fractions) were calculated by division.

Pharmacokinetics and Biodistribution: Female mice (female CD-1, 18–20 g, Charles River) were intravenously injected via tail vein with Dox loaded DOPC-containing PoP liposomes (10 mg kg^{-1} Dox), $n = 4$. Small blood volumes were sampled at submandibular and retroorbital locations at 0.5, 2, 4, 10, 24, and 28 h postinjection. Blood was centrifuged at 1,500×g for 15 min. 10 μL serum was collected and diluted 100 times in extraction buffer (0.075 N HCl, 90% isopropanol). Samples were stored at −20 °C overnight. Samples were removed and centrifuged for 15 min at 10 000×g. Supernatants were collected and analyzed by fluorescence. Dox concentrations were determined by a standard curve. Noncomparmental pharmacokinetics parameters were analyzed by PKsolver.

For biodistribution, female nude mice (Jackson labs, #007850) were inoculated with 5×10^6 MIA Paca-2 cells on both flanks ($n = 4$). 10 min following intravenous injection with 6 mg kg^{-1} Dox loaded DOPC-containing PoP liposomes, mice were anesthetized via inhalation of isoflurane and tumors (8–10 mm) on one flank were irradiated at 250 mW cm^{-2} for 40 min, tumors on the other flank were used as nonirradiated controls. Mice were sacrificed immediately after irradiation. Tumors and key organs were collected and washed in PBS, weighted, and homogenized in nuclear lysis bufffer [250 mM sucrose, 5 mM Tris-HCl, 1 mM $MgSO_4$,1 mM $CaCl_2$ (pH 7.6)]. Dox was extracted overnight in 0.075 N HCl 90% isopropanol and fluorometrically quantified using a fluorescence standard curve.

Tumor Growth Inhibition: Five week old female nude mice (Jackson Labs, # 007805) were inoculated with 5×10^6 MIA Paca-2 cells on one flank. When tumor sizes reached 6–8 mm, mice were randomly grouped into four groups with 5–6 mice per group: (1) Dox–PoP with laser; (2) Empty PoP with laser; (3) Dox–PoP without laser; (4) Saline. 200 μL of Dox–PoP (6 mg kg^{-1} Dox, 0.25 mg kg^{-1} PoP) or empty PoP liposomes (0.25 mg kg^{-1} PoP) were I.V. injected through the tail vein. For the dose response experiment, another two groups; Dox–PoP (2 mg kg^{-1} Dox) + laser or Dox–PoP (4 mg kg^{-1} Dox) + laser were included. 10 min following I.V. injection, mice were anesthetized via inhalation of isoflurane. Irradiated tumors were treated with 665 nm laser at 250 mW cm^{-2} for 40 min (600 J cm^{-2}). Tumor temperatures during laser treatment were monitored with a thermal camera. Tumor sizes were recorded 2–3 times per week by measuring tumor dimensions using a caliper. Tumor volumes were calculated with the ellipsoid formula: $\text{Volume} = \pi \cdot L \cdot W^2/6$ where L and W are the length and width of the tumor, respectively. Body weights of the mice were monitored for four weeks. Mice were sacrificed when the tumor volume exceeded 10 times initial volume or at the end of the study period (90 d).

Statistical Analysis: Data were analyzed by GraphPad Prism (version 5.01). Kaplan–Merier survival curves were analyzed with log-rank (Mantel-Cox) test. Median survival was defined as the time at which the staircase survival curve crosses 50% survival. Tumor volume curves were analyzed by one-way ANOVA test followed by Tukey's multiple comparison test. Differences were considered significant at $P < 0.05$. ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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