Strategies for Altering Lipid Self-assembly to Trigger Liposome Cargo Release

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Abstract

While liposomes have proven to be effective drug delivery nanocarriers, their therapeutic attributes could be improved through the development of clinically viable triggered release strategies in which encapsulated drug contents could be selectively released at the sites of diseased cells. As such, a significant amount of research has been reported involving the development of stimuli-responsive liposomes and a broad range of strategies have been explored for driving content release. These have included the introduction of trigger groups at either the lipid headgroup or within the acyl chains that alter lipid self-assembly properties of known lipids as well as the rational design of lipid analogs programed to undergo conformational changes induced by events such as binding interactions. This review article describes advances in the design of stimuli-responsive liposome strategies with an eye towards emerging trends in the field.

1. Introduction

The core attribute of lipid molecules that opens up imaginative possibilities is its ability to self-assemble into different supramolecular assemblies controlled by lipid structure and shape as well as conditions in the local environment. This is driven by the fundamental amphiphilic nature of lipids, in which incompatible polar and non-polar regions are driven to align with neighboring molecules in order to form favorable interactions (Cullis and Kruijff, 1979; Gruner et al., 1985; Israelachvili et al., 1980). To expand beyond this premise, the myriad shapes, properties, and functionalities of lipids enables interconversion between different lipid assemblages if one can alter lipid structure in a controlled manner.

1a. Liposome properties and applications

One of the most ubiquitous lipid assemblies is the liposome, which consists of a spherical superstructure containing an outer membrane bilayer surrounding an interior aqueous core

(Bangham and Horne, 1964; Bangham et al., 1965). Liposomes have been widely studied and applied for drug delivery applications due to their ability to encapsulate and deliver a wide range of therapeutic cargo (Gregoriadis, 1973; Gregoriadis and Florence, 1993; Gregoriadis and Ryman, 1971). Indeed, a number of liposomal formulations have been approved for clinical use (Barenholz, 2012; Bobo et al., 2016; Caracciolo, 2018), and crucial advancements have been made to improve characteristics including encapsulation efficiency (Alino et al., 1990; Gould-Fogerite and Mannino, 1985), circulation time (Gabizon et al., 1994; Gabizon and Papahadjopoulos, 1988; Klibanov et al., 1990), selectivity for diseased cells (Briones et al., 2008; Ghosh et al., 1982), cell entry (Qiu et al., 1998; Saesoo et al., 2016; Simões et al., 2005), and the ultimate subcellular localization of encapsulated cargo (Mustata et al., 2009; Pollock et al., 2010). However, in addition to drug delivery, liposomes are beneficial for applications including actions as sensors (Barba-Bon et al., 2019; Liu and Boyd, 2013; Mazur et al., 2017) and for enhancing catalysis by entrapping reagents (Cuccovia and Chaimovich, 2018; Song et al., 2006). The advancement of triggered release strategies has focused on the development of liposomes that respond to stimuli that are relevant to disease (Alam et al., 2017). While we won't categorize prior work based on this aspect in this review, we will point out that these approaches have typically fallen into two categories. Passive release exploits pathophysiological conditions such as increased acidity (Paliwal et al., 2015), reducing environment (McCarley, 2012), enzyme overexpression (Fouladi et al., 2017) or metabolite concentration associated with diseased cells (Lou et al., 2018; Lou et al., 2019; Zhang et al., 2018b). Active release strategies utilize external stimuli such as light (Leung and Romanowski, 2012; Puri, 2014; Shum et al., 2001), heat (Dicheva and Koning, 2014; Kneidl et al., 2014), and ultrasound (Boissenot et al., 2016; Sirsi and Borden, 2014) to drive release.

In general, a critical element needed to harness the potential of lipid membranes is to control the transport of materials across the bilayer, which in the case of liposomes and cells can entail

either the entry of exogenous material or the exodus of entrapped cargo. This is often accomplished in biological systems through the actions of complex channels and pumps that dictate inner- and extra-membrane composition (Gouaux and MacKinnon, 2005; Läuger, 1985). However, a simpler means to exert control over membrane transport, and particularly efflux in synthetic liposomes, involves the manipulation of lipid structure to dictate self-assembly and permeability properties. In particular, this can be exploited to trigger the release of contents entrapped within liposomes for therapeutic and theranostic applications (Figure 1). This approach requires the careful fine-tuning of lipid structure to control self-assembly properties before and after an external stimulus is introduced to alter membrane packing.

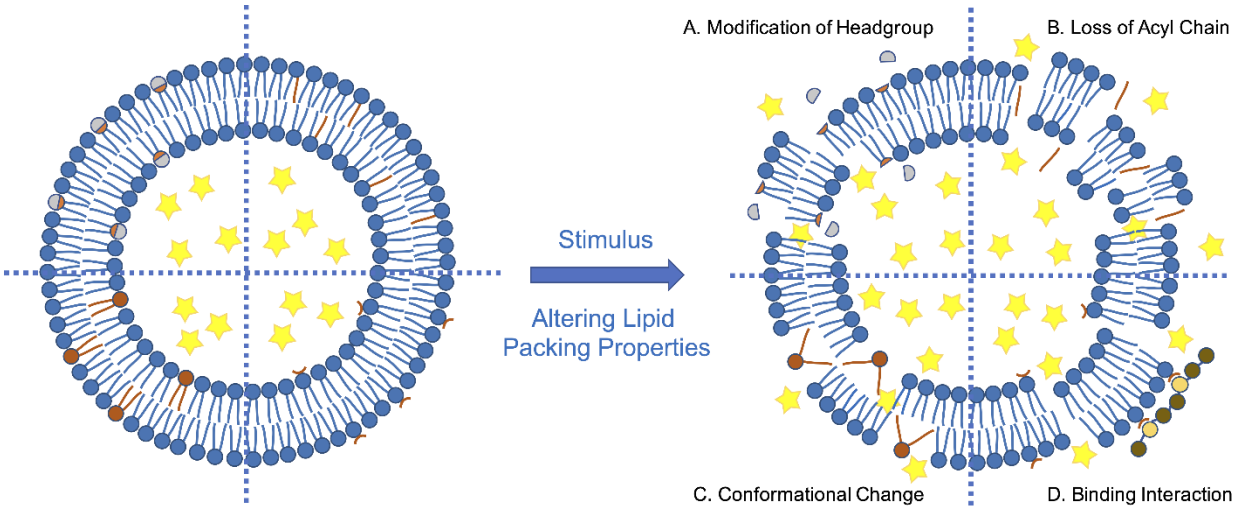


Figure 1. Triggered release of contents from liposomes by manipulating lipid structures and membrane properties. Cartoon illustration of common strategies reviewed herein, including **A.** modification of headgroup; **B.** loss of acyl chain; **C.** conformational change, and **D.** binding interaction.

1b. Lipid packing parameters and their relevance to liposome triggered release.

Fortuitously, pioneering work in understanding lipid packing parameters provides a head start for these endeavors, as will be briefly summarized herein. The preference of lipids for forming different self-assemblages can be understood based on the 3-dimensional structure of each particular structure (Israelachvili et al., 1977), as is shown in Figure 2. This is a function of the size of the lipid head group relative to the area occupied by the lipid acyl chains, with self-assembly characteristics dictated by the associated curvature (Tanford, 1974). Lipids that organize into membrane bilayers such as liposomes typically possess either cylindrical structures or slightly truncated cone structures. Particularly for spherical liposome structures, this is because such lipids must tolerate both the positive curvature of the outer leaflet and the negative curvature of the inner leaflet of the liposomal membrane, and thus a cone-shaped lipid of either curvature is not favorable. A prominent example is the lipid phosphatidylcholine (PC), for which the ability to form highly stable membrane bilayers likely correlates with this molecule being the most abundant lipid in eukaryotic membranes (Janmey and Kinnunen, 2006).

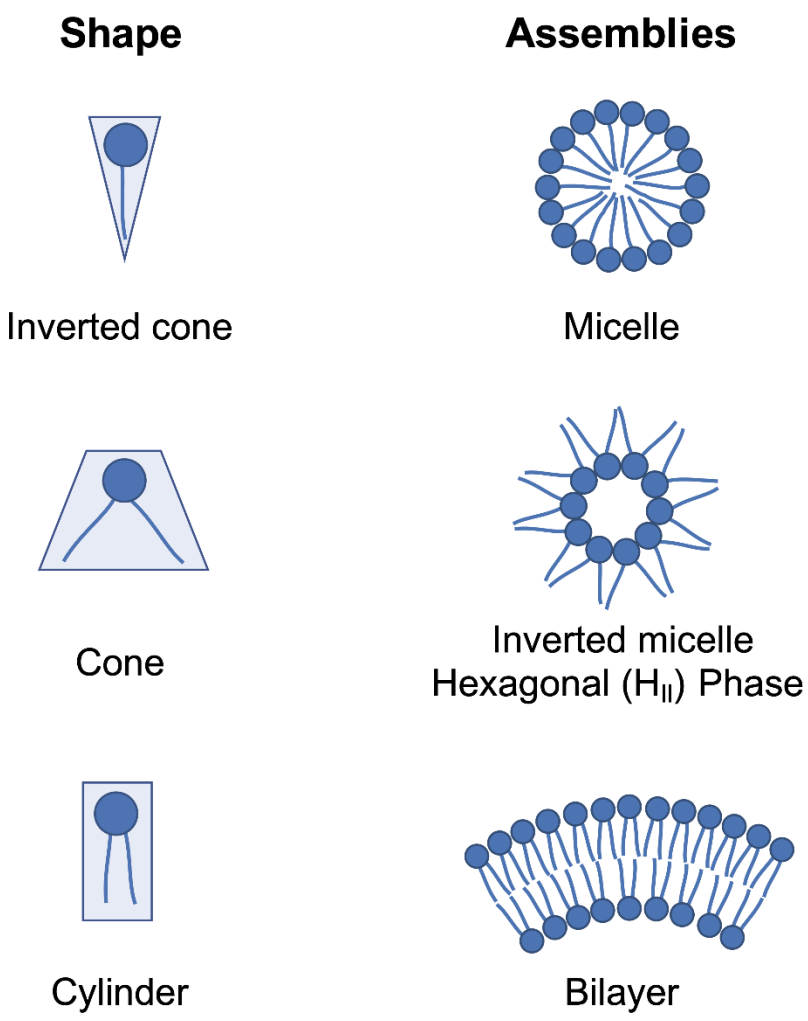


Figure 2. Self-assembly properties of lipids driven by their molecular shapes. Inverted cone-shaped lipids with positive curvature tend to form micelles. Cone-shaped lipids assemble into inverted micelles or hexagonal (H_{II}) phase, while cylindric lipids favor a lamellar phase.

Lipids possessing cone shapes of positive curvature, such as *lyso*-lipids containing only one acyl chain (i.e. *lyso*-phosphatidylcholine (LPC)), prefer to form micelles. In this case, the presence of a monolayer matches the tight curvature of the micellar structure. Lipids that exhibit cone-shaped geometries of negative curvature, such as different isomers of phosphatidylethanolamine (PE) including dioleoylphosphatidylethanolamine (DOPE), instead prefer to form inverted

micelles. However, due to the presentation of the hydrophobic chains on the periphery of these structures, they tend to self-assemble in aqueous solution into the inverted hexagonal (H_{II}) phase (Cullis and Kruijff, 1979). The self-assembly properties of these lipids are particularly affected by the extent of unsaturation in the lipid acyl chains since the *cis* double bonds present in natural lipids provide a kink in the structure that expands the area of the lipid acyl chains (Szule et al., 2002). Therefore, lipids in this category with unsaturated chains are more prone to form H_{II} phase than bilayers compared to their saturated counterparts. Due to these nuances in lipid self-assembly properties, interconversions between lipid structures driven by processes such as chemical reactions or conformational changes can be leveraged to switch membrane self-assembly properties and thereby trigger release of contents. Furthermore, relatively minor changes in lipid structure can be effective for triggering changes in supramolecular assemblies, as evidenced by the differing properties of PE and PC despite similarities in their chemical structures.

Another factor to consider pertains to the size range of liposome samples employed for studies/applications. Liposomes of smaller size are more prone to undergo transitions in assembly properties (as well as other processes such as fusion) since this correlates with enhanced curvature of the membrane bilayer (Gaber and Sheridan, 1982; Lentz et al., 1987). For drug delivery applications in particular this matches the small sizes (< 200 nm) of liposomes that are most common for clinical intervention (Maruyama, 2011). This results from the enhanced permeation and retention (EPR) effect, in which nanoparticles that are smaller than 200 nm exhibit selective delivery to tumors; the irregular cell growth associated with cancer causes leaky vasculature that can be infiltrated by particles of these sizes (Gabizon and Papahadjopoulos, 1988; Liu et al., 1992). Overall, to achieve triggered release, the self-assembly properties of lipid components before and after treatment with an appropriate stimulus must be carefully fine-tuned to overcome a stability threshold that is dependent on the size of the liposome under study.

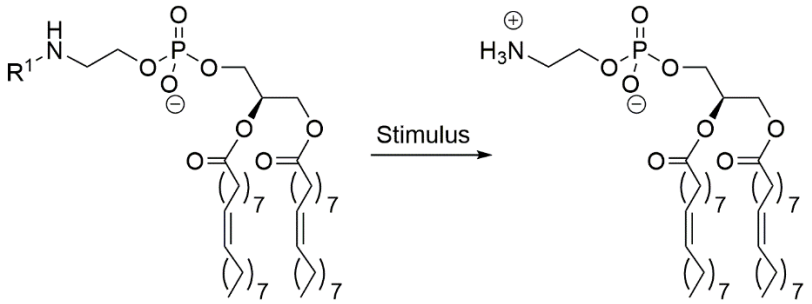
In this review, we will describe different approaches that have been taken for modulating lipid structure to alter self-assembly properties and trigger release of contents from liposomes. Rather than providing an exhaustive record in the field, we present a focused narrative by breaking successful strategies down into different categories and discussing representative examples. One successful strategy has entailed the modification of known non-bilayer lipids in a way that invokes a preference for bilayer membrane formation. Therefore, when the new group that is added is removed through stimulus treatment, a non-bilayer lipid with known properties is produced. However, alternate strategies have emerged in which completely artificial lipids are designed to undergo structural changes that drive liposome release. While this approach typically requires additional design and synthetic components, it offers the potential to expand beyond the scope of natural lipid structures to maximize release characteristics. Each of these areas will be discussed herein.

2. Triggered release through the generation of non-bilayer lipids

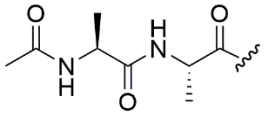
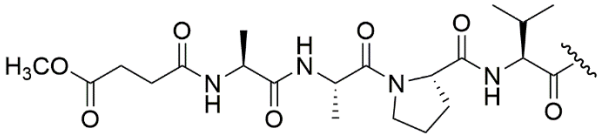
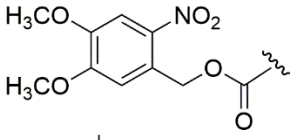
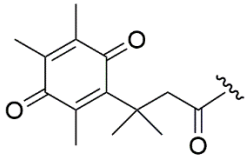
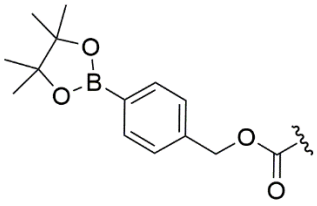
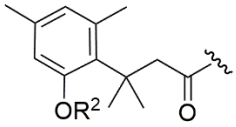
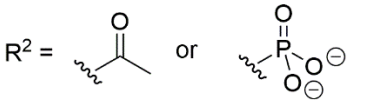
Since nature provides lipids with both bilayer and non-bilayer properties, a way to capitalize on this characteristic is to devise systems in which lipids in the former category are converted to those in the latter category as a result of a desirable stimulus. The most common lipid that is modified for this purpose is DOPE. This is because while DOPE itself exhibits non-bilayer properties, it is known that forming an amide bond by modifying the amino functionality increases the size of the head group and leads to significant stabilization of membrane bilayers including the resulting DOPE-conjugates (Domingo et al., 1994; Newman et al., 1986). Therefore, stimuli that remove the newly introduced group, which acts as the trigger, lead to release of non-bilayer forming DOPE resulting in destabilization of the membrane that can be harnessed to release contents from liposomes. PE is also a convenient synthetic building block for stimuli-responsive lipid development through acylation of the amino group.

2a. Modifications to lipid head groups

Due to the properties of DOPE and other non-bilayer lipids, a common strategy has been to design lipids that can be modified at the head group to produce them. In early work, Meers and co-workers developed liposomes that respond to the enzyme elastase by attaching an *N*-acylated dialanine peptide to DOPE to produce compound **1** (Figure 3) (Pak et al., 1998). Treatment with elastase activated vesicle fusion that was detected via a FRET mixing assay. This was later modified to *N*-methoxysuccinyl-Ala-Ala-Pro-Val conjugate **2** that exhibited greater sensitivity and selectivity for elastase cleavage (Pak et al., 1999). Smith and co-workers utilized this approach for the development of photoactivatable liposomes by introducing the nitroveratrylcarbonyl group onto DOPE to form compound **3** (Zhang and Smith, 1999). Here, UV irradiation of liposomes composed of 50% **3** in egg PE led to 90% uncaging of this compound and release of ~50% of encapsulated calcein dye. Lu and co-workers also developed a photocleavable nucleolipid by introducting an *o*-nitrobenzyl group in between a nucleotide and a dietherglycerol lipid scaffold, resulting in a lipid that formed stable bilayers (Sun et al., 2013). Upon light irradiation, the membrane was disrupted causing release of encapsulated carboxyfluorescein. McCarley and colleagues developed liposomes that are responsive to reducing environment using quinone-DOPE conjugate **4** (Loew et al., 2013; Ong et al., 2008). This compound was designed such that quinone reduction produces a phenoxide intermediate known as the trimethyl lock (TML) that is known to undergo fast kinetics for lactonization via intramolecular cyclization (Chandran et al., 2005; Wang et al., 1997), which in this case releases DOPE. Quantitative release of the encapsulated dye calcein was observed upon treatment with sodium dithionite as a reducing agent. Liposomes that respond to reducing conditions have also been developed by exploiting release of disulfide (Kirpotin et al., 1996; Koyanagi et al., 2017; Tang and Hughes, 1998, 1999; Zalipsky et al., 1999) and sulfonium (Dey et al., 2020) groups introduced within their structures.



Acylated DOPE - bilayer lipid **DOPE - non-bilayer lipid**

Compound #	R	Stimulus
1		Elastase enzyme
2		Elastase enzyme
3		Light
4		Reducing conditions
5		Oxidizing conditions
6	 	Esterase or phosphatase enzyme

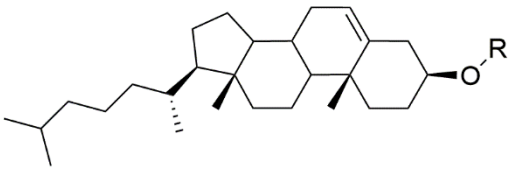
(esterase) (phosphatase)

Figure 3. Lipids designed for triggered release through the production of natural non-bilayer lipid products.

Our group has recently reported two different systems that rely upon DOPE release. In one platform, we developed boronate-PE conjugate **5** bearing a quinone methide (QM) self-immolating linker to respond to reactive oxygen species (ROS) such as hydrogen peroxide. Through studies including time-dependent NMR tracking, we determined that boronate oxidation on a short time scale was sufficient for release of the hydrophobic dye Nile red, while QM self-immolation was necessary for release of the polar dye pair ANTS/DPX, which required a longer timeframe and additional DOPE in the liposome formulation (Lou and Best, 2020b). Additional ROS-responsive liposome strategies will be discussed later in this article. Our group also developed a modular design strategy to target lipids that are responsive to different enzyme classes, in which we targeted esterase, phosphatase, and β-galactosidase enzymes (Lou and Best, 2020a). The former two lipids of type **6** also utilize the TML linker for self-immolation and DOPE release following hydrolysis of either an ester trigger by esterase or a phosphate trigger by phosphatase. The β-galactosidase-responsive liposome utilized a different design employing an aminodialkylglycerol scaffold due to synthetic considerations.

In addition to the direct formation of non-bilayer lipids, it is also possible to trigger modifications that exacerbate membrane instability caused by their presence. For example, it has been shown that liposomes containing the ganglioside G_{M1} released calcein upon treatment with the enzyme β-galactosidase (Pinnaduwage and Huang, 1988). In this strategy, the micellar lipid G_{M1} was initially used to stabilize DOPE liposomes due to its opposing curvature. However, truncation of the glycan headgroup negated this stabilization, thereby exposing the non-bilayer properties of DOPE and stimulating release. Glycolipid bolaamphiphiles have also been employed to develop pH-responsive liposomes for the release of nanoparticles driven by mesoscopic structural transitions caused by protonation state of appended carboxylate groups (Van Renterghem et al., 2019). Additionally, Davis and Szoka developed cholesterol phosphate analog **7** that engenders phosphatase-responsive properties in liposomes into which it is incorporated (Davis and Szoka,

1998). In this case, the authors exploited the ability of charged lipids such as **7** to stabilize DOPE liposomes, and thus the non-bilayer properties of DOPE emerge upon hydrolysis of the phosphate group by alkaline phosphatase, leading to release of the dye/quencher pair ANTS/DPX. A lipid that is sensitive to cholinesterase enzyme has also been reported by Menger and Johnston (Menger and Johnston Jr, 1991).



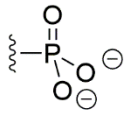
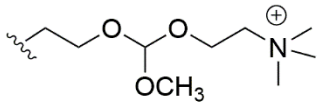
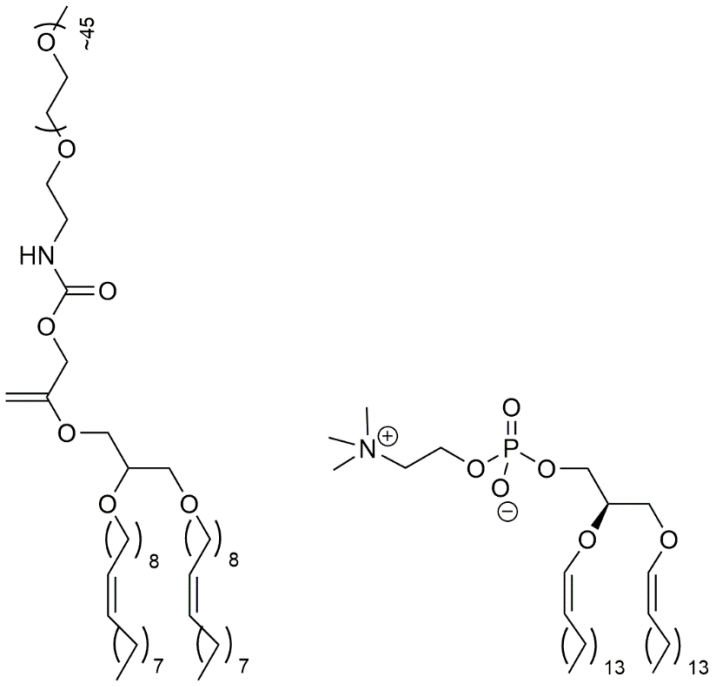
Compound #	R	Stimulus
7		Phosphatase enzyme
8		Acid

Figure 4. Lipids designed to undergo triggered release through modification of cholesterol analogs that stabilize membrane properties.

A similar approach was taken with acid-responsive lipid **8**, which is instead cationic (Guo et al., 2014). In this case, hydrolysis of the ortho ester linker upon decreasing the pH releases the cationic choline group and again magnifies the non-bilayer properties of DOPE separately incorporated in the liposomes. Other ortho ester-containing lipids have been developed for acid-responsive properties via hydrolysis (Guo et al., 2003; Zhu et al., 2000). Another acid-responsive functional group that has been exploited to cleave lipid structures and trigger liposome content release is the vinyl ether. Thompson and co-workers reported multiple compounds in which the hydroxyl group of the lipid diacylglycerol (DAG) was modified into a vinyl ether attachment connecting PEG groups (Shin et al., 2012). These compounds generate DAG derivatives upon

decomposition in acid, which also possess non-bilayer properties. Of these, compound **9** was found to elicit the greatest discrepancy between dye leakage at acidic and neutral pH, yielding ~50% release at pH 3.5 after 4 hours with minimal change at pH 7.5 when 10% of this compound was incorporated into DOPE liposomes. As has been the case with most functional groups that have been explored for liposome release, vinyl ether functionalities have also been incorporated into the lipid chains to modify hydrophobic portions in triggering release, which will be the focus of the next section. For example, the vinyl ether connections to hydrophobic chains (of plasmalogen and diplasmenyl lipids) such as in **10** can be used to trigger release either by increasing acidity or through photoirradiation (Anderson and Thompson, 1992; Boomer et al., 2003; Boomer and Thompson, 1999; Gerasimov et al., 1997; Rui et al., 1998; Thompson et al., 1996; Wymer et al., 1998). Dithiane-containing lipids have also been shown to be cleaved by photolysis (Li et al., 2003; Wan et al., 2002).



9 **10**

Figure 5. Lipids containing vinyl ether linkages at either the head group or within the lipid chains that are cleavable by acid or light.

2b. Modifications to lipid acyl chains

While alteration of lipid head group structure has been commonly exploited for liposome release, this is not the only approach that can be used to invoke non-bilayer lipid properties. This is because the size of the acyl chain region of the lipid also contributes to potential cone-shaped lipid character, and thus modifications to fatty acyl chains can also be harnessed to trigger content release. A way to achieve this is to exploit fatty acid hydrolase enzymes such as secretory phospholipase A2 (sPLA2) to remove the acyl chain from the *sn*-2 position of PC analogs, which truncates the area of the acyl chains, introducing micellar properties and perturbing membrane packing (Davidsen et al., 2003; Davidsen et al., 2001; Foged et al., 2007; Ghavami et al., 2020; Jorgensen et al., 1999; Mock et al., 2012; Thamphiwatana et al., 2014; Vermehren et al., 1998; Zhu et al., 2011). One strategy has involved development of phospholipid prodrugs that release compounds with antitumor properties such as ether lipids (Andresen et al., 2004; Andresen et al., 2005; Linderoth et al., 2009a), retinoids (Arouri and Mouritsen, 2011, 2012; Pedersen et al., 2010), chlorambicil (Pedersen et al., 2009), and tocopheryl succinate (Pedersen et al., 2012). In a clever approach, Andresen and co-workers modified the structure of phosphatidylglycerol (PG) by inserting extra carbons in the headgroup of sPLA2-responsive lipid **11** (Figure 6A) (Linderoth et al., 2009b). This was done such that removal of the *sn*-2 stearate chain by sPLA2 would unveil a hydroxyl group that is well-positioned for an intramolecular cyclization reaction since the remaining ester carbonyl is spaced 5-atoms away in this structure. Furthermore, the drug capsaicin is attached at this position, and so this strategy results in simultaneous destruction of the phospholipid structure and direct release of a covalently appended drug.

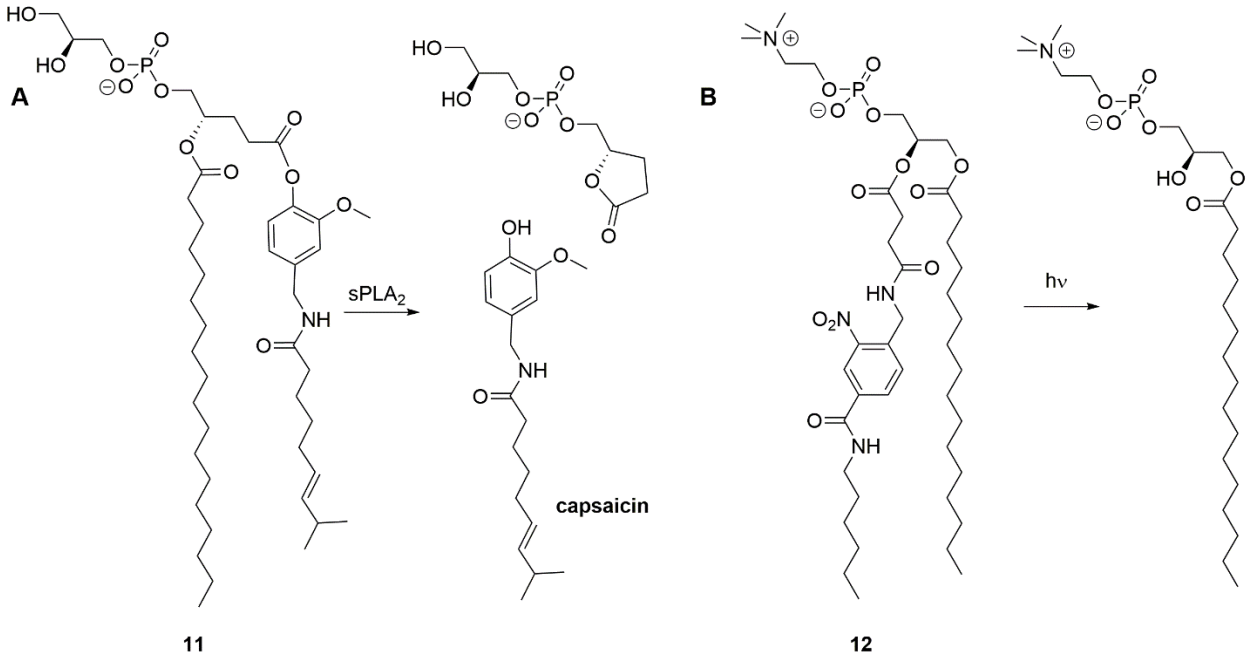
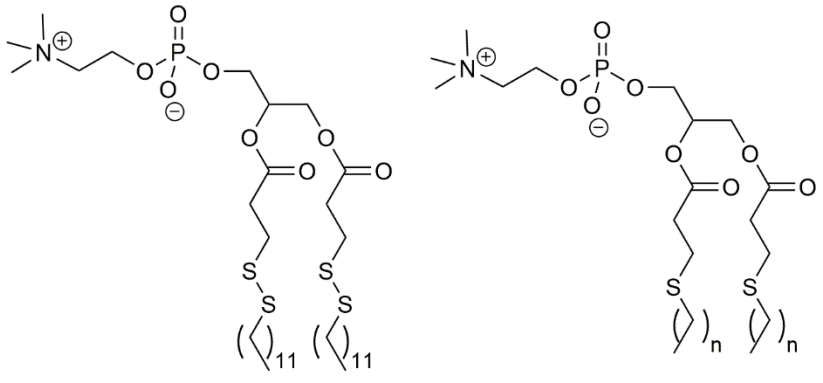


Figure 6. Lipid switches driven by alterations to lipid acyl chain structure.

Our group has developed photocleavable liposomes by incorporating a photocaged acyl chain at the *sn*-2 position of PC analog **12** (Figure 6B) (Bayer et al., 2014). This compound was designed to produce the non-bilayer forming lipid LPC through photo-uncaging followed by cyclization to form succinimide. This process led to a dose-dependent release of the dye Nile red from liposomes containing **12**. This work was inspired by the development of photocleavable amphiphiles containing amino acid headgroups (Chandra et al., 2005; Chandra et al., 2006; Subramaniam et al., 2010). A coumarin-linked PC analog has also been reported for release driven by irradiation with near-infrared (NIR) light (Sun et al., 2016). An intriguing strategy that has been developed involves the in situ formation and subsequent light-triggered decomposition of liposomes (Zhang et al., 2018a; Zhang et al., 2019), which has been applied for photoinduced pinocytosis (Konetski et al., 2018). In these examples, click chemistry is exploited to introduce acyl chains onto lipids to induce self-assembly, after which the resulting membranes can be cleaved.

While it was previously described that redox-responsive functionality has been introduced at the headgroups of lipids, more recent work has shown that this approach is also effective when introducing groups within the acyl chains. Li and co-workers have shown that disulfide-containing PC analogs of type **13** self-assemble into liposomes that are disrupted by thiol exchange reactions (Figure 7) (Du et al., 2019b). Treatment of liposomes composed of **13**:PC:cholesterol in a 9:1:3 molar ratio with dithiothreitol (DTT) led to dramatic decomposition of liposomes as judged by TEM and doxorubicin (DOX) release. These liposomes were also found to be highly effective for DOX delivery to cells and for in vivo antitumor efficiency. The encapsulation and release of paclitaxel (PTX) (Du et al., 2020) has also been described, as well as chemical attachment of camptothecin (CPT) (He et al., 2019) and PTX (Wang et al., 2019) to the disulfide linkage within the acyl chains. A similar approach was taken to generate lipids that are sensitive to reactive oxygen species by instead incorporating the thioether functionality of compounds of type **14** (Du et al., 2019a). Results showed that treatment of liposomes containing **14**:DSPE-PEG$_{2000}$:cholesterol in a 9:1:3 molar ratio with hydrogen peroxide led to sulfur oxidation and subsequent liposome degradation, as judged by TEM studies and DOX release. The oxidation of sulfide-linked polymer–lipid conjugates has also been shown to be effective for oxidatively driven release (Kim et al., 2020).



13 **14**

n = 9, 11, 13

Figure 7. Lipids containing disulfide and thioether functionalities within the acyl chains for release driven by redox conditions.

3. Triggered release through the design of artificial lipid structures

While nature provides non-bilayer lipids whose properties can be exploited to fine tune liposome stability and drive release, this toolbox is limited to a few lipids with unusual self-assembly properties. As such, a new approach is to design artificial stimuli-responsive lipids in which chemical and conformational changes are programmed in to alter liposome stability. While this often requires additional effort in synthesis, it dramatically opens up the structural possibilities for modulating membrane properties. In particular, many of the liposome release strategies in the prior sections require the addition of a significant amount of non-bilayer lipid additives such as DOPE and DOTAP to the formulation. This is done to destabilize membranes and bring them to the precipice of the threshold between interconversion of self-assembled states. However, this destabilization is likely not ideal for clinical drug delivery applications. Through the development of artificial responsive lipids, there exists the potential to overcome this challenge by designing compounds that undergo more dramatic changes in lipid structure, thereby circumventing the need to introduce instability into the initial liposome formulation.

A recent movement in the development of artificial lipid switches for content release has been spearheaded by the development of pH-responsive liposomes, which rely on groups that undergo programmed conformational changes upon protonation. These are of great interest due to the enhanced acidity associated with cancer cells (Gillies et al., 1994; Van Sluis et al., 1999) and endosomal compartments (Geisow and Evans, 1984; Lee et al., 1996), the latter of which is useful for endosomal escape strategies that enhance delivery of drugs to the cytosol (Martens et al., 2014; Shete et al., 2014). Guo, Samoshin and co-workers developed lipids of type **15** designed based on the *trans*-2-aminocyclohexanol moiety and associated analogs, which undergo a pH-induced chair flip that causes divergence of the hydrophobic chains (Figure 8A) (Brazdova et al., 2008; Liu et al., 2012; Ruyonga et al., 2019; Samoshin et al., 2013; Samoshin, 2014; Samoshin

et al., 2017; Samoshina et al., 2011; Yaroslavov et al., 2015; Zheng et al., 2012; Zheng et al., 2015, 2018). This design has been shown to be highly effective for release, with liposomes containing 25% of **15** doped into 1-palmitoyl-2-oleyl-*sn*-glycerophosphocholine (POPC) yielding release of ~50% of dye upon dropping of the pH to 5.5. Leblond and co-workers developed di(methoxyphenyl)pyridine **16**, in which protonation of the central pyridine nitrogen leads to aryl group rotation that causes the two hydrophobic chains to point in opposite direction and thereby counteract membrane bilayer-forming properties (Figure 8B) (Viricel et al., 2015). Following the testing of multiple substituents, the dimethylaminopyridine (DMAP) analog was identified as possessing a beneficial pK_a (5.28) to cater to enhanced acidities of endosomes and cancer cells. Inclusion of **16** within liposomes composed of distearoylphosphatidylcholine (DSPC) along with 5 mole % of DSPC-PEG_{2000} led to 88% release of encapsulated sulforhodamine B dye upon decreasing of the pH from 7.4 to 5.

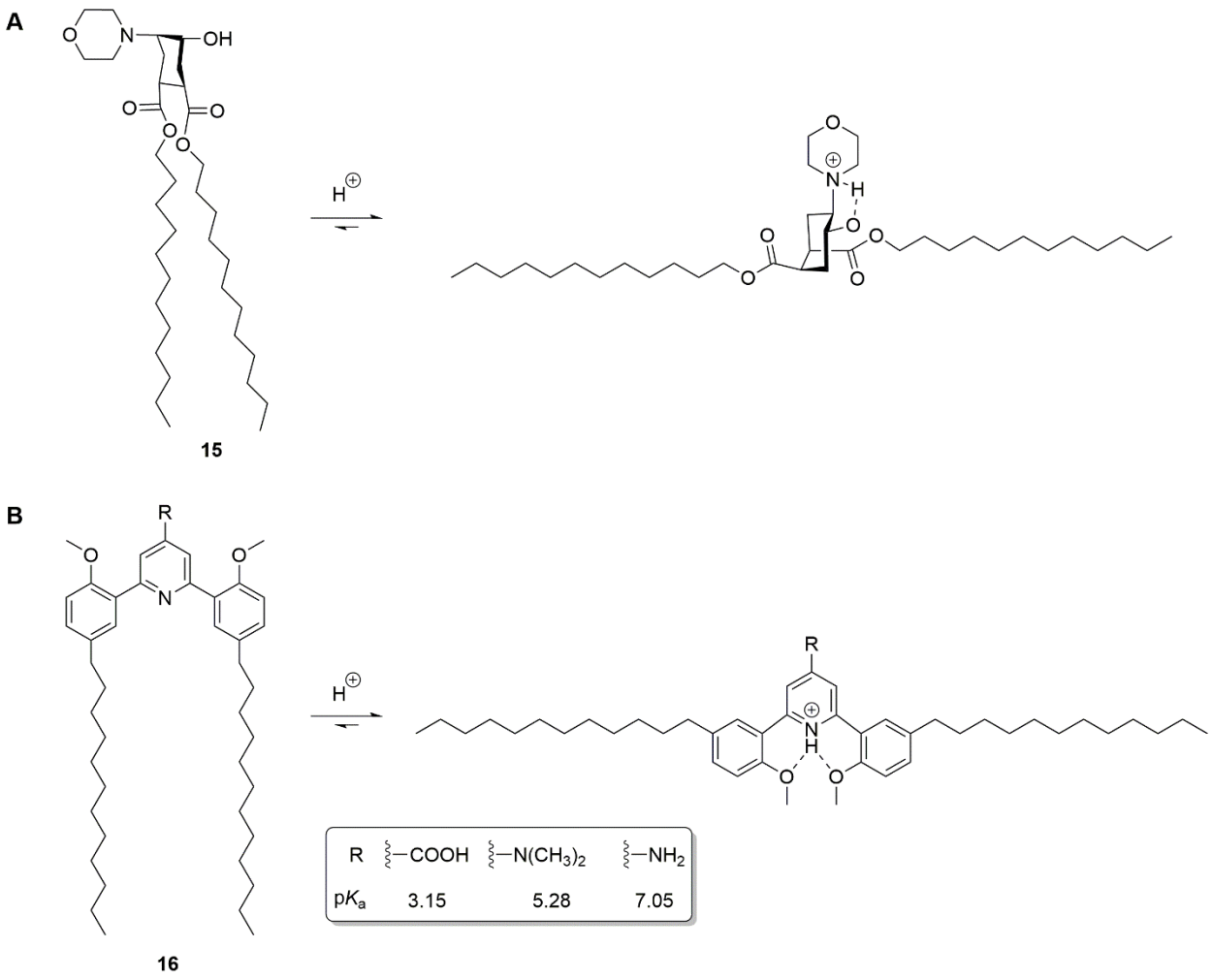


Figure 8. pH-responsive lipids driven by conformational changes upon protonation.

Another area that has recently emerged for the development of artificial lipid switches pertains to ion-responsive liposomes. While presenting a similar scenario to pH-responsive lipids in that the coordination of a target atom is designed in such a way to drive conformational changes, it comes with the added issue of tailoring the recognition to selectively bind a particular ion. Fortunately, advancements in metal ion coordination chemistry and sensing have provided a number of ligands that can be modified in the production of ion-responsive liposomes. Zefirov and co-workers reported bispidinone-lipid **17** (Figure 9A) in which interconversion of an initial chair-boat conformation into chair-chair upon copper chelation leads to a significant modulation of alkyl chain geometry upon copper binding (Veremeeva et al., 2014a; Veremeeva et al., 2014b).

Inclusion of 25% of **17** within liposomes otherwise composed of egg yolk lecithin led to release of up to greater than 80% of encapsulated carboxyfluorescein upon treatment with copper sulfate.

Yuasa and co-workers developed lipid **18** based on a 2,4-diaminoxylopyranoside group that interacts with zinc (Figure 9B) (Takeuchi et al., 2015). This compound works in a different manner, in which initial zinc binding forms a 2:1 **18**:Zn complex that drives the molecule into a structure that forms stable liposomes. This process is then reversed, thus instilling acid-responsive properties into the system, which was applied to the release of glycine-fluorescein dye from cells and the delivery of liposomes to gastric cancer cells. Our group developed calcium-responsive lipid **19** (Lou et al., 2018), which was inspired by the calcium sensor indo-1 developed by Tsien and co-workers (Grynkiewicz et al., 1985). Compound **19** was designed to undergo a conformational change that introduced cone-shaped characteristics and non-bilayer properties upon binding of calcium to the lipid head group (Figure 9C). This lipid enabled release of > 60% of the dye Nile red when only 10% of **19** was incorporated into liposomes otherwise composed of PC.

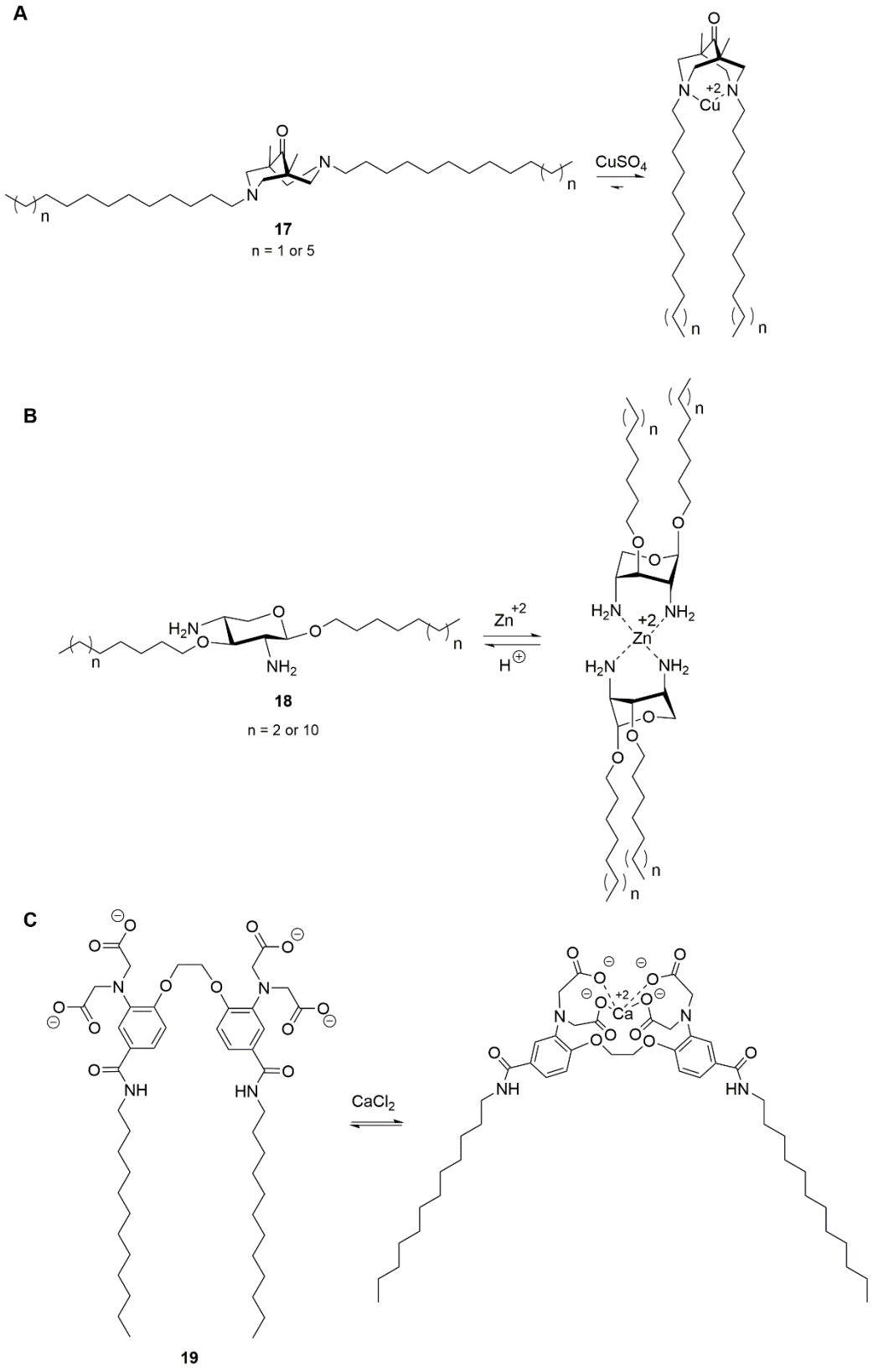


Figure 9. Artificial ion-responsive lipids driven by conformational isomerism.

Another approach that has been taken exploits molecules that bind to liposome surfaces to disrupt lipid packing and trigger release of contents. Smith and co-workers implemented this strategy in the application of bis(zinc(II)-dipicolylamine) (ZnBDPA) sensors to trigger release from liposomes containing phosphatidylserine (PS) (Plaunt et al., 2012; Plaunt et al., 2014). These sensors had previously been developed for imaging cancer cells due to the irregular presentation of PS on the outer leaflets of diseased cells (Hanshaw and Smith, 2005; Smith and Smith, 2012). In these studies, it was shown that treatment of liposomes containing 5% PS with ZnBDPA led to release of greater than 80% of encapsulated fluorescein. In addition, our group has developed boronic acid liposomes for the binding of complex carbohydrates, which we envisioned would enhance both cell entry while also triggering release of contents due to the perturbation of membrane properties upon formation of multivalent carbohydrate-boronic acid interactions (Zhang et al., 2018b). The latter properties were demonstrated through dye release assays, DLS analysis and STEM imaging. Finally, an additional approach has entailed polymers that engulf the liposome surface and are then uncaged through acid addition (Lee et al., 2007). These are composed of poly(acrylic acid) functionalized with cholesterol groups that anchor the polymers onto the liposome surface, with pH-responsive properties driven by the protonation state of carboxylic acid moieties. These examples showcase the wide-ranging avenues that have been explored for controlling liposome stability and release towards drug delivery applications.

Conclusion

While it is clear that numerous creative and chemically effective approaches have been developed for liposome release, the reality is that these platforms have not progressed through clinical trials due to significant challenges. While many reported examples rely on the manipulation of non-bilayer lipid structures, this approach appears to suffer from the need to introduce additional non-bilayer lipid content that places the liposome on the brink of the

stability/release threshold. One potential way to circumvent this issue would be to explore lipid anchors other than DOPE with different self-assembly properties and/or extents of unsaturation to probe for those that exhibit greater changes in membrane properties. Nevertheless, artificially designed lipid switches show strong prospects for enhancing release properties due to the ability to engineer more dramatic changes in lipid structures upon the introduction of stimuli. As a result, a benefit of these lipids could involve incorporation at lower percentages within liposomes that don't require additional non-bilayer lipids, thereby stabilizing the membrane during circulation. Another issue is that since many release strategies revolve around destabilization of the membrane and/or vesicle fusion, lipid molecules are retained afterwards that continue to self-assemble, which can hinder total release of contents. Therefore, the advancement of platforms in which lipid structures are completely undone to produce non-lipid products is also beneficial for complete liposome disintegration and content release. These promising approaches provide exciting avenues for building on the wealth of cleavable liposomes that have been reported to culminate in clinically applicable drug release platforms.

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