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Thiol-Triggered Release of Intraliposomal Content from Liposomes Made of Extremophile-Inspired Tetraether Lipids

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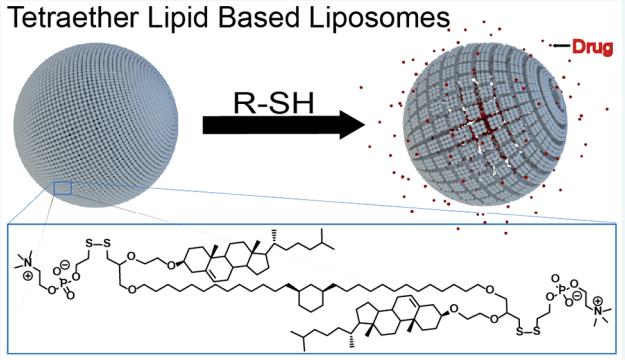
Supporting Information

ABSTRACT: Liposomal drug-delivery systems have been used for delivery of drugs to targeted tissues while reducing unwanted side effects. DOXIL, for instance, is a liposomal formulation of the anticancer agent doxorubicin (DOX) that has been used to address problems associated with nonspecific toxicity of free DOX. However, while this liposomal formulation allows for a more-stable circulation of doxorubicin in the body compared to free drug, the efficacy for cancer therapy is reduced in comparison with systemic injections of free drug. A robust liposomal system that can be triggered to release DOX in cancer cells could mitigate problems associated with reduced drug efficacy. In this work, we present a serum-stable, cholesterol-integrated tetraether lipid comprising of a cleavable disulfide bond, {GcGT(S−S)PC−CH}, that is designed to respond to the reducing environment of the cell to trigger the release intraliposomal content upon cellular uptake by cancer cells. A cell viability assay revealed that DOX- loaded liposomes composed of pure GcGT(S−S)PC−CH lipids were ∼20 times more toxic than DOXIL, with an IC_{50} value comparable to that of free DOX. The low inherent membrane-leakage properties of GcGT(S−S)PC−CH liposomes in the presence of serum, combined with an intracellular triggered release of encapsulated cargo, represents a promising approach for developing improved drug-delivery formulations for the treatment of cancer and possibly other diseases.



Liposomes have been extensively studied in drug delivery formulations due to their low toxicity, good biocompatibility, high encapsulation efficiency, and ease of chemical structural modifications.[1−4] Specifically, stimuli-responsive liposomes have gained interest for their capability to selectively release drugs at a specific target site, which potentially leads to reduced side effects and lower drug resistance.[5] Stimuli-responsive liposomes have been developed to respond to acidic environments, UV light, enzymes, biological thiols and other redox-active agents.[6−8] In particular, thiol-responsive liposomes have garnered interest as a delivery system to cancer cells, in which the intracellular thiol level (i.e., thioredoxin, thioredoxin reductase, cysteine, dihydrolipoic acid, and glutathione) is found to be significantly higher than the extracellular fluid, allowing for potential rapid release of encapsulated cargo upon internalization of liposomes in cancer cells (Figure 1).[9,10] Liposome technologies using cleavable linkers responsive to biological thiols are traditionally mixed with commercial diacyl lipids that acts as the foundation for forming liposomes.[1,11] However, the instability of diacyl lipids in serum often limits the clinical translation of thiol-responsive liposomes generated using this mixed-lipid approach.[12]

To address liposomal stability in serum, we have previously reported a synthetic hybrid lipid, namely, glycerol cholesterol-

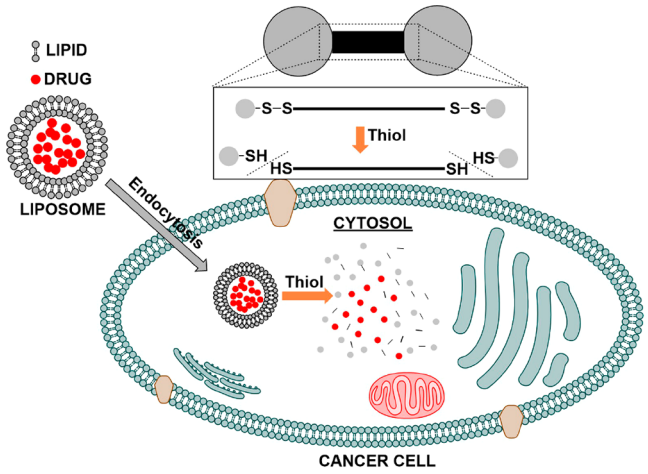


Figure 1. Schematic illustration of intracellular liposomal drug release triggered by thiol.

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integrated glycerol tetraether lipid with phosphocholine headgroups (GcGTPC−CH),[13] which incorporates cholesterol groups used by eukaryotes and covalent tethering of lipid tails[14] used by extremophile Archaea to increase lipid packing in membranes. Liposomes derived from pure GcGTPC−CH were stable in serum (exhibiting 80% retention of encapsulated cargo over 5 days) and displayed a 35−50-fold improvement in retention of small ions and a neutrally charged drug compared with liposomes composed of pure 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC, a commercial diacyl lipid).[13] However, despite the enhanced stability and reduced permeability of GcGTPC−CH liposomes, the liposomes lacked the capability for accelerated release of intraliposomal content in response to an external stimulus, which could be advantageous for drug delivery applications.

To incorporate stimuli-responsive functionality into GcGTPC−CH lipids, here we chose to integrate a disulfide linker near the polar headgroups of the lipid (Figure 2) with the

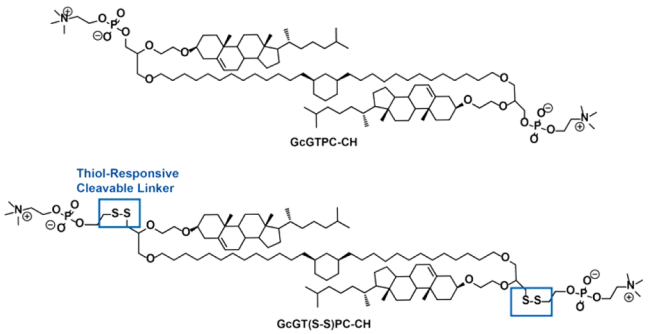

Figure 2. Molecular design of a thiol-responsive hybrid lipid.

hypothesis that the relatively small addition of the disulfide outside of the hydrophobic core would not perturb membrane packing of the lipid,[15] whereas cleavable linkers that typically involve an addition of bulky aromatic groups or heteroatoms could affect lipid organization within the membrane.[16−18] Incorporation of the disulfides near the polar lipid headgroups, rather than within the hydrophobic portion of the lipid, would also presumably facilitate access of free thiols to the disulfide bond for triggered response of the lipid. In this work, we show that this lipid can form serum stable liposomes without the need for mixing with other lipids and exhibits accelerated release of encapsulated cargo in the presence of thiols. We also examine the efficacy of this thiol-responsive GcGT(S−S)PC−CH lipid for delivery of encapsulated doxorubicin (DOX) to mammalian cancer cells. To the best of our knowledge, stimuli-responsive tetraether lipids that form serum stable liposomes by themselves (i.e., without mixing with other lipids) have not been reported.

We synthesized the GcGT(S−S)PC−CH lipid (Figure 2) using the route summarized in Scheme 1. We first synthesized phosphocholine derivative **3** that began by the selective thiol Boc protection of 2-mercaptoethanol **1** to form **2**. An initial nucleophilic substitution of the free alcohol of **2** with 2-choloro-2-oxo-1,3,2-dioxaphospholane, followed by a selective ring opening of dioxophospholane by addition of trimethylamine afforded the S-Boc-protected phosphocholine derivative **3**. The lipid scaffold was synthesized by beginning with a selective protection of the terminal alcohol and terminal thiol of 3-mercaptopropane-1,2-diol to afford the dimethoxytrityl (DMTr) protected glycerol derivative **5**. The free secondary alcohol of **5** participates in a nucleophilic displacement of the 3β-(2-tosylethoxy)cholesterol derivative **6**[13] to afford the protected glycerol scaffold **7**. After the addition of cholesterol to the glycerol scaffold **7**, the terminal O−DMTr was selectively deprotected[19] with aqueous acetic acid to form **8**, which successively reacted with dibromoalkane **9** to form the S-DMTr protected lipid **10**. The DMTr groups on the thiols of **10** were removed with trifluoroacetic acid to afford dithiol **11**. After deprotection of the S-Boc group from the phosphocholine derivative **3** with HCl to generate thiol **4**, the formation of the disulfide bonds in GcGT(S−S)PC−CH were completed by an initial activation of the free thiols of compound **11** by 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD), followed by a nucleophilic addition of the free thiol **4** to displace the triazolone auxiliaries and generate the disulfides (see the Supporting Information and Figure S1 for details on the synthesis of GcGT(S−S)PC−CH).

Next, we examined whether the GcGT(S−S)PC−CH lipid could be formulated into stable liposomes. Differential scanning calorimetry (DSC) measurements revealed that GcGT(S−S)PC−CH liposomes did not undergo a phase transition and remained in a liquid phase in the temperature range of 5 to 65 °C (see Figure S2). Dynamic light-scattering (DLS) measurements showed that pure GcGT(S−S)PC−CH lipids readily formed stable liposomes with an average radius of ∼80 nm when extruded through a 100 nm polycarbonate membrane (see Figure 3A).

After confirming that GcGT(S−S)PC−CH lipids formed stable liposomes in aqueous buffer and remained in a liquid state at room temperature or 37 °C, we next probed whether the addition of disulfide bonds to the lipid scaffold affected liposomal stability in serum containing buffer by using a self-quenching leakage assay of encapsulated carboxyfluorescein (CF).[20] Briefly, liposomes containing 100 mM CF (self-quenched fluorescence) were incubated in HEPES buffer containing 10% fetal bovine serum at 37 °C for 48 h (additional experimental details can be found in the Supporting Information). The fluorescence of CF was monitored to estimate the percent leakage of CF over time. Figure 3B shows that the addition of disulfide bonds to the lipid scaffold in GcGT(S−S)PC−CH did not affect stability in serum compared with liposomes made with GcGTPC−CH (a similar lipid that does not contain disulfide bonds; Figure 2). In contrast, liposomes made with the conventional diacyl lipid POPC had released ∼80% of CF within 2 h, suggesting liposomal membrane destabilization in the presence of serum.

To examine whether the GcGT(S−S)PC−CH liposomes exhibited accelerated release of encapsulated cargo in the presence of free thiols in solution, we again employed a standard self-quenching leakage assay[20] of encapsulated CF in buffers containing various concentrations of dithiothreitol (DTT) at 37 °C for 1 h (Figure 3C; see also Figure S3). As expected, a dose-dependent leakage of CF was observed when the concentration of DTT was increased from 0.1 to 20 mM, which presumably is caused by the destabilization of the liposomes after cleavage of the polar lipid headgroup from the glycerol lipid backbone. To determine whether the destabilization of GcGT(S−S)PC−CH liposomes were caused by the reduction of the disulfide bonds rather than the high concentration of DTT, liposomes composed of pure GcGTPC−CH[13] (which lacked a cleavable disulfide) were incubated in a solution of 20 mM DTT under the same conditions. Satisfyingly, Figure 3C shows that liposomes made

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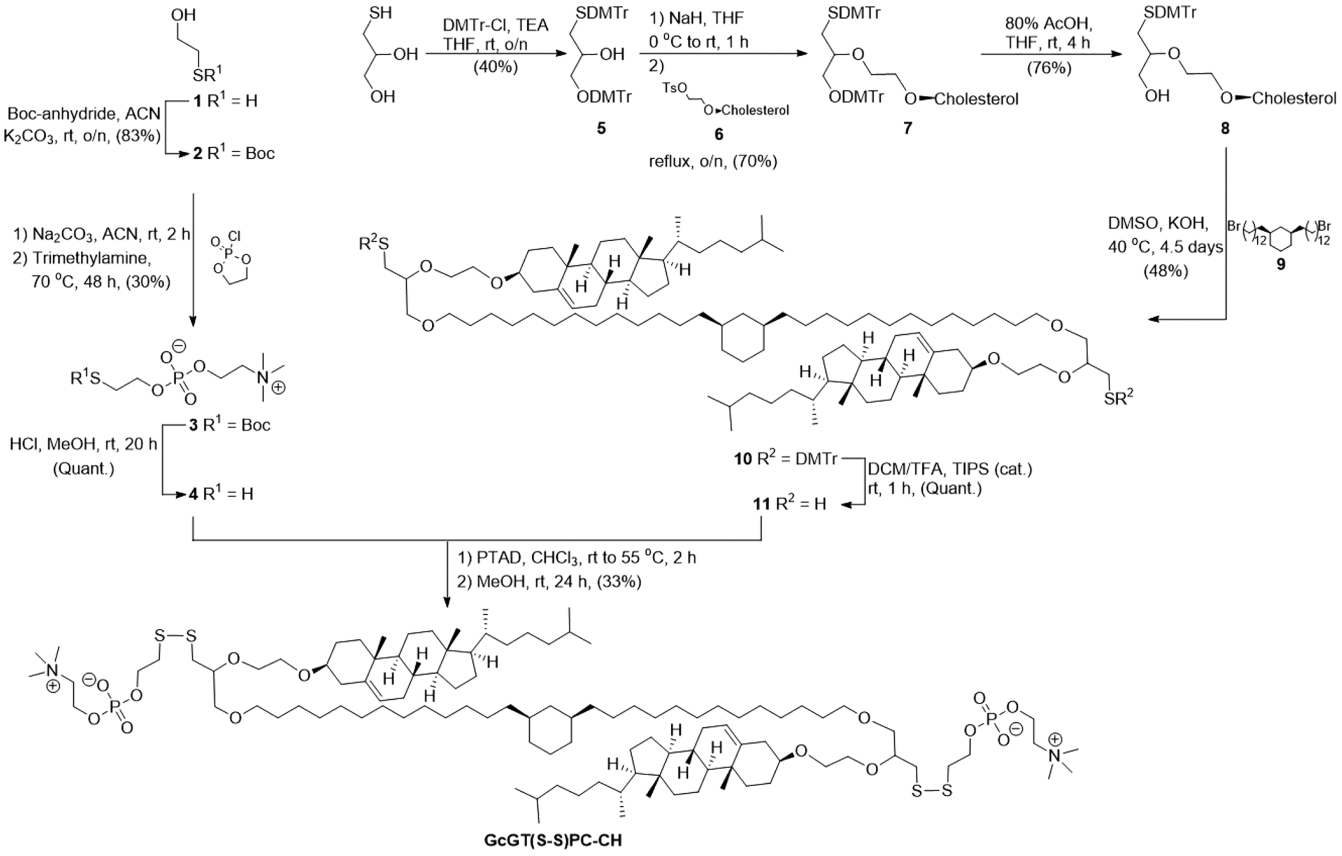
Scheme 1. Synthesis of GcGT(S−S)PC−CH Lipids



from pure GcGTPC−CH remained stable in the presence of buffer containing 20 mM DTT (represented by the blue bar in Figure 3C). Additionally, DLS measurements showed that liposomes made with GcGT(S−S)PC−CH lipids after incubation in HEPES buffer (pH 7.4) containing 20 mM DTT at 37 °C for 60 min underwent a change in morphology, while the morphology of liposomes made from GcGTPC−CH lipids remained static under the same conditions (see Figure S4). Next, to probe whether biologically relevant thiols could trigger the release of cargo from GcGT(S−S)PC−CH liposomes, GcGT(S−S)PC−CH liposomes were introduced to buffers containing 20 mM concentrations of cysteine or glutathione. When we incubated the GcGT(S−S)PC−CH liposomes in a buffer containing 20 mM of cysteine for 2 h at 37 °C, ∼40% of CF was released compared with liposomes in buffer without cysteine (see Figure 3C). However, when we incubated GcGT(S−S)PC−CH liposomes in buffer containing 20 mM of glutathione (GSH), we did not observe significant leakage of CF after 2 h at 37 °C (see Figure 3C). We hypothesize that the lack of observed response of the GcGT(S−S)PC−CH liposomes to GSH could be due to (1) the lower reducing potential of glutathione[21,22] compared with DTT or (2) the increased negative charge on membrane impermeable glutathione compared with cysteine or DTT, causing limited access to the disulfide bonds within the lipid.[23] While these experiments using different thiols in cell free assays produced mixed results regarding the capability of thiols to trigger the release of encapsulated cargo from GcGT(S−S)PC−CH liposomes, we hypothesized that the complex mixture of different thiol-containing molecules within a cell might have enough reducing potential to cause accelerated release of intraliposomal content.

To examine whether thiol-responsive GcGT(S−S)PC−CH liposomes could form stable liposomes using active loading of drugs, we examined whether DOX could be actively loaded inside liposomes using a previously reported method employing an acid gradient.[24] Briefly, 300 mM citrate buffer was passively encapsulated inside ∼160 nm diameter extruded liposomes and incubated in a solution of free DOX at 60 °C for 15 min. Unencapsulated DOX was separated from liposomes by filtration using Sephadex G-100 and the DOX-loaded liposomes were used immediately. Remarkably, DOX was successfully loaded inside the liposomes with the encapsulation efficiency of DOX in GcGT(S−S)PC−CH liposomes and GcGTPC−CH liposomes of 86% (3.5% w/w DOX/lipid) and 84% (3.9% w/w DOX/lipid), respectively (the encapsulated DOX concentration was determined using high-performance liquid chromatography [HPLC], and the lipid concentration was determined using the Bartlett assay).[25] In addition, no significant leakage of DOX was observed from GcGTPC−CH and GcGT(S−S)PC−CH liposomes after a 4 h incubation at 37 °C in HEPES buffer (pH 7.4), as estimated by HPLC using a dialysis assay[26] (shown in Figure 3D).

We next confirmed that both GcGTPC−CH and GcGT(S−S)PC−CH liposomes (without encapsulated cargo) were not toxic to HeLa cells at concentrations up to 300 μM of total lipid concentration (see Figure S5). When DOX-loaded liposomes were incubated with HeLa cells for 4 h, confocal imaging of native DOX fluorescence revealed that liposomes readily internalized in the cells (Figure 4A; additional images of cellular uptake of free DOX and Doxil are provided in Figure

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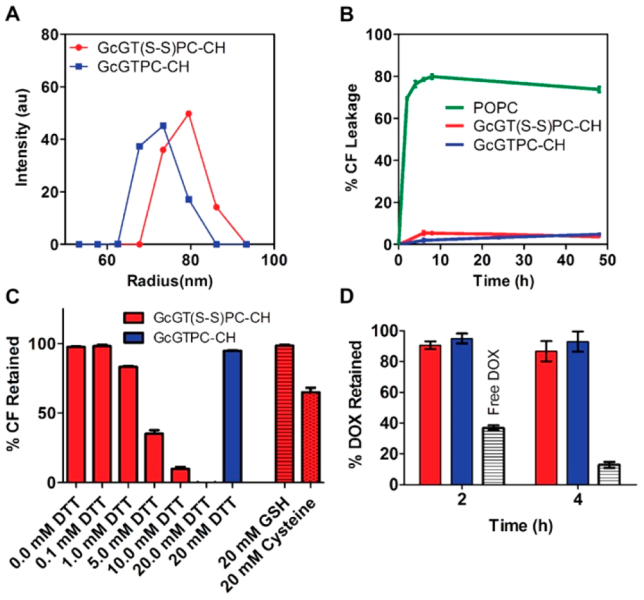

Figure 3. Characterization of liposomes composed of pure GcGTPC−CH or GcGT(S−S)PC−CH lipid. (A) Hydrodynamic radius (nm) distribution of liposomes measured using DLS. (B) Percent leakage of carboxyfluorescein (CF) from liposomes after incubation with serum in HEPES buffer (10 mM HEPES, 100 mM NaCl, 1 mM EDTA, pH 7.4) at 37 °C over 48 h (monitored at $\lambda_{ex}/\lambda_{em}$ = 485/517 nm). (C) Dose-dependent liposomal release of CF triggered by dithiothreitol (DTT) in HEPES (pH 7.4) buffer at 37 °C over 1 h (monitored at $\lambda_{ex}/\lambda_{em}$ = 485/517 nm); effect of 20 mM glutathione (GSH) or 20 mM cysteine in HEPES buffer (pH 7.4) on liposomal release of CF after 2 h of incubation at 37 °C (monitored at $\lambda_{ex}/\lambda_{em}$ = 485/517 nm). (D) Percent leakage of DOX from liposomes after incubation at 37 °C over 2 or 4 h. Free DOX released through the dialysis membrane is shown in gray (monitored at λ_{abs} = 280 nm).

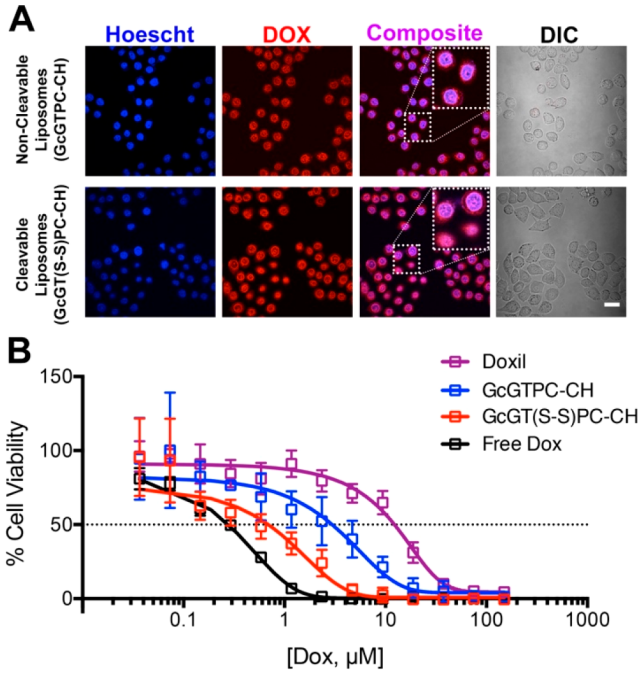

Figure 4. (A) Cellular uptake of doxorubicin into Hela cells after short-term (4 h) exposure of GcGT(S−S)PC−CH and GcGTPC−CH liposomes encapsulated with doxorubicin. Scale bar: 25 μm. (B) Cellular toxicity of DOX as either the free drug or encapsulated into various liposomal delivery systems on Hela cells after short-term exposure (4 h) and 48 h of total incubation. $n \geq 3$ for each concentration. The data are shown as normalized to the total concentration of encapsulated or free DOX.

S6). After confirming successful cell uptake of DOX loaded liposomes after a 4 h incubation, we performed a cell viability assay to investigate whether GcGT(S−S)PC−CH liposomes loaded with DOX exhibited improved efficacy for cellular toxicity compared with DOX-loaded GcGTPC−CH liposomes (which lack reducible disulfides). Briefly, HeLa cells were incubated with various concentrations of DOX-loaded GcGTPC−CH liposomes, DOX-loaded GcGT(S−S)PC−CH liposomes, DOXIL (a Food and Drug Administration [FDA]-approved liposomal formulation of DOX), or free DOX for 4 h. The cells were then washed and incubated at 37 °C for an additional 48 h, followed by analysis by an MTT cell viability assay. Figure 4B shows that DOX-loaded GcGT(S−S)PC−CH liposomes exhibited good toxicity toward HeLa cells with an IC_{50} of 0.5 μM, which was comparable to the toxicity observed for free DOX (IC_{50} of 0.2 μM) (Figure 4B). However, DOX-loaded GcGTPC−CH (which lack reducible disulfides) exhibited an IC_{50} of 1.9 μM, which is approximately an order of magnitude less toxic than the free DOX under these experimental conditions. DOXIL, however, had an IC_{50} value of 9.8 μM under the same conditions, which is ∼20 times less toxic than the DOX-loaded GcGT(S−S)PC−CH liposomes. These results support that the natural reducing environment within the HeLa cancer cells is sufficient to accelerate the release of DOX cargo from liposomes containing a thiol-cleavable linker compared with DOX-loaded GcGTPC−CH liposomes or DOXIL, which contain lipids that do not have disulfide groups.

We have, thus, successfully designed and synthesized the first tetraether lipid that contains thiol-reactive disulfide groups and that is capable of forming serum stable liposomes. Liposomes made from pure disulfide-containing lipids can be actively loaded with drugs such as DOX and can readily internalize in cancer cells. These disulfide-containing liposomes loaded with DOX showed good toxicity in HeLa cells, with an IC_{50} value similar to that of free DOX and a ∼4-fold lower IC_{50} value than an analogous DOX-loaded liposome that did not contain disulfide groups. Moreover, the DOX-loaded GcGT(S−S)PC−CH liposomes were ∼20 times more toxic than DOXIL, which is an FDA-approved liposomal formulation of DOX. This work represents a first step toward development of stimuli-responsive tetraether lipids, which may offer important advantages of improved stability and reduced passive leakage of weakly basic and charged drugs compared with current liposomal drug-delivery formulations used for cancer therapy and other systemically treated diseases.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.7b00342.

Details of the synthesis, formation and characterization of GcGT(S−S)PC−CH liposomes, kinetic analysis, and cell-uptake studies. (PDF)

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Notes

The authors declare no competing financial interest.

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