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FULL PAPER

A General Approach to Enzyme-Responsive Liposomes

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Dedicated to Professor Eric V. Anslyn on the occasion of his 60th birthday

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Abstract: Liposomes are effective nanocarriers due to their ability to deliver encapsulated drugs to diseased cells. Nevertheless, liposome delivery would be improved by enhancing the ability to control the release of contents at the target site. While various stimuli have been explored for triggering liposome release, enzymes provide excellent targets due to their common overexpression in diseased cells. We present a general approach to enzyme-responsive liposomes exploiting targets that are commonly aberrant in disease, including esterases, phosphatases and β-galactosidases. Responsive lipids correlating with each enzyme family were designed and synthesized bearing an enzyme substrate moiety attached via a self-immolating linker to a non-bilayer lipid scaffold, such that enzymatic hydrolysis triggers lipid decomposition to disrupt membrane integrity and release contents. Liposome dye leakage assays demonstrated that each enzyme-responsive lipid yielded significant content release upon enzymatic treatment compared to minimal release in controls. Results also showed that fine-tuning liposome composition was critical for controlling release. DLS analysis showed particle size increases in the cases of esterase- and β-galactosidase-responsive lipids, supporting alterations to membrane properties. These results showcase an effective modular strategy that can be tailored to target different enzymes, providing a promising new avenue for advancing liposomal drug delivery.

Introduction

Liposomes are spherical structures formed by lipid self-assembly that have proven to be effective for encapsulating and delivering a wide variety of drugs with different properties in order to enhance therapeutic attributes.[1] Comparing to free drugs, liposomal delivery systems exhibit advantages including protection against degradation, reduction of drug toxicity,[2] and side effects[3] as well as optimization of pharmacokinetic properties[4] and therapeutic indices of drugs.[5] As a result, various designer liposome platforms have been intensely studied in recent years in order to further enhance delivery characteristics.[6] Indeed, there are approximately fifteen liposomal drugs currently approved by FDA that are commercially available, including the Doxil/Caelyx (Johnson & Johnson) formulation for the delivery of the anti-cancer drug doxorubicin, and AmBisome (Gilead) encapsulating amphotericin B to treat fungal infections.[7] Drug release from clinically used formulations currently relies upon liposome carrier breakdown, which is not ideal for maximizing activity.[8] Therefore, controlling the timing and location of cargo release is a key point in advancing liposomal drug delivery.

In modern liposome research, different strategies for achieving control over drug release have been reported, which can be divided into two main categories: passive release (by internal stimuli) and active release (by external stimuli).[8-9] For passive release, variations in biological conditions between healthy and diseased cells have been used to differentiate release, such as pH profiles,[10] redox environment,[11] metabolites[12] and enzyme expression.[13] For active release, external stimuli including light,[14] heat,[15] and ultrasound[16] have been investigated for disrupting liposomes and cause encapsulated cargo release. Though a variety of triggered release approaches have been pursued, many challenges remain to be overcome in order to develop clinically applicable controlled release strategies. Active release protocols commonly suffer from challenges associated with selective delivery of stimuli to diseased cells. For example, while light-initiated liposomal release has been extensively studied, this typically entails irradiation with UV light, for which disadvantages include limited tissue penetration and damage to healthy tissue due to photodecomposition of biomolecules.[17] Considering passive release, minimal variations between diseased and healthy cells often offers only a narrow window for differentiation. For example, for pH-triggered release, reported pH differences between normal cells and cancer cells are quite small (6.5-6.9 for cancer cells and 7.2-7.4 for normal tissue),[18] which makes it difficult to develop systems that respond to these specific pH variations.

Among the potential stimuli for triggered release, the targeting of enzymes is particularly promising due to the significant overexpression of enzyme abundance commonly associated with diseased cells. Despite this potential, the development of enzyme-responsive liposomes has received less attention than other strategies, and has been limited to a small group of enzyme targets.[19] Most previously reported enzyme-responsive systems have targeted matrix metalloproteinase (MMP)[13a,13b], cholinesterase[13c] or phospholipase enzymes.[20] The latter approach has exploited the fact that phospholipase enzymes directly modify lipids that compose the liposome. As an example, the Andresen group[20a] developed a class of prodrugs incorporated within lipid scaffolds, for which drug release is driven by lipid hydrolysis catalyzed by secretory phospholipase A_2

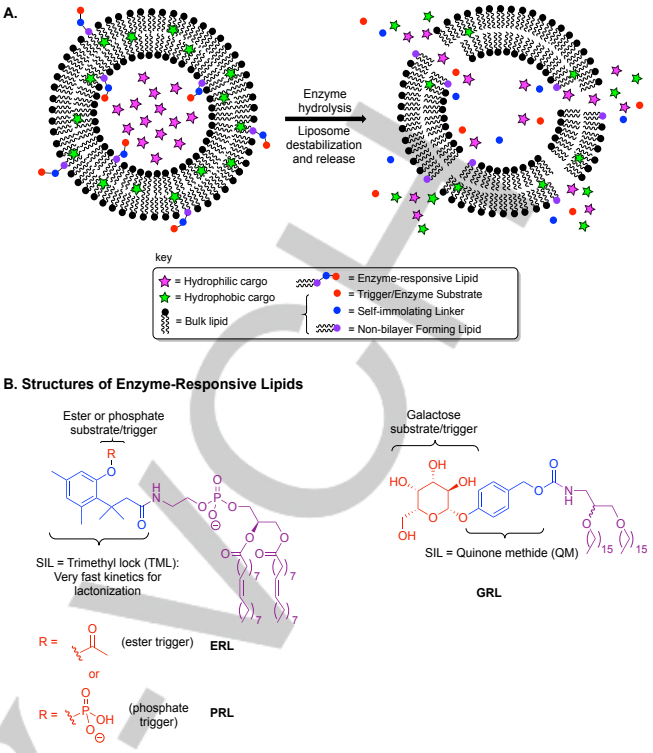
(sPLA2). These prior studies support the hypothesis that enzymatic reactions can be harnessed to drive liposome release. However, there are many other enzymes that are significantly overexpressed in different types of cancerous and otherwise diseased cells that provide exciting targets.

In this work, we report a general, modular strategy for developing liposomal drug delivery platforms that is effective for targeting a range of enzymes that exhibit dramatic upregulation in diseases such as cancer, including esterases, phosphatases, and β-galactosidases. Esterase concentrations have been reported to be enhanced by two to three orders of magnitude in cancer cells,[21] and these enzymes have been implicated in other diseases such as neuronal,[22] liver[23] and Alzheimer's diseases.[24] Overabundance of multiple phosphatase enzymes, such as alkaline phosphatase, has also been correlated with cancer[25] as well as liver,[26] cardiovascular[27] and kidney diseases.[28] β-Galactosidases are also overexpressed in cancer, and thus have been targeted for cancer imaging.[29] Glycosidase enzymes in general are involved in numerous diseases including diabetes, cancer, viral infections such as HIV, and lysosomal storage disorders.[30] These particular enzyme targets have been the subject of few studies. In a rare example, the ganglioside GM1 has been reported for triggered release using β-galactosidase based on the modulation of membrane properties upon truncation of the carbohydrate head group.[31] While this is an elegant strategy, it relies upon a complex glycolipid structure for release. In addition, the Szoka group developed a cholesterol-based phosphate lipid analog, which was able to stabilize the DOPE bilayer.[13d] Addition of alkaline phosphatase and hydrolysis of the phosphate head group disrupted the bilayer and caused encapsulated cargo release.

Results and Discussion

Herein, we report a versatile stimuli-responsive lipid design by exchanging appended substrate moieties to target multiple enzymes that are commonly overexpressed in cancer cells. The design strategy is shown in Scheme 1. Each lipid analog contains three functional regions. First, a variable substrate moiety corresponding to each target enzyme is included that acts as the trigger for liposome release. This includes an ester that can be cleaved by an esterase (esterase-responsive lipid (**ERL**)), a phosphate group that is hydrolyzed by a phosphatase (phosphatase-responsive lipid (**PRL**)) or a β-galactose moiety for enzymatic cleavage by a β-galactosidase (galactosidase-responsive lipid (**GRL**)). Secondly, a self-immolating linker (SIL) is present in between the trigger head group and the lipid scaffold to be released, a strategy that has been used in stimuli-responsive systems including sensors[32] and nanomedicine.[33] Upon trigger removal, the SIL is designed to quickly undergo a disassembly reaction that results in release of an appended leaving group. Specifically, the SILs for our responsive lipids include the trimethyl lock (TML, *o*-hydroxydihydrocinnamic acid)[34] for **ERL** and **PRL** or the quinone methide (QM)-generating 4-hydroxylmethylphenol group for **GRL**,[35] both of which are well known for fast kinetics of decomposition upon trigger removal.

Finally, these responsive lipids are designed to form stable liposome membranes that are perturbed upon enzymatic removal of the trigger, in this case through the release of a non-bilayer lipid



Scheme 1. Design of enzyme-responsive liposomes. **A.** Cartoon depicting hypothetical model for liposome release. Enzyme-responsive lipids contain three major regions including enzyme substrate head group, SIL and a non-bilayer lipid scaffold. After hydrolysis of the substrate by the appropriate enzyme, SIL decomposition will cause the release of a non-bilayer lipid to disrupt the membrane integrity and release of encapsulated cargo. **B.** Structures of enzyme-responsive lipids. Esterase- and phosphatase-responsive lipids (**ERL** and **PRL**, respectively) include TML as the SIL and DOPE as the lipid scaffold. β-Galactosidase-responsive lipid **GRL** instead bears a QM-generating SIL and an aminodialkylglycerol analogue as the lipid scaffold.

that will destabilize the membrane and trigger release of liposome contents. The lipids dioleoylphosphatidylethanolamine (DOPE) and aminodialkylglycerol were employed for this purpose, with the latter selected to avoid synthetic challenges associated with introduction of the sugar head group of **GRL**. It has been well-studied that due to the small head-to-tail volume ratio, lipids such as DOPE typically prefer to form hexagonal phase (H_{II}) lipid assemblies in aqueous solution at physiological conditions.[36] However, increasing the head-to-tail volume ratio through *N*-acylation with a bulky group results in the self-assembly of resulting lipids into stable membrane bilayers.[37] Therefore, the release of DOPE has previously been harnessed for liposome release strategies. For example, the Smith group reported photocleavable liposomes using a light-responsive lipid analogue that generates DOPE upon UV light irradiation resulting in release of the entrapped dye calcein.[14a] In an example more closely related to this work, McCarley and co-workers[11a] developed redox-responsive liposomes by coupling DOPE to a quinone redox switch using TML as a SIL. Addition of $Na_2S_2O_4$ initiated the self-immolating process of the quinone head group and the release of DOPE, which ultimately perturbed the membrane and released the loaded dye calcein. Putting all of these groups together, our systems are designed such that enzymatic removal of trigger groups will stimulate decomposition of the SIL to produce non-bilayer lipids that disrupt the membrane and release encapsulated cargo.

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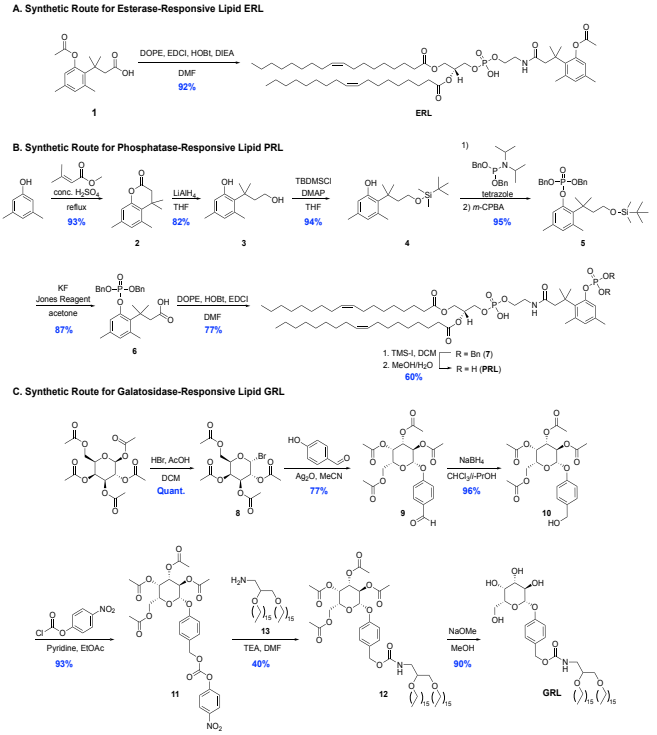

Scheme 2. Synthetic routes developed to access **ERL** (**A**) **PRL** (**B**) and **GRL** (**C**).

The synthetic routes used to access the three enzyme-responsive lipids are shown in Scheme 2. The synthesis of **ERL** benefitted from commercially available esterified TML-carboxylic acid **1**, which allowed for convenient access to this product through a one-step amide-bond coupling reaction with DOPE (Scheme 2A). To access **PRL**, compounds **2**-**4** were synthesized from 3,5-dimethylphenol as previously reported[38] through a Michael addition with methyl 3-methylbut-2-enoate, spontaneous lactonization to **2**, reductive ring opening to **3**, and silyl protection to **4**. Phosphoramidite chemistry was next performed to produce the phosphodiester of **5**, followed by Jones oxidation and simultaneous silyl deprotection to afford **6**.[38a] Finally, an amide coupling reaction with DOPE yielding **7** was followed by benzyl deprotection to produce **PRL**. For **GRL**, the design and synthetic route were different, as shown in Scheme 2C. As discussed above, to overcome synthetic issues associated with the use of acetyl protecting groups, 4-hydroxy benzyl alcohol was instead used as the SIL and dialkylglycerol as the non-bilayer forming lipid scaffold. Compounds **8**-**11** were synthesized from β-D-galactose pentaacetate as previously reported by Toth et al[39] through HBr treatment to form α-D-galactopyranosyl bromide (**8**), glycosylation with 4-hydroxyl benzaldehyde to produce **9**, aldehyde reduction to **10**, and conversion of the resulting alcohol to *p*-nitrophenyl carbonate **11**. Dialkyl aminoglycerol lipid **13** was synthesized from solketal in five steps as we previously reported[40] through tosylate introduction, acetonide deprotection, substitution of tosylate with azide, Williamson ether synthesis to introduce lipid alkyl chains, and reduction of azide to the primary amine **13** (not shown). Amine **13** and carbonate **11** were combined to produce the carbamate moiety of protected lipid **12**, followed by deprotection of the acetyl groups on galactose access **GRL**.

Following the successful synthesis of all three lipids, we next tested their enzyme-responsive properties. Considering the accessibility of **ERL** compared to the other two lipids, we initiated studies with this compound to determine conditions for liposomal release. Prior to incorporating this compound into liposomes, we set out to evaluate esterase hydrolysis of the free lipid using a TLC assay in a manner similar to a previous report.[13e] For this experiment, 2 μg of **ERL** was dissolved in 50 μL of TBS buffer and was then incubated with 0.45 U of commercially available porcine liver esterase at room temperature for three hours. After this, the reaction mixture was spotted on a silica gel TLC plate, which was run with 20% MeOH-chloroform as eluant and visualized with potassium permanganate stain.[41] As is shown in Figure S1, this plate indicated complete disappearance of the **ERL** spot in the product coupled with the appearance of a new spot attributed to DOPE. This result indicates that **ERL** acts as an appropriate substrate and provided evidence that the esterase hydrolyzed only the TML head group while leaving the DOPE fatty acid ester chains intact. In addition, the catalytic activities of phosphatase and β-galactosidase enzymes were confirmed via colorimetric assays using *p*-nitrophenylphosphate and *p*-nitrophenylgalactose, respectively (Figure S2).

With evidence that **ERL** acts as a substrate for esterase enzyme, we next moved on to evaluate liposome triggered release properties using fluorescence-based dye leakage assays. These assays can be used to evaluate the release of either hydrophobic or hydrophilic dyes encapsulated within the membrane bilayer or aqueous core, respectively, as judged by changes in dye emission properties. The first assay we pursued utilized Nile red (NR), which is a hydrophobic dye that can mimic common non-polar drugs. NR is widely used in cell biology studies[42] since it only fluoresces in aqueous media when it is solubilized through encapsulation within membrane bilayers. Therefore, following triggered release into aqueous media, NR will no longer fluoresce due to precipitation,[43] and the resulting decrease in fluorescence intensity can be used to track release. We incorporated responsive lipids at varying percentages into liposomes primarily composed of bulk lipids including phosphatidylcholine (PC) and DOPE. Other lipid additives such as 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and phosphatidic acid (PA) were also included to probe their effects on liposome membrane stability before and after enzyme treatment. Unilamellar liposomes were prepared using standard thin-film hydration methods including lipid film preparation, hydration, freeze-thaw cycles and extrusion through 200 nm polycarbonate membranes. Dynamic light scattering (DLS) experiments were performed to verify the successful formation of the stable liposome vesicles, as will be later discussed.

During the course of these studies, we systematically varied lipid composition to analyze liposomes containing a wide range of percentages of different mixtures of PC, DOPE, DOTAP and PA. The results of these studies are summarized in Table S1. Many of these liposome formulations were found to not release contents or to not be sufficiently stable before enzyme treatment, indicating that the stability of the liposome before and after enzymatic release has to be carefully fine-tuned. Our initial success in esterase-responsive liposome release was achieved using liposomes composed of PC, DOPE, and DOTAP. DOTAP is a cationic lipid that is widely used in transfection,[44] and has been reported to render membranes more fusogenic, which is why we began introducing this compound into liposomes in an effort to induce cargo release.[13e] After screening a variety of different lipid mixtures, liposomes composed of 30% **ERL**, 50% DOPE, 10% PC and 10% DOTAP treated with 0.45 U of porcine liver esterase and incubated in a 30 °C water bath in between measurements

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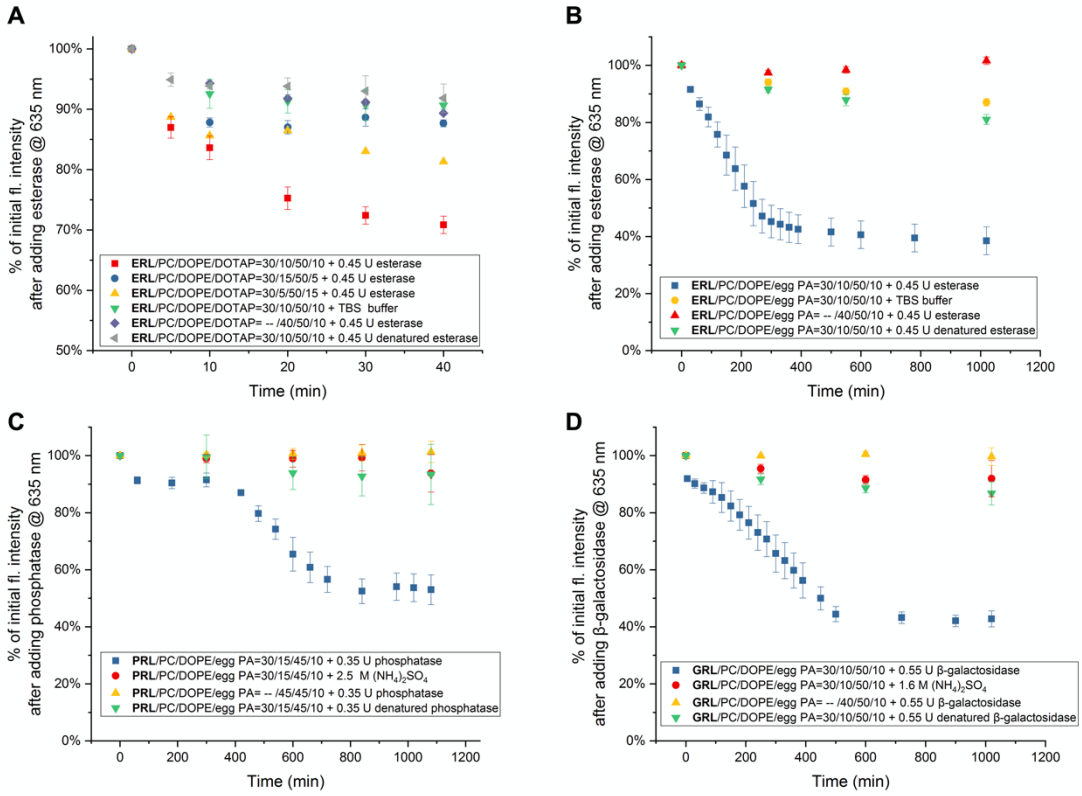

Figure 1. Release of NR over time from different formulations of enzyme-responsive liposomes. **A.** Data for **ERL** liposomes composed of 30% **ERL**, 50% DOPE and varying percentage of PC or DOTAP addition. **B.** Data for **ERL**-containing liposomes containing PA. A 30/10/50/10 ratio of **ERL**/PC/DOPE/PA yielded ~60% release over time upon esterase treatment. **C.** Data for **PRL**-containing liposomes. A 30/15/45/10 ratio of **PRL**/PC/DOPE/PA gave ~50% release overtime upon phosphatase treatment. **D.** Data for **GRL**-containing liposomes. A 30/10/50/10 ratio of **GRL**/PC/DOPE/PA resulted in ~60% release overtime upon galactosidase treatment. Control experiments involving treatment with buffer or heat-denatured enzyme, along with removal of responsive lipid, did not show significant fluorescent decrease in all cases. Error bars indicate standard errors from at least three studies.

showed an ~30% decrease in fluorescent intensity upon esterase treatment within 40 minutes (Figure 1A and Table S1, Entry 1). We have previously found that heating of liposomes can be necessary to achieve effects such as liposome fusion.[45] Different control sets were also tested using the exact same liposome formulation by treating with only buffer or with heat-denatured enzyme, leading to diminished fluorescence changes (background release ranging from ~8-12%, Figure 1A). Similar results were also observed for liposomes lacking **ERL** in which this lipid was replaced by PC. To drive home the sensitive nature of liposome composition, we will note that liposomes containing 30% **ERL**, 50% DOPE, 15% PC and 5% DOTAP showed almost the same fluorescent intensity decrease (~10%) as the control sets, indicating that trading only 5% of DOTAP for PC was sufficient to stabilize the membrane and shut down release of encapsulated dye. On the other hand, liposomes containing 30% **ERL**, 50% DOPE, 5% PC and 15% DOTAP only showed ~15% release. In this way, the liposome composition needed to be carefully fine-tuned in order to optimize release properties.

The sensitive nature of the DOTAP liposomes led us to next explore another lipid additive, PA. PA is an ionic lipid that is known to exaggerate the non-bilayer properties of DOPE within membranes.[46] After evaluating different lipid percentages (Table S1, Entries 15-17), we identified that the formulation in which DOTAP was simply replaced by PA showed significantly enhanced activity (30% **ERL**, 10% PC, 50% DOPE and 10% egg PA). NR release curves for these experiments are shown in Figure 1B. Upon treatment with esterase and heating at 30 °C in between measurements, the fluorescence intensity gradually decreased by ~60%, although this process took a significantly longer time (approximately five hours) to run to completion. Control experiments were also done as before, either treating the exact same liposome solution with buffer or heat denatured esterase or by treating liposomes in which **ERL** was replaced by PC with esterase. All of these, again, showed only minimal background release. We will also note that the percent change in fluorescence may not indicate the total percentage of dye release, for example since the released hydrophobic dye may be re-encapsulated into other lipid assembly structures after being initially released. These results indicate that **ERL** enables effective enzyme-responsive liposome properties for the release of hydrophobic cargo.

To pursue our goal of developing a general strategy that could be adapted to target different enzymes, we next evaluated triggered release conditions driven by **PRL** and **GRL**. For the former, we again tested different formulations (Table S2) and arrived at an effective composition of 30% **PRL**, 15% PC, 45% DOPE and 10% PA. After adding 0.35 U alkaline phosphatase from *Escherichia coli* and incubating at 30 °C in between measurements, a fluorescence intensity decrease of ~50% was observed after about thirteen hours, as is shown Figure 1C. Interestingly, this release curve appeared to exhibit an induction

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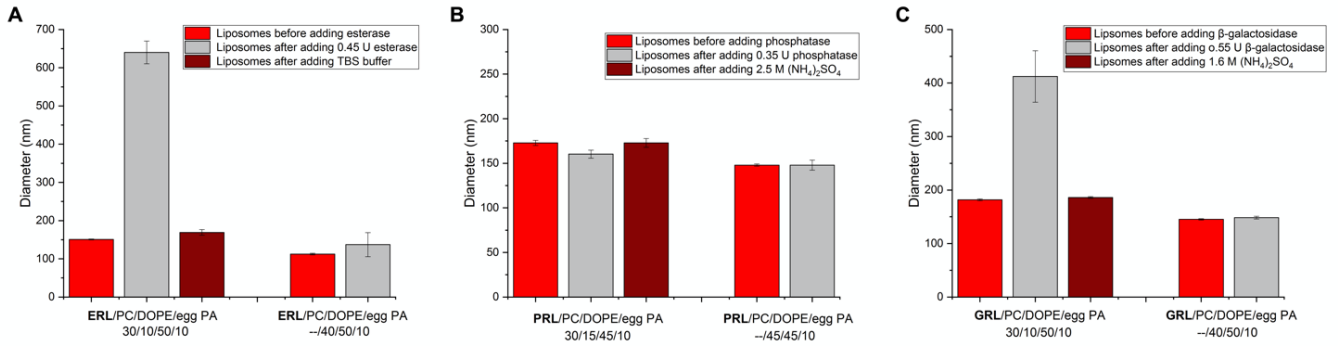

Figure 2. DLS results from enzyme-responsive liposomes used in NR release experiments. Data for **ERL**-containing liposomes (**A**) and **GRL**-containing liposomes (**C**) yielded significant increases in average particle sizes upon esterase or β-galactosidase treatment, respectively. For **PRL**-containing liposomes (**B**), minimal changes were observed under any conditions. Control experiments in all three cases did not show any significant change. Error bars indicate standard errors from at least three studies.

period of approximately 5 hours before a steeper drop in fluorescence intensity. This result was only observed with **PRL**, and since this is the only anionic enzyme-responsive lipid tested, it is possible that the altered charge of the membrane affects enzymatic modification and/or release properties. Also, as mentioned above, a key point we encountered with each of these responsive systems is that initial and final stability of the membrane must be carefully tuned to maximize release. In this case, this was done by dropping the percentage of DOPE from 50% for **ERL** to 45% for **PRL**, which was perhaps needed to compensate for the introduction of the negatively charged **PRL** lipid. **GRL** exhibited release properties that were quite similar to **ERL**, despite being composed of entirely different structural attributes (altered enzymatic substrate, SIL and lipid scaffold that is released). Again, different formulations were screened (Table S3), and a 30/10/50/10 ratio of **GRL**/PC/DOPE/PA resulted in an ~60% decrease in fluorescent intensity within nine hours upon addition of β-galactosidase from *Escherichia coli* (Figure 1D). For both **PRL and GRL**, multiple control experiments were again carried out. Liposomes containing responsive lipids didn't show significant release upon treatment with either buffer or heat-denatured enzyme addition. Similar results were again observed for liposomes in which responsive lipids were replaced by PC and treated with enzyme. These dye release results showcase that these enzyme-responsive liposome systems are effective for controlling the release of encapsulated contents in a manner driven by enzyme treatment. These also indicate our design indeed provides a general strategy for targeting different enzymes by simply exchanging the enzymatic substrate trigger incorporated into the head groups of enzyme-responsive lipid structures. The stabilities of liposomes formed by incorporating each of these three responsive lipids as well as PA in the formulation were separately tested via tracking encapsulated Nile red fluorescence over time (Figure S3). All of them were stable for at least three days when being kept at 4 °C, which is common of stabilized liposomal nanoparticles.

In order to understand potential structural changes to self-assembled lipid structures caused by the introduction of enzymatic stimuli, we next performed DLS studies using each system. For these experiments, liposome formulations that yielded optimal NR release results in the prior studies were analyzed by DLS before and after enzyme treatment, with results shown in Figure 2. In all cases, the original liposome samples prior to enzyme treatment showed uniform vesicle sizes with average diameters ranging from 150 nm – 200 nm, which is expected for particles prepared via extrusion by passing through membranes of 200 nm. This indicates that these formulations containing responsive lipids form stable liposomes. As can be seen in Figure 2A as well as Figure S4, incubation of **ERL** containing liposomes with esterase resulted in a dramatic increase in average particle size. In the corresponding control experiments, liposomes containing **ERL** but instead treated with TBS buffer or liposomes without **ERL** subjected to esterase only showed minimal change. These results are in line with previous stimuli-responsive liposomes we have developed,[40b,47] and indicate that liposomes underwent structural changes upon esterase treatment. Possible explanations include that lipids adopt different assembly properties after enzyme modification such as the inverted hexagonal phase, which is favored by DOPE at physiological conditions.[36] Considering the complicated lipid composition, another reasonable explanation would be that after enzyme treatment, the membrane was destabilized, ultimately leading to fusion and concomitant release of encapsulated cargo.

Liposomes containing **GRL** yielded comparable increases in average particle sizes after adding β-galactosidase and null results in controls (Figure 2C and Figure S5), suggesting that this system underwent alterations to lipid self-assembly properties that were similar to **ERL** liposomes. However, somewhat surprisingly, **PRL** liposomes did not yield any significant size changes in any of these experiments, suggesting that **PRL** containing liposomes did not undergo significant changes in lipid assembly properties (Figure 2B and Figure S6). While **PRL** liposomes did exhibit NR release, the response curve was unique from the others since it showed an induction period and it ultimately yielded less of a decrease in percentage of fluorescence. Again, the most significant difference in this compound is the presence of the charged phosphate group, which may impact either liposome membrane packing or the interaction of phosphatase enzyme with the liposomes. Nevertheless, we have previously observed similar results in which a light-triggered release liposomal platform yielded significant NR released but no size changes were detected via DLS.[14d]

A benefit of the unique structure of liposomes is that they are quite versatile in being able to encapsulate both hydrophobic cargo within the membrane bilayer but also hydrophilic contents within the aqueous core. The ability of such liposomal platforms to deliver hydrophilic molecules is also important for polar therapeutics such as siRNA. However, this is usually more challenging as this requires polar contents to escape through the hydrophobic bilayer region to ultimately achieve release. As a

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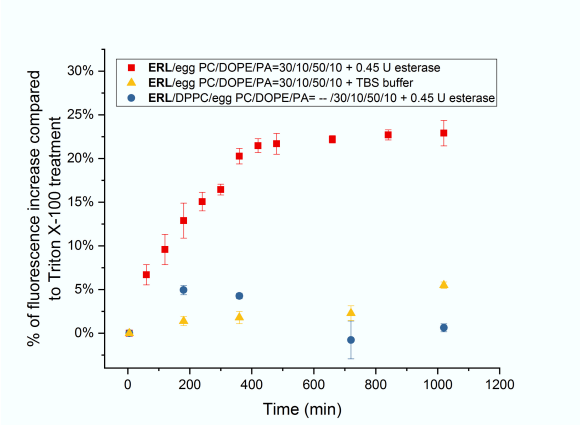

Figure 3. SRB release data over time upon esterase addition to liposomes containing 30% **ERL**, 50% DOPE, 10% PC and 10% PA or control experiments of buffer treatment or liposomes lacking **ERL**. Only liposomes containing **ERL** with esterase treatment showed significant fluorescence increases. Error bars indicate standard errors from at least three studies.

result, significant membrane disruption is needed to induce hydrophilic cargo release. In our studies, we selected Sulforhodamine B (SRB) to evaluate polar dye release because it is a water soluble fluorescent dye commonly used in release[10b] and cytotoxicity assays.[48] The concentration of SRB used for encapsulation was carefully chosen to make sure it is high enough for fluorescence to be self-quenched due to collisional effects when entrapped within liposomes. Therefore, when the dye is released from liposomes, it will be diluted leading to a fluorescence turn-on effect. When preparing these liposomes, similar methods were used that were modified by the addition of purification through a size exclusion column (SEC), which is needed after extrusion to remove unencapsulated dye. The successful preparation of liposomes was again determined by DLS.

To assess the hydrophilic cargo release properties of esterase-responsive liposomes, SRB was encapsulated within liposomes containing 30% **ERL**, 10% PC, 50% DOPE and 10% PA as well as control liposomes containing 30% DPPC, 10% egg PC, 50% DOPE and 10% PA. After treating with esterase and incubating in a 30 °C water bath for 17 hours when not performing measurements, the detergent triton X-100 was added into the liposome solution as a measure of the total release of encapsulated contents. The normalized results are reported for each point as a percentage of fluorescent increase compared to Triton X-100 treatment. As is shown in Figure 3, liposomes containing **ERL** exhibited a significant increase in fluorescence compared to control experiments in which liposomes containing **ERL** were instead treated with buffer or liposomes without **ERL** were subjected to enzyme, which both showed minimal fluorescence change. These results showcase that enzyme-responsive systems are also capable of releasing hydrophilic contents.

Conclusion

In conclusion, we have designed and synthesized three enzyme-responsive lipids targeting unique enzyme families that are commonly aberrant in disease, including esterase, phosphatase and β-galactosidase. These lipids shared similar design strategies including an enzyme substrate/trigger at the head group, a self-immolating linker and a non-bilayer forming lipid scaffold. These novel lipids were incorporated into liposomes, and after careful fine-tuning of membrane stability based on composition, each compound was found to be successful for achieving triggered release of the hydrophobic dye NR upon treatment with the appropriate enzyme. These results showcase that this approach provides a general strategy for enzyme-responsive liposomes in which different enzymes can be targeted by modifying the substrate trigger displayed at the lipid headgroup. However, despite the generality of this approach, there were differences observed in release properties based on structural nuances. In particular, **PRL** was found to exhibit NR release curves (less release and induction period) and DLS results (no observed change) that were different from **ERL** and **GRL**, despite the fact that the structures of **ERL** and **PRL** only differ by one functional group, and indeed they are expected to produce the same lipid product upon release. This exception indicates that response properties can vary based on changes to the structures, in this case most likely due to the charge of the resulting enzyme-responsive liposomes. Finally, **ERL**-containing liposomes also showed ability to release hydrophilic cargo. Enzymes provide exciting targets for controlling release of encapsulated drugs from nanocarriers using stimuli-responsive materials since these are commonly overexpressed in many diseases. However, the existence of numerous families of enzymes that catalyze wide-ranging reactions by which varying substrates are modified provides a grand challenge for exploring different potential therapeutic targets. The modular strategy reported herein provides an efficient approach to overcoming this barrier as an initial step for advancing enzyme-responsive liposomal therapeutics.

Experimental Section

General experimental

Reagents and solvents were generally purchased from Acros, Sigma-Aldrich, or Fisher Scientific and used without further purification. PC (L-α-Phosphatidylcholine, mixed isomers from chicken eggs), PA (L-α-phosphatidic acid sodium salt from chicken eggs), DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine), DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) and DOTAP (1,2-dioleoyl-3-trimethylammonium-propane, chloride salt) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Esterase from porcine liver was purchased from Sigma-Aldrich (lyophilized powder, ≥15 units/mg solid, SKU: E3019). β-Galactosidase from *Escherichia coli* was purchased from Abnova (in 1.6 M ammonium sulfate, Catalog #: P5270). Alkaline phosphatase from *Escherichia coli* was purchased from Sigma-Aldrich (in 2.5 M ammonium sulfate, SKU: P4252). β-Galactosidase substrate, 4-nitrophenyl β-D-galactopyranoside was purchased from Carbosynth. Phosphatase substrate, 4-nitrophenyl phosphate disodium salt hexahydrate (pNPP) was purchased from Sigma-Aldrich (5 mg tablet, SKU S0942). Compounds **4**,[38] **11**,[39] and **13**[40] were synthesized as previously reported with matching characterization data. Dry solvents were obtained from a Pure Solv MD-7 solvent purification system purchased from Innovative Technology, Inc (Newburyport, MA). Column chromatography was performed using 230-400 mesh silica gel purchased from Sorbent Technologies. NMR spectra were obtained using Varian 300 MHz, 500 MHz or 600 MHz spectrometers. Mass spectra were obtained with JEOL DART-AccuTOF and Waters Synapt G2-Si mass spectrometers (Milford, MA). Liposome extruder and polycarbonate membranes were obtained from Avestin (Ottawa, Canada). Ultrapure water was purified via a Millipore water system (≥ 18 MW·cm triple water purification system). Small quantities (< 5 mg) were weighed on an OHRUS analytical-grade mass balance. Fluorescence studies were

performed using a Cary Eclipse Fluorescence Spectrophotometer from Agilent Technologies. Plots were generated using Origin Pro 2018. All error bars in plots show the standard errors of at least three experimental replicates.

Synthesis

ERL. DOPE (100 mg, 0.1344 mmol), Compound **1** (42.63 mg, 0.1613 mmol) and hydroxybenzotriazole (HOBt, 24.7 mg, 0.1613 mmol) were added into a small vial under N_2. Then, 1 mL dry DMF was added into the vial and the reaction mixture was cooled to 0 °C. After being stirred for 5 min, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI, 30.92 mg, 0.1613 mmol) and *N*,*N*-Diisopropylethylamine (DIEA, 55 µL, 0.336 mmol) were added. The reaction was then stirred at rt for 5 h before being quenched by pouring into 100 mL 1 M HCl. The aqueous layer was extracted three times with 25 mL chloroform. The combined organic layer was washed with 20 mL water five times and once with 20 mL brine. After being dried over Na_2SO_4, filtered and concentrated, the crude was subjected to column chromatography using gradient elution from 100% chloroform to 20% methanol-chloroform, which yielded **ERL** as a colorless oil (123 mg, 0.124 mmol, 92 % yield). Rf=0.48 (10% methanol-chloroform). ^{1}H NMR (300 MHz, Chloroform-*d*) δ 6.80 (s, 1H), 6.55 (d, *J* = 2.0 Hz, 1H), 5.37 – 5.29 (m, 4H), 5.20 (s, 1H), 4.36 (d, *J* = 11.8 Hz, 1H), 4.11 (dd, *J* = 12.2, 7.2 Hz, 1H), 3.89 (s, 2H), 3.68 (s, 2H), 3.33 (d, *J* = 10.6 Hz, 2H), 2.52 (d, *J* = 25.0 Hz, 5H), 2.33 – 2.23 (m, 8H), 2.21 (s, 3H), 2.00 (q, *J* = 6.6 Hz, 8H), 1.56 (s, 10H), 1.37 – 1.20 (m, 46H), 0.91 – 0.84 (m, 6H). ^{13}C NMR (75 MHz, cdcl$_3$) δ 173.70, 173.32, 171.29, 149.50, 138.23, 136.49, 133.45, 132.50, 129.89, 129.51, 129.49, 123.11, 70.24, 63.62, 62.48, 39.69, 39.39, 34.06, 33.91, 31.76, 31.28, 29.60, 29.37, 29.17, 29.01, 27.06, 27.03, 24.75, 22.52, 13.84. ^{31}P NMR (121 MHz, cdcl$_3$) δ 1.46. ESI-MS: $[M-H]^-$ calcd for $C_{56}H_{95}NO_{11}P$, 988.6643, found 988.6609.

Dibenzyl (2-(4-((*tert*-butyldimethylsilyl)oxy)-2-methylbutan-2-yl)-3,5-dimethylphenyl) phosphate (5)

Compound **4** (0.1 g, 0.31 mmol) was dissolved in 4 mL dry DCM under nitrogen followed by addition of tetrazole (2.1 mL, 0.93 mmol, 0.45 M in MeCN). The reaction mixture was cooled down to 0 °C and then dibenzyl *N*,*N*-diisopropylphosphoramidite (0.155 mL, 0.456 mmol) was added. After the reaction was allowed to warm up to rt and further stirred for 1.5 h, it was brought back to 0 °C again. *m*-CPBA (0.281 g, 0.93 mmol, 57% purity) was added and the reaction was stirred for another 1.5 h. After completion, the reaction was quenched by adding 100 mL saturated $NaHCO_3$ and the aqueous phase was extracted three times with 25 mL $CHCl_3$. The combined organic layer was then washed with 50 mL water, 50 mL brine and dried over Na_2SO_4. After being filtered and concentrated under reduced pressure, the crude was purified through column chromatography using gradient elution from hexane to 20% EtOAc-hexane. Compound **5** was obtained as a yellow oil. (0.1714 g, 0.3 mmol, 95% yield). Rf=0.24 (10% EtOAc-hexane). ^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.43 – 7.26 (m, 10H), 7.11 (d, *J* = 2.1 Hz, 1H), 6.75 – 6.69 (m, 1H), 5.12 (d, *J* = 8.2 Hz, 4H), 3.49 (dd, *J* = 7.9, 6.9 Hz, 2H), 2.50 (s, 3H), 2.18 (s, 3H), 2.13 – 2.05 (m, 2H), 1.53 (d, *J* = 0.8 Hz, 6H), 0.84 (d, *J* = 0.8 Hz, 9H), -0.04 (d, *J* = 0.8 Hz, 6H). ^{13}C NMR (75 MHz, cdcl$_3$) δ 150.44, 150.35, 138.66, 136.21, 135.82, 135.73, 132.86, 132.75, 131.09, 128.62, 128.59, 128.04, 118.95, 118.93, 69.80, 69.73, 61.11, 45.91, 39.63, 32.24, 26.05, 25.69, 20.42, 18.31, -5.22. ^{31}P NMR (121 MHz, cdcl$_3$) δ -6.98. HRMS-DART: $[M+H]^+$ calcd for $C_{33}H_{48}O_5PSi$: 583.3009, found: 583.2745.

3-(2-((bis(benzyloxy)phosphoryl)oxy)-4,6-dimethylphenyl)-3-methylbutanoic acid (6)

Compound **5** (0.43 g, 0.74 mmol) was dissolved with 4 mL acetone in a 50 mL RBF followed by addition of KF (47.34 mg, 0.815 mmol) and stirred briefly before being cooled down to 0 °C. Jones reagent (~1 mL, containing 1 mL H_2O, 0.23 mL concentrated H_2SO_4 and 0.27 g CrO_3) was then added dropwise to obtain a clear orange solution. The reaction was allowed to warm up to rt and further stirred for 3 h. After this time, the reaction mixture was filtered through a column packed with florisil and the filtrate was concentrated under reduced pressure. The crude was loaded onto silica gel and subjected to column chromatography. Gradient elution from DCM to 5% MeOH-DCM containing 1 drop of acetic acid was used to obtain **6** as a yellow oil. (0.31 g, 0.64 mmol, 87% yield). Rf=0.4 (5% MeOH-DCM)/0.625 (50% EtOAc-hexane). ^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.31 (t, *J* = 1.8 Hz, 10H), 7.05 (d, *J* = 1.9 Hz, 1H), 6.72 – 6.68 (m, 1H), 5.12 (s, 2H), 5.09 (s, 2H), 2.89 (s, 2H), 2.50 (s, 3H), 2.13 (s, 3H), 1.59 (s, 6H). ^{13}C NMR (126 MHz, cdcl$_3$) δ 150.13, 150.08, 138.72, 136.68, 135.53, 135.48, 132.08, 132.02, 131.56, 128.77, 128.71, 128.18, 128.17, 119.13, 119.11, 70.17, 70.12, 47.50, 39.39, 31.79, 29.84, 25.69, 20.43. ^{31}P NMR (202 MHz, cdcl$_3$) δ -6.77. HRMS-DART: $[M+H]^+$ calcd for $C_{27}H_{32}O_6P$, 483.1936, found 483.1644

(2*R*)-3-(((2-(3-(2-((bis(benzyloxy)phosphoryl)oxy)-4,6-dimethylphenyl)-3-methylbutanamido)ethoxy)(hydroxy)phosphoryl)oxy)propane-1,2-diyl dioleate (7)

DOPE (29.9 mg, 0.04 mmol) and HOBt (7.35 mg, 0.048 mmol) were dissolved in 0.8 mL of dry DMF containing **6** (23.16 mg, 0.048 mmol) in a small vial. The reaction was cooled down to 0 °C and stirred for 5 min followed by addition of EDCI (9.2 mg, 0.048 mmol) and DIEA (17 µL, 0.1 mmol). The mixture was stirred overnight before being quenched by pouring into 100 mL 1 M HCl. The aqueous layer was extracted three times with 20 mL chloroform. The combined organic layer was washed five times with 50 mL water, once with 50 mL brine, dried with Na_2SO_4, filtered and concentrated under reduced pressure. Column chromatography using gradient elution from chloroform to 10% MeOH-chloroform was needed to obtain **7** as a yellow oil. (37.22 mg, 0.03 mmol, 77% yield). Rf=0.18 (10% MeOH-chloroform). ^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.27 (s, 11H), 6.84 (s, 1H), 6.65 (s, 1H), 5.38 – 5.29 (m, 5H), 5.14 (d, *J* = 8.4 Hz, 6H), 4.29 (d, *J* = 12.1 Hz, 1H), 4.03 (s, 1H), 3.88 (s, 2H), 3.77 – 3.59 (m, 2H), 3.29 (s, 2H), 2.70 (s, 2H), 2.47 (s, 4H), 2.26 – 2.10 (m, 5H), 2.09 – 1.92 (m, 14H), 1.27 (h, *J* = 9.3, 8.3 Hz, 57H), 0.86 (d, *J* = 6.9 Hz, 6H). ^{31}P NMR (121 MHz, cdcl$_3$) δ -3.98, -9.02.

PRL

Compound **7** (34.4 mg, 0.0285 mmol) was dissolved in 1 mL dry DCM in a small vial at 0 °C. TMS-I (10.2 µL, 0.0712 mmol) was then added. The reaction was stirred for 40 min followed by removal of the solvent under reduced pressure. After further drying under high vacuum for 30 min, 2 mL of MeOH/H_2O (95/5, v/v) was added into the vial and the reaction was stirred for another one hour. Upon removal of the solvent, the crude was subjected to column chromatography. Gradient elution from chloroform to 30% methanol-chloroform and final elution with MeOH/chloroform/H_2O (25/65/4, v/v) was performed to purify **PRL** as a yellow oil. (17.6 mg, 0.0171 mmol, 60% yield). ^{1}H NMR (300 MHz, 20% CD_3OD-$CDCl_3$) δ 6.42 (m, 2H), 5.21 – 5.17 (m, 4H), 5.01 (s, 1H), 4.19 (d, J = 11.5 Hz, 2H), 3.72 (d, J = 33.7 Hz, 6H), 2.29 (s, 4H), 2.12 (t, J = 15.6 Hz, 8H), 1.86 (d, J = 6.2 Hz, 8H), 1.44 (s, 4H), 1.13 (t, J = 6.0 Hz, 46H), 0.75 – 0.69 (m, 6H). ^{31}P NMR (121 MHz, 20% CD_3OD-$CDCl_3$) δ 0.63, -4.55. ESI-MS: $[M-H]^-$ calcd for $C_{54}H_{94}NO_{13}P_2$, 1026.6200, found 1026.6173. $[M-2H]^{2-}$ calcd for $C_{54}H_{93}NO_{13}P_2$, 512.8061, found 512.8077. $[M+I]^-$ calcd for $C_{54}H_{95}NO_{13}P_2I$, 1154.5323, found 1154.5304.

(2*R*,3*S*,4*S*,5*R*,6*S*)-2-(acetoxymethyl)-6-(4-((((2,3-bis(hexadecyloxy)propyl)carbamoyl)oxy)methyl)phenoxy)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (12)

In a 50 mL RBF, **11** (172 mg, 0.278 mmol), and **13** (125 mg, 0.23 mmol) were added under nitrogen and the flask was cooled down to 0 °C in an ice bath. 2 mL DMF containing triethylamine (96.2 µL, 0.69 mmol) was then added into the flask. After overnight stirring, the reaction was quenched by pouring into 100 mL water. The aqueous layer was extracted three times with 25 mL chloroform. After washing the combined organic layer with 50 mL water five times, and 50 mL brine once, the resulting

solution was dried over Na_2SO_4, filtered and concentrated under vacuum, the crude was purified by column chromatography using gradient elution from 10% EtOAc-hexane to 40% EtOAc-hexane to yield **12** as a white solid. (174 mg, 0.17 mmol, 74% yield). Rf=0.7 (50% EtOAc-hexane). ^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.34 – 7.28 (m, 2H), 7.04 – 6.92 (m, 2H), 5.55 – 5.41 (m, 2H), 5.16 – 4.98 (m, 5H), 4.28 – 3.99 (m, 3H), 3.60 – 3.36 (m, 8H), 3.29 – 3.17 (m, 1H), 2.18 (s, 3H), 2.06 (s, 6H), 2.01 (s, 3H), 1.54 (t, *J* = 7.3 Hz, 4H), 1.25 (s, 56H), 0.93 – 0.84 (m, 6H). ^{13}C NMR (75 MHz, cdcl$_3$) δ 170.46, 170.37, 170.25, 169.49, 156.95, 156.56, 131.81, 129.94, 117.06, 99.81, 71.96, 71.29, 71.20, 70.96, 70.41, 68.76, 67.02, 66.97, 66.27, 61.49, 42.60, 32.07, 30.18, 29.85, 29.81, 29.77, 29.63, 29.51, 26.24, 22.84, 20.87, 20.82, 20.74, 14.27. HRMS-DART: $[M+H]^+$ cacld for $C_{57}H_{98}O_{14}N$: 1020.6987, found: 1020.6404.

GRL

Compound **12** (20 mg, 0.0196 mmol) was dissolved in 2 mL MeOH in a large 4 dr vial at 0 °C. NaOMe (27 µL, 0.1176 mmol, 4.375 M in MeOH) was next added slowly. The reaction mixture immediately turned yellow and was stirred overnight before being neutralized with 1 N HCl. The solvent was evaporated, and the crude was purified via a column chromatography using gradient elution from 100% chloroform to 30% MeOH-chloroform. **GRL** was obtained as a white solid. (15 mg, 0.0176 mmol, 90% yield). Rf=0.2 (10% MeOH-chloroform). ^{1}H NMR (600 MHz, 20% CD_3OD-Chloroform-*d*) δ 7.12 (d, *J* = 7.9 Hz, 2H), 6.92 – 6.86 (m, 2H), 4.92 – 4.83 (m, 2H), 4.70 (dd, *J* = 7.7, 1.9 Hz, 1H), 3.80 (t, *J* = 2.6 Hz, 1H), 3.70 – 3.59 (m, 3H), 3.49 (t, *J* = 6.1 Hz, 1H), 3.46 – 3.38 (m, 2H), 3.38 – 3.20 (m, 7H), 3.05 (dd, *J* = 14.3, 6.1 Hz, 1H), 1.39 (q, *J* = 6.6 Hz, 4H), 1.09 (d, *J* = 2.0 Hz, 58H), 0.71 (td, *J* = 7.0, 2.0 Hz, 6H). ^{13}C NMR (126 MHz, 20% CD_3OD-Chloroform-*d*) δ 157.21, 156.88, 130.45, 129.43, 116.56, 101.18, 77.36, 76.97, 75.00, 73.33, 71.69, 70.88, 70.23, 68.53, 66.22, 61.07, 49.24, 49.13, 49.07, 48.96, 48.90, 48.78, 48.73, 48.69, 48.61, 48.56, 48.44, 48.27, 48.10, 41.93, 31.75, 29.79, 29.52, 29.50, 29.48, 29.46, 29.39, 29.30, 29.18, 25.90, 25.87, 22.49, 13.79. ESI-MS: $[M+H]^+$ cacld for $C_{49}H_{90}O_{10}N$: 852.6565, found 852.6561, $[M+NH_4]^+$ cacld for $C_{49}H_{93}O_{10}N_2$: 869.6830, found: 869.6847. $[M+Na]^+$ cacld for $C_{49}H_{89}O_{10}NNa$:874.6384, found: 874.6409.

Preparation of liposomes for Nile red (NR) release studies

Enzyme-responsive lipids, bulk lipids and NR stock solutions were prepared in either chloroform or MeOH/chloroform mix and stored in -20 °C freezer. Proper volumes of each stock solution were pipetted into a small vial to obtain the desired percentage of each lipid. NR was added as 5% of the total lipid content. The solvents were then evaporated under nitrogen stream and the resulting lipid film was further dried under vacuum for at least one hour. After that, the appropriate buffer or water was added into the vial. For **ERL** and **GRL** containing liposomes, 1×TBS (pH=8, containing 25 mM Tris/Tris HCl, 0.13 M NaCl, 0.0027 M KCl) was used. For **PRL** containing liposomes, 1×TBS with cation activators (pH=8, containing 25 mM Tris/Tris HCl, 0.13 M NaCl, 0.0027 M KCl, 0.25 mM $MgCl_2$ and 0.25 mM $ZnCl_2$) was used. The concentration of the total lipid content used in this release assay was 2 mM. The film was hydrated in a 60 °C water bath for 4 sets of 15 min with vortexing after each set, followed by ten freeze-thaw cycles using a dry ice-acetone bath and 60 °C water bath. Finally, the solutions were extruded through a 200 nm polycarbonate membrane for 15 passes with a LiposoFast extruder (Avestin, Inc.). The resulting liposomes were stored at 4 °C and were used up within two days.

Enzyme stock preparation

The esterase stock solution was prepared in 1×TBS (pH=8, containing 25 mM Tris/Tris HCl, 0.13 M NaCl, 0.0027 M KCl) at a concentration of 3 mg/mL. According to LOT bioactivity analysis and unit definition provided by Sigma-Aldrich, the unit concentration was ~45 U/mL. The stock solutions were divided into small aliquots in Eppendorf tubes and stored in a -20 °C freezer until their use. Alkaline phosphatase was used directly after purchasing. According to the LOT bioactivity analysis, the unit concentration was 350 U/mL. The solution was stored in a 4 °C fridge. β-Galactosidase was also used directly after purchasing. According to the LOT bioactivity analysis, the unit concentration was 550 U/mL. The solution was stored in 4 °C fridge. Denatured enzyme solutions were prepared by heating the above-mentioned stock solutions in a 60 °C water bath for one hour and then slowly cooling back down to room temperature.

Nile red release studies with enzyme addition

A 100 µL aliquot of the prepared 2 mM liposome solution was added into a sub-micro quartz cuvette. Enzyme was added directly into the cuvette (esterase: 10 µL enzyme stock (0.45 U), β-galactosidase: 1 µL enzyme stock (0.55 U), alkaline phosphatase: 1 µL enzyme stock (0.35 U)). The cuvettes were heated in a 30 °C water bath in between measurements. Buffer control sets were also done by switching enzyme solution into either TBS or ammonia sulfate buffer based on the buffers used for the different enzymes used in study. Fluorescence intensity was then measured over time (excitation wavelength=552 nm). For **GRL** and **ERL**, spectrometer settings were: excitation slit=5 nm, emission slit=5 nm. For **PRL**, these were: excitation slit=5 nm, emission slit=10 nm. When processing the data, fluorescence intensities at 635 nm were selected. Experiments were run at least 3 times, each with different batches of liposomes, and averaged data were reported with error bars showing standard error.

Preparation of liposomes for sulforhodamine B (SRB) studies

The same lipid stocks prepared for NR study were used. SRB sodium salt was dissolved with 1×TBS (pH=8, containing 25 mM Tris/Tris HCl, 0.10 M NaCl, 0.0027 M KCl) to produce a 20 mM solution. Proper volumes of each stock solution were pipetted into a small vial to obtain the desired percentage of each lipid. The solvents were evaporated under nitrogen stream and the resulting lipid film was further dried under vacuum for at least one hour. After that, the lipid film was hydrated with 20 mM SRB stock solution in a 60 °C water bath for 4 sets of 15 min with vortexing after each set, followed by ten freeze-thaw cycles in a dry ice-acetone bath and a 60 °C water bath. Then, the solutions were extruded through a 200 nm polycarbonate membrane for 15 passes with a LiposoFast extruder (Avestin, Inc.). Finally, the unencapsulated dye was removed with a size exclusion column (SEC). Sephadex G-50 used for SEC was pre-equilibrated with 1×TBS (pH=8, containing 25 mM Tris/Tris HCl, 0.13 M NaCl, 0.0027 M KCl) for at least one hour prior to use. A micro-column was used for separation and gravity elution was sufficient for separation. Fractions were collected every ~1mL, and the first fraction showing pink color was usually the liposome solution, which was further checked by adding Triton X-100 under a UV lamp, after which an increase in fluorescent intensity was observed denoting the release of encapsulated dye.

SRB release assays with esterase addition

A 100 µL aliquot of the prepared liposome solution was added into a sub-micro quartz cuvette. Esterase stock solution (10 µL, 3 mg/mL, 0.45 U) was added directly into the cuvette to a final concentration of 0.27 mg/mL. The cuvette was heated in a 30 °C water bath in between measurements. Buffer control sets were also performed by switching enzyme solution into TBS buffer and performing the same readings. Fluorescence intensity was then measured over time (excitation wavelength=550 nm, excitation slit=10 nm, emission slit=2.5 nm). After completion, 1 µL of an aqueous 20% Triton X-100 solution was added to trigger complete release. When processing the data, fluorescence intensities at 590 nm were selected and fluorescence increases were reported as a percentage of the fluorescence after triton X-100 treatment for each sample. Experiments were run at least 3 times each with different batches of liposomes, and averaged data were reported with error bars showing standard error.

DLS analysis of particle sizes before and after the enzyme treatment

DLS measurements were carried out with a Malvern Zetasizer Nano ZS instrument equipped with a 4.0 mW laser operating at λ=633 nm. Samples were prepared by diluting the liposome solutions before or after adding enzyme by 10x with the proper matching buffer. As an example, a 5 µL of the liposome solution was added into 45 µL of proper buffer in a micro cuvette for measurement. All samples were determined at a scattering angle of 173° at 25 °C. The reported data were the average of three tests with error bars showing standard error.

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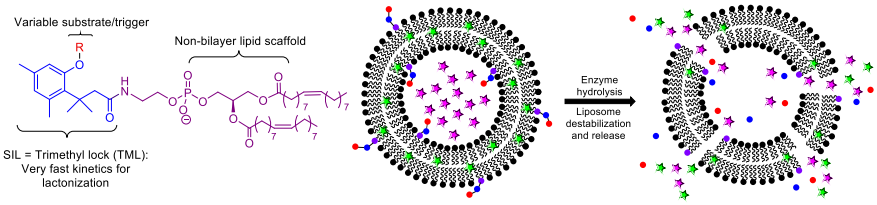

A modular approach is reported for the development of enzyme-responsive liposomes. These exploit synthetic lipid switches containing variable enzyme substrates that, when removed, yield decomposition of a self-immolating linker producing a non-bilayer lipid that perturbs the membrane and triggers release of contents. This approach enables the targeting of a range of enzymes that are overexpressed in diseased cells for drug delivery applications.

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