Demolish and Rebuild: Controlling Lipid Self-Assembly Toward Triggered Release and Artificial Cells

Megan L. Qualls[‡], Ruhani Sagar[‡], Jinchao Lou, and Michael D. Best[*]

Department of Chemistry, University of Tennessee, 1420 Circle Drive, Knoxville, TN, 37996, United States

ABSTRACT: The ability to modulate the structures of lipid membranes, predicated on our nuanced understanding of the properties that drive and alter lipid self-assembly, has opened up many exciting biological applications. In this perspective article, we focus on two endeavors in which the same principles are invoked to achieve completely opposite results. On one hand, controlled liposome decomposition enables triggered release of encapsulated cargo through the development of synthetic lipid switches that perturb lipid packing in the presence of disease-associated stimuli. In particular, recent approaches have utilized artificial lipid switches designed to undergo major conformational changes in response to a range of target conditions. On the other end of the spectrum, the ability to drive the *in situ* formation of lipid bilayer membranes from soluble precursors is an important component in the establishment of artificial cells. This work has culminated in chemoenzymatic strategies that enable lipid manufacturing from simple

components. Herein, we describe recent advancements in these two unique undertakings that are linked by their reliance on common principles of lipid self-assembly.

Introduction

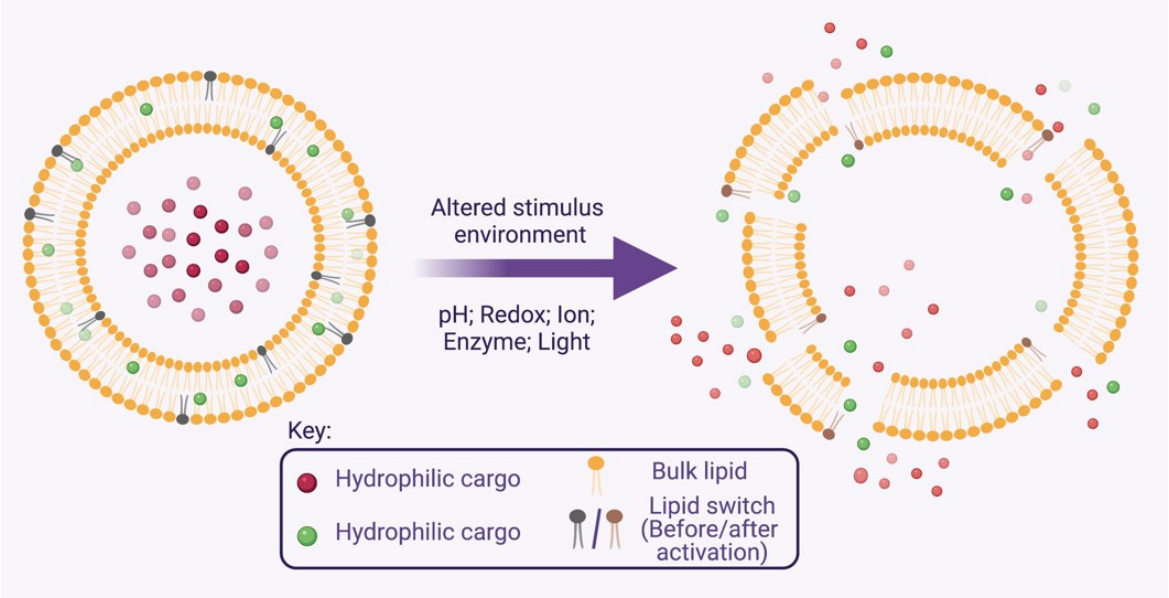
Research focusing on lipid membranes has intensified in recent years due to the critical roles that membranes play in driving biological processes as well as the applications that are enabled by the unique environments of lipid bilayers.[1,2] Beyond understanding the impacts of biological membranes, the ability to carefully manipulate and refine the self-assembly properties of lipids has emerged as a powerful chemical tool that is applicable to wide-ranging endeavors. These abilities stem from an advanced understanding of lipid assembly properties, and particularly the propensity of different amphiphilic molecules to form variable supramolecular morphologies based on their precise structural features.[3,4] For example, this enables the design of lipid switches, by which self-assembly properties are reprogrammed in response to a target stimulus such as a chemical reaction or non-covalent binding event.[5]

These abilities have spawned a wide range of creative applications by which lipids can be rearranged into a variety of shapes for different pursuits. In this perspective, we will group together the odd couple of approaches in which the same principles are applied to achieve completely opposite results. On one hand, the introduction of defects within membrane bilayer assemblies can be harnessed for controlled decomposition, for which a primary application is the triggered release of encapsulated contents from liposomal membranes to enhance drug delivery.[6-8] In the opposing direction, the ability to promote the assembly of previously soluble precursors into membrane bilayers has been of significant interest, particularly toward the development of artificial cells. Further, we have reached the point where these typically independent pursuits have begun to merge

by controlling both the assembly and then disassembly of lipids in one pot. Herein, we will discuss the commonalities and differences between these two opposing endeavors by describing recent examples that exploit common principles of self-assembly.

1. Demolition: Triggered Release of Encapsulated Cargo from Liposomes

A primary application that is enabled by the ability to remotely control membrane decomposition entails the triggered release of drug cargo.[9,10] Liposomes are highly effective therapeutic nanocarriers due to their ability to encapsulate and enhance the pharmacokinetic properties of both hydrophobic and hydrophilic drugs.[11] However, conventional liposome delivery could be improved by advancing the selectivity of delivery to diseased cells and the controlled release of contents in desired subcellular locations. Triggered release strategies seek to overcome these challenges by exploiting abnormalities associated with diseased cells to achieve site-specific delivery. These can be categorized into passive and active release protocols, wherein passive release avenues exploit aberrant conditions intrinsic to disease including acidity, enzyme overexpression, reducing environment and hypoxia to trigger release, while active release employs external stimuli such as light, heat, and ultrasound to drive escape. Triggered release can be achieved by perturbing membrane packing through processes including structural modifications and conformational changes involving lipid switches (Scheme 1). A traditional approach has entailed modification of naturally existing lipids, typically in a manner that produces non-bilayer forming products through the actions of stimuli. However, there has been a recent push to design artificial lipid structures to magnify alterations to membrane properties.[12] The latter will be our primary focus in this perspective on recently emerging approaches for stimuli triggered release of contents from liposomes. We will group recent examples based on stimuli that are harnessed to trigger release.



Scheme 1. Cartoon illustration for triggered release from liposomes containing lipid switches. Encountering of stimulus leads to the activation of lipid switches that disrupt the bilayer membrane driving the escape of encapsulated hydrophobic and/or hydrophilic cargo that were initially encapsulated within the membrane bilayer and aqueous core, respectively.

1.1. pH-Responsive lipid switches

Variations in pH in and around diseased cells provide a primary condition that has been exploited for liposome triggered release. Cancer cells, in particular, exhibit enhanced acidity in their environment resulting from increased anaerobic metabolism.[13] In pioneering work, Guo, Samoshin and co-workers rendered liposomes pH-sensitive through a protonation-induced conformational switch of hydrocarbon chains in artificial lipids.[14-16] These "flipids" (**1**, Figure 1) leverage a *trans*-2-aminocyclohexanol moiety as well as derivatives, which undergo a proton-induced chair flip and simultaneous spreading of the lipophilic tails, termed the "peacock effect." These events disrupt the liposomal membrane, inducing content release in response to increased acidity of the environment. Using ~200 nm liposomes (stable for months to years), release via an

8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt (ANTS)/DPX assay occurred within seconds to minutes upon pH changes. Recently, structure-activity relationship studies on these lipids showed that larger sized and more hydrophilic headgroups resulted in smaller liposomes while increased basicity of the headgroup induced release at higher pH-ranges.[17] Also, higher encapsulation efficiencies resulted from longer "flipid" tails, while shorter tails enhanced the extent of leakage. As an extension of this work, a series of *trans*-2-(azaaryl sulfanyl) cyclohexanol derivatives were designed to undergo pH-induced change to a more stable conformer with an intramolecular hydrogen bond and electrostatic interactions between azaaryl and hydroxy groups in equatorial positions.[18] Leblond and co-workers developed a pH-responsive lipid switch based on bis(methoxyphenyl)pyridine units (**2**).[19,20] This compound is designed to undergo a major conformational change in environments with enhanced acidity due to intramolecular hydrogen bonding interactions. ~200 nm polyethylene glycol- (PEG)-ylated liposomes containing **2** and distearoylphosphatidylcholine (DSPC) exhibited high stability at physiological pH, while ~88% encapsulated sulforhodamine B cargo release was observed within 15 min when the pH was dropped to 5. This system also demonstrated cytosolic delivery in HeLa cells with minimal toxicity.

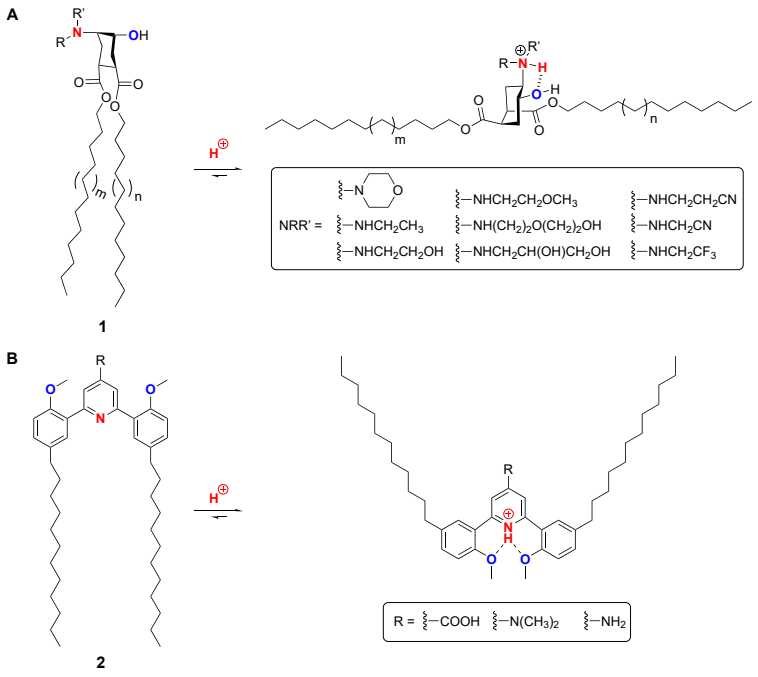


Figure 1. pH-Responsive lipid systems driven by conformational switches

Omolo and co-workers reported a system that acts as a pH-responsive on/off-switch based on oleic acid and a cationic oleic acid quaternary amine lipid derivative to deliver antibiotics.[21] This hinges upon oleic acid initially being deprotonated at physiological pH and therefore electrostatically attracted to cationic lipid, which is reversed upon protonation to oleic acid in a more acidic environment. The surface charge switching enhanced the activity and targeted delivery of loaded drugs. Yaroslavov and co-workers developed pH-sensitive switches derived from lithocholic acid.[22] This activator contains both carboxylate and triazole moieties and changes its orientation in the lipid bilayer as a function of pH to expose an ionic group to the outer environment. Incorporation of only 3 mol% of this compound in 30-50 nm liposomes resulted in approximately 50-60% cargo release within the first minute as compared to 50 mol% “flipid” required in the earlier example. In addition, pH variations have also been exploited to form liposomes and encapsulate cargo as opposed to release.[23] In more recent studies, pH triggered

release from liposomes has expanded to include alternatives to lipid switches such as polypeptides to drive release.[24]

1.2. Ion-responsive Liposomes

A recently emerging strategy is to develop liposomes that respond to chemical agents other than protons, such as ions. This approach hinges upon engineering molecular recognition principles into lipid switch design through the need for host structures that will bind to a target chemical agent. The first lipid switch we will discuss is in fact a pH-responsive system, but we will include it in this section since the approach takes advantage of ion-binding properties. Here, Takeuchi and co-workers developed a sugar-derived lipid switch based on 2,4-diaminoxylopyranoside that undergoes a conformational change in the absence/presence of zinc.[25] Under initial conditions, the pyranose ring of this compound exists in a $^4C_1$ conformation in which the lipid chains point in opposite directions, which is not beneficial for forming bilayer membranes. However, upon the binding of zinc, a conformational change to $^1C_4$ is triggered, in which the lipid chains converge, thereby enabling assembly into stable liposomes of ~100-400 nm measured by dynamic light scattering (DLS). Release of contents is then driven by increasing acidity, which counteracts this process by protonating lipid-Zn adduct, releasing zinc and causing this compound to revert to the original $^4C_1$ confirmation. Therefore, while this work culminates in a pH-responsive lipid switch, it harnesses metal ions to drive conformational changes in lipid switches toward ion-responsive liposomes. Holmstrøm and co-workers reported a similar approach in which zinc chelation by compound **3** was used to form liposomes ~ 150 nm and contents were released within minutes by adding the metal chelator EDTA to reverse zinc chelation.[26]

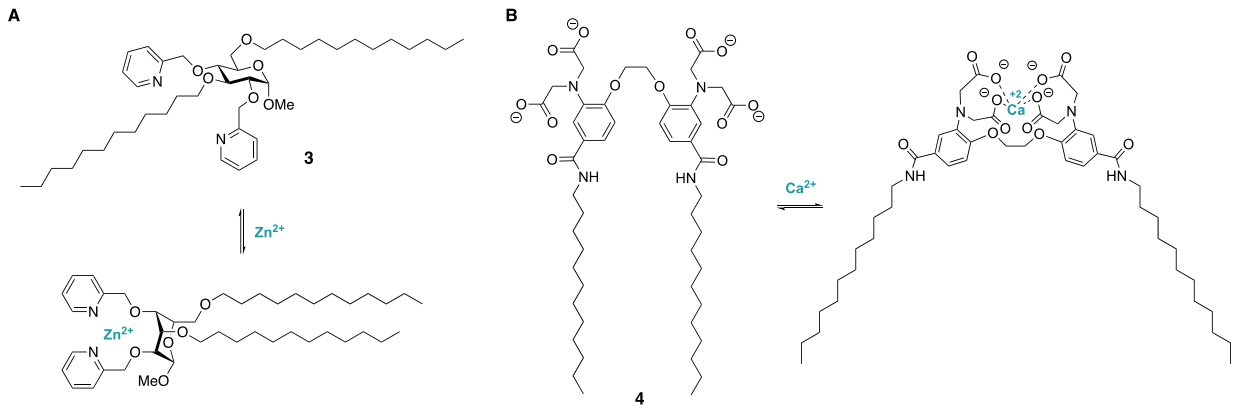


Figure 2. Lipid switches designed to undergo conformational changes upon ion binding

Our group has reported ion-responsive liposomes in a manner that the ion binding event instead triggers a lipid switch conformation that destabilizes the liposomal membrane and enables ion-mediated cargo release. Calcium was selected as the target for this work due to the overabundance of this cation in disease, and particularly in malaria. To this end, calcium-responsive lipid **4** was designed to undergo a conformational change upon interaction with this ion that would cause the lipid chains to diverge, producing a conical shape that is not favorable in membrane bilayers. Compound **4** was effective for driving release of both hydrophobic and hydrophilic cargo within seconds upon calcium addition when incorporated into ~200 nm liposomes at low percentages (5-10 mol %).[27] This opens up new avenues for controlled release utilizing chemical agents/metabolites as the trigger by invoking molecule recognition principles. For example, we have additionally developed boronic acid liposomes to bind complex carbohydrates which would enhance cell entry and trigger content release.[28]

1.3. Redox-responsive liposomes

Overly reducing environments associated with tumors and inflammatory diseases have also been exploited to trigger liposome cargo release. The approaches in this section and beyond are

fundamentally different since they generally exploit chemical reactions to alter lipid switch properties rather than molecular recognition events. One such strategy entails the development of disulfide-containing variants of phosphatidylcholine (PC), the membrane properties of which can be disrupted through thiol-exchange reactions. Using this approach, a redox-responsive prodrug formulation based upon paclitaxel-SS-lysophosphatidylcholine (PTX-SS-PC) was developed to improve drug loading and paclitaxel delivery.[29] Liposomes with diameters of ~240 nm were prepared and exhibited ~80% paclitaxel (PTX) release in 12 hours in the presence of glutathione (GSH). Modified PC analogs bearing disulfide groups (i.e., **5**, Figure 3) have been further exploited to generate stealth liposomes[30] and encapsulate and release doxorubicin (Dox).[31] An additional approach involved incorporation of sulfonium moieties into lipid structures, in part to address challenges including high reactivity and low cellular uptake abilities associated with disulfide-based liposomal systems. Dey and co-workers synthesized a series of novel sulfonium lipids (**6**) that underwent reductively responsive cargo release that was driven by dealkylation.[32] Vesicles of ~100-200 nm composed of 100% **6** resulted in ~57% and 88% release of encapsulated Dox after 60 hours in the absence and presence of GSH, respectively. These lipids also exhibited anti-microbial activities against Gram-positive and Gram-negative bacteria in addition to drug release characteristics. Also, a sulfonium-containing proanionophore has been employed to effect controlled transport of chloride ions in a cellular environment in response to reducing environment.[33]

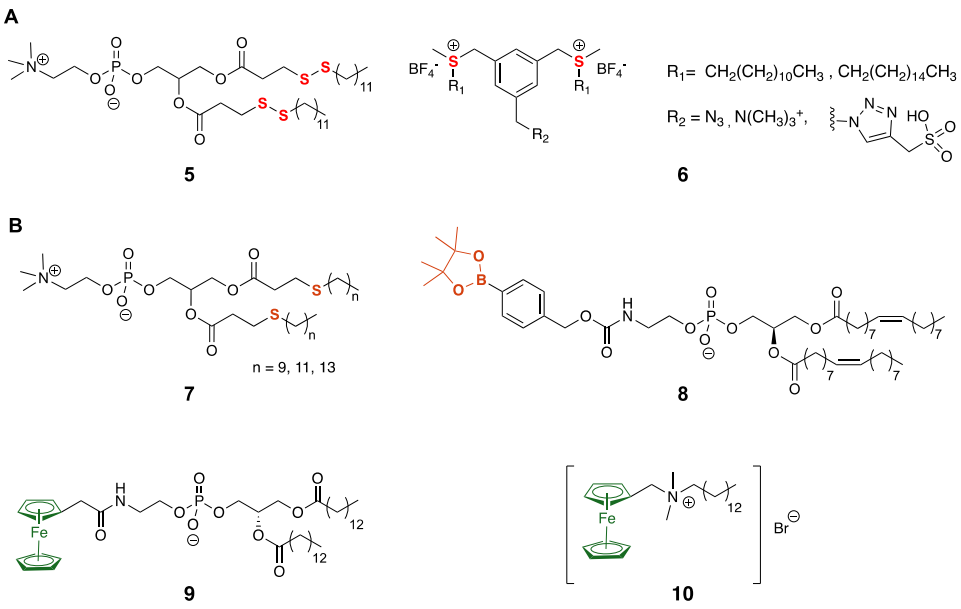


Figure 3. Redox-responsive lipid systems designed by modification of either lipid acyl chains or headgroups. Lipids **5**-**6** are designed to be GSH-responsive, while lipids **7**-**10** are sensitized to oxidants.

On the other end of the spectrum, increased abundance of reactive oxygen species (ROS) also correlates with disease and therefore has been explored for triggering liposome release. In a recent approach, thioether functionalities were incorporated within the acyl chains of PC analog **7** to result in ROS-responsive liposomes.[34] Here, oxidation to sulfoxide groups was employed to alter lipid polarity and drive content release. Liposomes composed of **7** with diameters around 100-120 nm exhibited ~80% of encapsulated Dox release within the first 24 hours. While the prior example consists of phospholipid analogs with trigger groups introduced within the acyl chains, modifications to lipid headgroups have also been effectively exploited to drive release. Our group developed ROS-responsive conjugate **8** designed to release the non-bilayer lipid DOPE upon treatment with hydrogen peroxide as a representative ROS.[35] This compound contains a boronate trigger that is oxidatively cleaved by ROS, feeding into immolation of a quinone methide linker to

generate DOPE, and was capable of driving liposome release. Liposomes composed of 100% **8** with sizes of 150 nm released 50% hydrophobic cargo within 5 minutes upon H_2O_2 treatment. Odette and co-workers developed ferrocene-phospholipid conjugate **9** and monoalkylated ferrocene amphiphile **10** for ROS-induced disassembly.[36,37] Inclusion of 10 mol% of ferrocene-lipid **9** or **10** was sufficient to initiate vesicle disruption while addition of 30 mol% cholesterol lengthened the time course of vesicle breakdown.[36] In addition to the redox-sensitive functional groups mentioned above, selenium-containing nanocarriers have also been intensively studied.[38-41]

A condition related to redox state is hypoxia, which arises because of an imbalance between low oxygen supply and rapid oxygen utilization that is not only a consequence of tumor invasion, but also plays an important role in tumor development as well.[42,43] Low oxygen levels also limit the delivery of therapeutics to tumor regions resulting in treatment failure. Nitroaromatics and azo moieties have emerged as the most commonly employed hypoxia-responsive moieties (Figure 4). Li and co-workers incorporated nitroaromatic **11** into liposomes of 160-200 nm, which underwent structural changes upon reduction in the hypoxic environment to destabilize the bilayer and cause 83% release of encapsulated Dox within 12 hours.[44] Another hypoxia-responsive system was developed by Long and co-workers using azo-inserted lipid analogue **12**. Reductive cleavage of azo compounds and their derivatives was responsible for hypoxia triggered release.[45] **12**-Lipid nanoparticles with ~300 nm in sizes yielded over 80% Dox release over 24 hours.

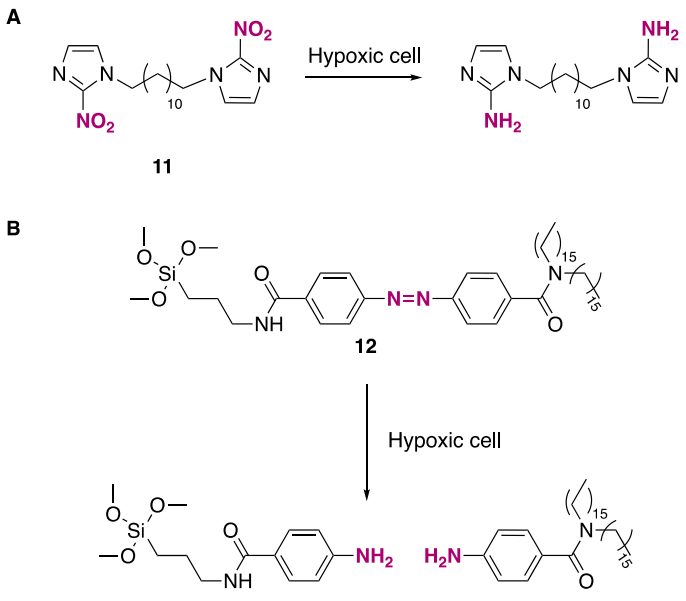


Figure 4. Structures for synthetic lipids that can respond to hypoxic environment.

1.4. Enzyme-responsive liposomes

Enzyme overexpression is another common marker for diseased cells that can be exploited to trigger content release. However, enzyme-responsive systems have generally targeted a subset of prospective enzymes, primarily phospholipases and other lipid-modifying enzymes.[12] To enhance the versatility of enzyme-responsive lipids, our lab developed a modular design strategy represented by generalized structures **13**-**14** in Figure 5. In this work, lipid switches that respond to esterase, phosphatase, and β-galactosidase enzymes were developed by incorporating a variable substrate moiety corresponding to each enzyme target linked via a self-immolating linker (trimethyl lock or quinone-methide generating moiety) designed to undergo decomposition upon trigger removal. The final liposome disruption was brought about by release of non-bilayer lipids including DOPE and aminodialkylglycerol.[46] Liposomes composed of **13**/**14**, PC, DOPE and PA with ~150 nm diameters released cargo over 8 hours upon enzyme treatment. This approach could

enable the targeting of a wide array of disease-associated enzymes simply by exchanging the trigger group.

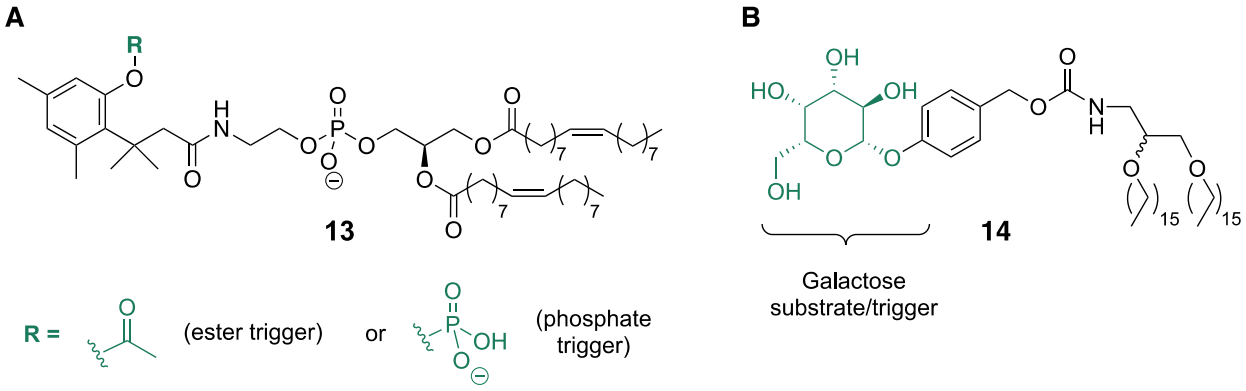


Figure 5. Enzyme-responsive lipids developed by Best and co-workers

1.5. Liposome release triggered by biorthogonal reactions

The prior examples of reactivity-driven liposome release each harness reactive tags that respond to a target biological entity (i.e., ROS, enzyme). However, a more recent approach instead exploits biorthogonal conjugation reactions for release by an exogenously added reagent. In particular, Kannaka and co-workers reported a new strategy for initiating drug release via liposome destabilization using an inverse electron demand Diels Alder (IEDDA) reaction between tetrazine and 2-norbornene.[47] In this work, ~80 nm liposomes containing a tetrazine-lipid were first administered and allowed to accumulate in tumors via the enhanced permeation and retention (EPR) effect. Subsequently, norbornene reagents were added to react with tetrazine via IEDDA, which altered liposome properties and drove ~22% content release after 24 hours of incubation. While initial platforms yielded insufficient release rates that compromised activity, redesigns of norbornene reagents culminated in accelerated drug release (~80% release over 24 hours).[48]

Specifically, the introduction of carboxylic acid moieties on norbornene enhanced the drug release rate, which is attributed to changes in membrane fluidity and inter-molecular repulsion forces.

1.6. Light responsive liposomes

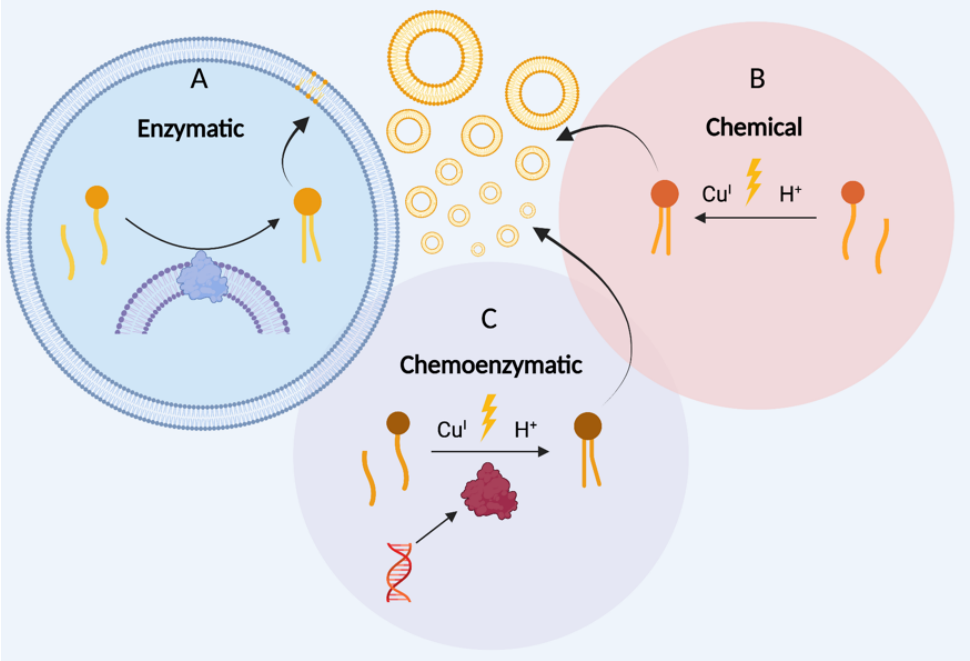
While this perspective article focuses primarily on emerging passive release strategies, we will briefly describe recent advancements pertaining to active release, focusing on light-responsive liposomal systems. In addition to site-specific delivery, actively triggered systems can help achieve controlled release rates at specified time points. Commonly explored photochemical activation strategies include photoisomerization with azobenzene units or photocleavable linkers, such as *o*-nitrobenzyl moieties.[49,50] Witzigmann and co-workers recently reported an optimized photoactivable liposomal delivery system based on *cis-trans* azobenzene isomerization. Irradiation induced 65%-70% Dox release from ~50 nm lipid nanoparticles within 24 hours.[51] Azobenzene isomerization has also been exploited for the development of photo-responsive micelles that allowed the spectroscopic analysis of the physical behavior of antimicrobial peptides within a membrane environment, as reported by Roberson et al.[52] The *o*-nitrobenzyl moiety has been employed to develop liposomes with dual responsive release capabilities by incorporating both light-sensitive and acid-sensitive units into a single system.[53] Liposomes of ~120 nm diameters were formed, and maximum release was achieved within 30 minutes under pH 5. Various multi-responsive drug delivery systems have been developed combining both passive and active release stimuli into one carrier with light responsive moieties serving a major role in order to enhance drug delivery capabilities.[54,55] Finally, while tissue penetration and damage have typically been disadvantages of light-responsive liposomes, the development of near IR-mediated release using porphyrin-lipid conjugates has aided in alleviating these issues.[56-59] Cargo release from porphyrin-lipid containing liposomes involves transient pore formation in the membrane resulting from

photo-oxidation of unsaturated lipids. To obtain a better understanding of the pores formed on membrane, the Lovell group recently reported the release of different sizes of biomacromolecule cargos from these systems, underscoring their feasibility and potential in future protein delivery applications.[60] We will discuss further light-responsive liposomes in section 3 focusing on strategies that combine spontaneous liposomes assembly followed by triggered release.

In these ways, progress in our understanding of lipid self-assembly properties has led to clever advancements in liposome triggered release properties. Furthermore, these same principles can be applied for the applications that require the exact opposite approach, which is to take non-lipid precursors and spontaneously drive their assembly into complex membrane architectures. This exciting realm of research will be the focus of the next section of this perspective.

2. Rebuilding: *In situ* Membrane Construction

A prominent recent emphasis of lipid research has been to develop methods for spontaneously constructing bilayer membranes. This is being pursued to advance a broad range of applications, ranging from the formation of artificial cells pertinent to the origins of life to the advancement of functionalized liposomes for drug delivery. A variety of approaches have been pursued for the *in situ* synthesis of membrane-forming lipids from soluble precursors facilitated by enzymes, chemical catalysts, and catalyst-free chemical reactions. Current research is trending towards combining biocompatible enzymatic and chemical methods for *in situ* membrane synthesis in order to combine the benefits of both approaches (Scheme 2).



Scheme 2. Cartoon illustration for various strategies utilized for *in situ* membrane construction. Primary strategies that have been explored include enzymatic, chemical and chemoenzymatic membrane synthesis.

2.1. Enzyme-catalyzed phospholipid synthesis

Enzymatic catalysis has provided one primary approach for *in situ* membrane generation and expansion. Pioneering work in this area showed that new vesicles can be formed within existing vesicles through an enzyme injection method.[61] When vesicles were injected with the enzyme *sn*-glycerol-3-phosphate-acyltransferase (GPAT), encapsulated palmitoyl-coenzyme A (palmitoyl-CoA) and *sn*-glycerol-3-phosphate (G3P) were coupled to form 1-palmitoyl-*sn*-glycerol-3-phosphate. However, since the newly formed lipids self-assembled inside of the original liposome, the phospholipid formation did not result in membrane expansion or creation of new individual liposomes.

Expanding on this concept, Scott and co-workers developed a versatile cell-free gene expression system in which eight lipid biosynthetic enzymes were generated and reconstituted within liposome membranes to produce different lipid products.[62] For example, the enzymes GPAT and lysophosphatidic acid acyl transferase (LPAAT) were expressed to convert G3P and fatty acyl coenzyme A (FA-CoA) into *lyso*-phosphatidic acid (LPA) and subsequently phosphatidic acid (PA) products. Additional enzymes were reconstituted to generate products including cytidine diphosphate diacylglycerol (CDP-DAG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phosphatidylserine (PS) lipids. While this is impressive work, the phospholipid formation was rather inefficient, resulting in membrane growth of <1% that was too small to cause membrane expansion. Also, the significant cost of FA-CoA is a drawback in constructing a widely employable synthetic cell, which could be addressed by incorporating a mechanism for recycling CoA.

Exterkate et. al circumvented the FA-CoA supply issue by incorporating the *in situ* synthesis of this precursor. This approach utilized a long-chain fatty acid-CoA ligase (FadD) enzyme to convert fatty acids (FAs), adenosine-triphosphate (ATP), and CoA into FA-CoAs for downstream lipid production. A cascade of phospholipid biosynthetic pathways containing eight enzymes was then constructed to synthesize numerous phospholipids.[63] In the final steps, phospholipids including PE and PG were formed and then incorporated into the pre-existing liposomes, resulting in expansion of the membrane.

A major issue with enzymatic synthesis of lipids involves challenges associated with controlling these reactions. A recent advancement harnesses genetic control of lipid biosynthesis by incorporating a minigenome within a plasmid to encode for the Kennedy Pathways in *E. coli*. In this groundbreaking work, Blanken et. al. incorporated a metabolic feedback mechanism to exert

control over phospholipid synthesis. Specifically, they employed the enzyme CDP-DAG—serine *O*-phosphatidyltransferase (PssA) to maintain the ratio between acidic and zwitterionic lipids.[64] Mass spectrometry studies using isotopically labeled lipid standards confirmed that this strategy enabled regulation of phospholipid synthesis. For example, a correlation was observed between the initial amount of PG incorporated within liposomes and the amount of PE that was synthesized.

These advances in membrane construction take advantage of the natural phospholipid biosynthesis by using integral membrane proteins to synthesize membrane-forming lipids. One disadvantage of the typical enzymatic approaches is the need for membrane bound or transmembrane proteins, which are difficult to express and reconstitute. This approach also generally requires pre-existing liposomes within which new lipids are synthesized. Additionally, low yields in phospholipid synthesis leading to minimal vesicle growth remain a pitfall for enzymatic lipid membrane synthesis. To address these challenges, alternative processes are being developed to form liposomes *in situ* that do not require pre-existing membranes, primarily using chemical and chemo-enzymatic coupling strategies.

2.2. Membrane synthesis via chemical reactions

The implementation of chemical reactions for *in situ* membrane construction has gained popularity because of the potential for controlled synthesis, high yields, and quick conversion. There are two primary categories of reactions utilized for this purpose including catalyzed and non-catalyzed strategies. Common catalytic strategies include copper catalyzed alkyne-azide cycloaddition (CuAAC), photocatalytic reactions, and acid-catalyzed reactions. The catalyst-free approaches have harnessed reactions including imine formation, native chemical ligation, thioester exchange, thiol-Michael click chemistry, histidine ligation, and transacylation.

2.2a Membrane synthesis through catalyzed chemical reactions

Copper catalyzed alkyne-azide cycloadditions

A prominent catalytic reaction that has been harnessed for *in situ* bilayer synthesis is CuAAC, which provided a pioneering example of chemoselective phospholipid membrane formation.[65] This biomimetic reaction entailed the coupling of an oleyl azide (**16**) to a PC analog bearing a short alkyne-containing acyl chain (**15**) in the presence of a copper (I) catalyst, inducing self-assembly of the resulting triazole-containing PC to form vesicles (Figure 6A). Although the limiting copper catalyst was not regenerated in this system, a later CuAAC approach incorporated a self-reproducing copper-chelating oligotriazole catalyst that was synthesized from tripropargylamine and 1-azidododecane (**17**).[66] This catalyst could also drive triazole phospholipid synthesis, which culminated in membrane expansion. The resulting vesicles also underwent division due to the increase in curvature stress, which created new vesicles. Continual synthesis of membranes through serial transfers showed repeated and long-term (> 500 h) phospholipid formation. However, similar to the enzyme-catalyzed reactions, this method required a pre-existing membrane in order to synthesize new lipids. Another unique report described the use of CuAAC to form membranes followed by locking through crosslinking. In this case, CuAAC coupling of azide precursor **18** containing a triethoxysilane group to **15** formed a triethoxysilane triazole-phosphatidylcholine, which self-assembled into liposomes *in situ*.[67] Subsequently, sol–gel condensation led to the formation of a silica network in the bilayer of the liposomes, thereby reinforcing their structures with silica shells.

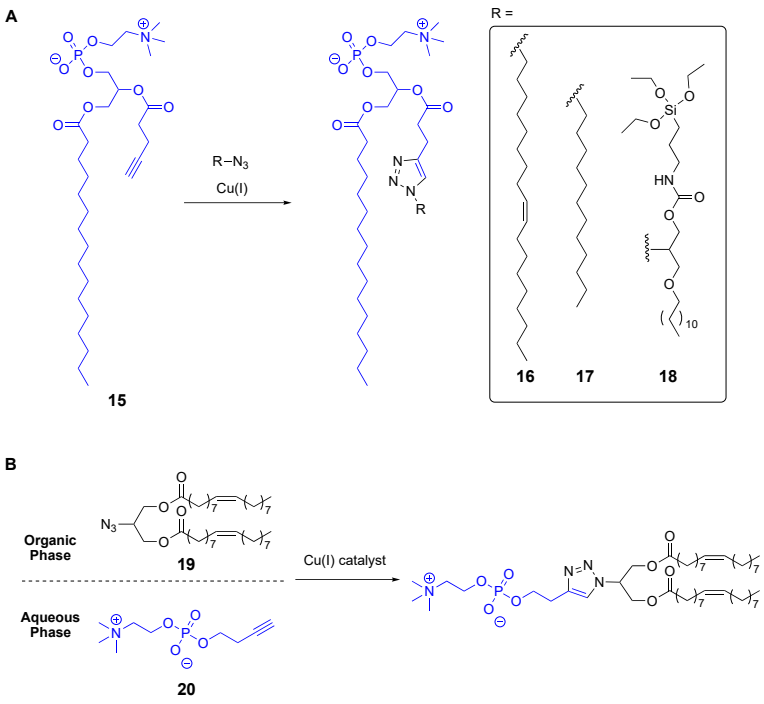


Figure 6. Strategies for CuAAC-driven formation lipids that spurred self-assembly into membrane bilayers.

Another recent study showed how phase separation could be used to control CuAAC during catalysis of lipid formation, resulting in the production of self-reproducing phospholipid vesicles (Figure 6B).[68] In this work, a hydrophobic azide (**19**) in the organic phase of a biphasic solvent mixture reacted at the phase interface with a hydrophilic alkyne-phosphocholine (**20**) via CuAAC, resulting in phospholipid formation. The CuAAC catalyst consisted of a hydrophobic copper–ligand complex, causing it to remain in the organic phase. Vesicles formed by the self-assembly of generated phospholipids then acted as phase-transfer catalysts by taking up alkyl azide **19** and catalyst from the organic phase within their bilayers, which enabled interaction with alkyne-phosphocholine **20**. This accelerated phospholipid formation and closed the autocatalytic cycle.

Photocatalytic Reactions

Photocatalytic reactions have also been explored due to their potential for spatiotemporal control over membrane formation. Many of these methods exploit the precise activation of UV irradiation for photo-initiation of click reactions such as CuAAC and thiol-yne to controllably form vesicles.[69,70] It has also been shown that membrane-forming fatty acids can be synthesized by a ruthenium-based photocatalyst driven by a photoinduced electron transfer (PET).[71] While this reaction formed a simple fatty acid, a more recent example extended ruthenium photocatalysis to synthesize a triazole phospholipid that self-assembled into vesicles.[72] Photochemical initiation of ruthenium-(II) tris-(bipyridine) ($[Ru\text{-}(bpy)_3]^{2+}$) catalyst has also been used to convert resting copper-(II) species into active copper-(I) catalyst via intermolecular PET. The resulting copper(I) then mediated CuAAC between alkyne-lipid and alkyl azide to synthesize triazole-phospholipids that self-assembled into vesicles. A drawback to this system is that it requires additives, photosensitizers, and sacrificial electron donors to function. Enomoto et. al. set out to simplify this system by using an intramolecular PET reaction that only required a single molecular species with no additives or sacrificial reductants.[73] To this end, they developed a method for photochemical activation of a CuAAC catalyst using an intramolecular PET process to initiate biomimetic *in situ* phospholipid membrane formation by phospholipid synthesis. This approach exhibited spatiotemporal control over membrane formation through irradiation.

While these photoinduced reactions were controllable, they did still require catalyst, which limited their biocompatibility. To overcome this issue, Zhou et.al. developed a new catalyst-free method using photoligation chemistry to convert non-membrane molecules to phospholipids (Figure 7).[74] In this work, the precursors *lyso*-sphingomyelin (**22**) and amphiphiles containing nitrobenzyl alcohols (**21**) formed aggregates that brought them into close proximity. Light

irradiation then activated the NBs through alcohol conversion into aldehydes, triggering ligation with the amine groups of **22** to form imine linkages, which was encouraged by their close proximity. When using C10 precursor **21** in HEPES buffer, the irradiation induced the generation of final product immediately without appearance of aldehyde intermediate, and the reaction yield was determined to be ~62%. The synthesized phospholipids then self-assembled into vesicles *in situ*, which were shown to encapsulate cargo and function as bio-reactors. Future photoinduced vesicle work can take note of this catalyst-free approach in order to enhance control, biocompatibility, and efficiency.

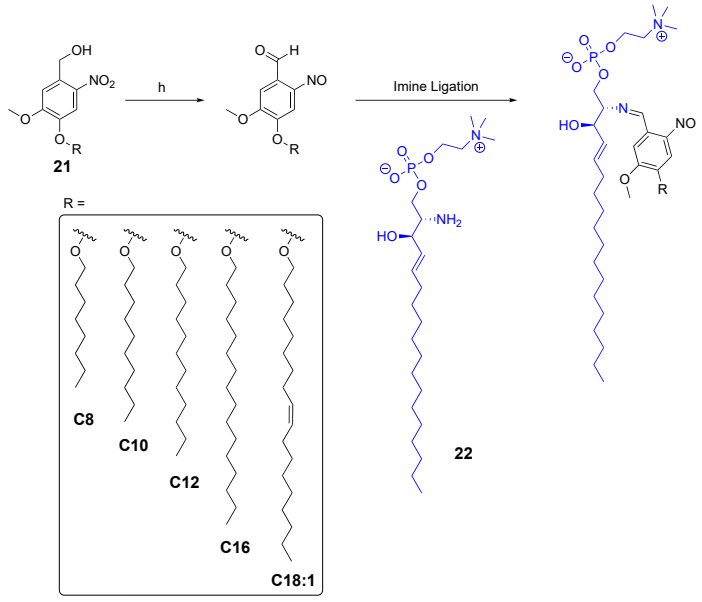


Figure 7. Bilayer forming lipid generation via a photocatalyzed ligation reaction.

Acid-catalyzed membrane formation

Another stimulus that has been exploited for catalysis of *in situ* membrane formation is enhanced acidity. In early work, it was shown that acid-catalyzed imine hydrolysis could result in autocatalytic membrane amplification on the surfaces of pre-existing vesicles, resulting in the

generation of new vesicles.[75] This utilized a synthetic lipid containing a charged choline group linked at the terminus of an acyl chain via an imine group. Imine hydrolysis led to release of this choline group, which generated new vesicles. Vesicle formation was pH-dependent, with more vesicles being formed in lower pH environment. Furthering this work, it was shown that the polymerase chain reaction (PCR) could take place inside of these self-reproducing vesicles.[76] A drawback of this approach is that the liposomes incorporated cholesterol and were formed in an alcohol-containing aqueous environment, which is not ideal for biological applications and would challenge enzyme function. In a more recent example that addresses these drawbacks, Matsuo et. al. developed a synthetic phospholipid that repeatedly self-reproduces without using helper lipids, inorganic catalysts, or membrane proteins.[77]

While these catalytic reactions are controllable, fast, and efficient, an issue with all of these reactions is that they require some form of catalyst, which introduces limitations such as the need to deliver catalyst to the site of membrane production and can make them non-biocompatible. To overcome these limitations, an evolving area of research is catalyst-free chemical reactions.

2.2b. Catalyst-free chemical reactions

Imine chemistry

Many approaches to catalyst-free *in situ* membrane synthesis hinge upon dynamic covalent chemistry through controlled functional group exchange. For example, imine bonds have been used to generate vesicles using cationic surfactants, resulting in self-assembly that can be switched back and forth between the bilayer state and the reactive precursor through pH changes and dilution.[78] The latter example is part of a strategy for one-pot membrane assembly and then release that will be further discussed in section 3 on this topic. It has also been shown that imine hydrolysis

generating migration of dodecylamine from a single chain imine-containing amphiphile to another containing an aldehyde was effective for triggering *in situ* formation of giant vesicles.[79] Seoane et. al. demonstrated that imine formation between sphingomyelin and oleylaldehyde was capable of driving spontaneous self-assembly into bilayers. Due to the reversibility of the imine bond, the vesicles responded to external stimuli including temperature or aldehyde addition that triggered controlled release of encapsulated cargo.[80] The reaction between **22** and (Z)-9-octadecenal in HEPES buffer proceeded rapidly. Oil droplets could be observed immediately after mixing precursors, and vesicles started to show up after 10 minutes under microscope. While vesicles formed using imine chemistry are typically in equilibrium with their precursors and lack robustness, stability was improved through imine reduction to lock in bilayer lipid products.

Native chemical ligation

A more robust and chemoselective catalyst-free chemical reaction is native chemical ligation (NCL), a reaction originally devised for coupling of intact peptides, in which the sulfhydryl moiety of an *N*-terminal cysteine reacts with a *C*-terminal thioester (Figure 8A). This reaction was originally harnessed by Brea et. al. in an impressive report of spontaneous *de novo* formation of phospholipids from reactive thioesters (**23**) and lyso lipid **24a** for self-assembly into vesicles.[81] Building off of that work, it was shown that reversible NCL (RNCL), a reaction that occurred spontaneously between tertiary amides (specifically, lyso-lipid **24b**) and thioesters (**23**), could assist in the exchange and remodeling of both lipid acyl chains and head groups,[82] resulting in changes to self-assembly, spatial organization, composition, and vesicle morphology. Under typical NCL conditions, **23** and **24b** afforded aminophospholipid over 30 min. Subsequent RNCL proceeded rapidly, generally within several minutes. This exciting approach using non-enzymatic, chemoselective reactions enabled spontaneous formation of dynamic membranes in a manner that

physical and chemical properties could be directly modulated. Another impressive display exploited NCL for the *de novo* formation of synthetic proteoliposomes inside which the adenosine A_{2A} receptor ($A_{2A}R$) was simultaneously reconstituted.[83] This technique harnessed NCL to rapidly generate $A_{2A}R$-embedded liposomes from receptor solubilized in *n*-dodecyl-β-D-maltoside analogs (**25**, Figure 8B). This commendable method of reconstituting membrane proteins was orthogonal, fast, and biocompatible.

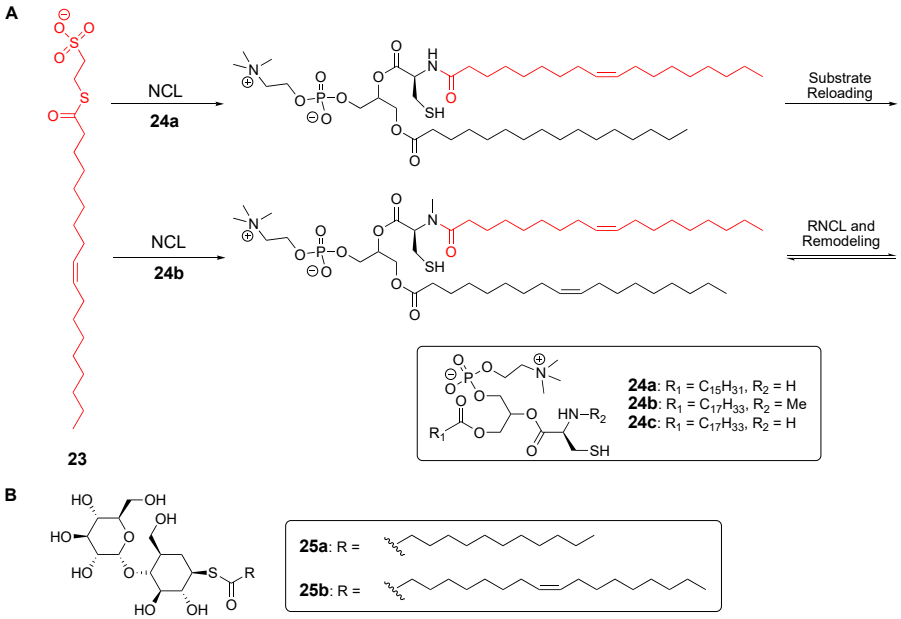


Figure 8. *In situ* generation of membrane bilayers using NCL reaction.

Other catalyst-free chemical reactions

A number of other chemical reaction types have thus far been used to generate liposomes. Thioester exchange chemistry between thiol *lyso*-lipids (**26a,** Figure 9) has been used to form dynamic membranes, and changes in the reaction conditions caused differences in exchange rates,

temperature sensitivity, permeability, and exchange behavior.[84] Similarly, catalyst-free thiol-Michael click chemistry between **26a** and an acrylate-functionalized tail has been used to drive the formation of lipid vesicles.[85] Work by Devaraj et. al. showed that histidine ligation could be used to couple histidine-functionalized lysolipid **26b** with fatty acyl thioester **23**, forming phospholipids that self-assembled into vesicles,[86] a method that was efficient, catalyst-free, and chemoselective. An additional approach utilized transacylation to synthesize natural diacylphospholipids.[87] A combination of ion pairing and self-assembly between lysophospholipids and acyl donors (**27**) resulted in an *in situ* self-assembled membrane. Instead of chemical reactions, binding interactions can also be harnessed to generate bilayer assemblies *in situ*. As an example, Jiang and co-worker took advantage of imine formation as well as boronic acid-carbohydrate binding interactions to form vesicles.[88] Selective glucose sensing was achieved by simply mixing commercially available 4-formylphenylboronic acid and octylamine.

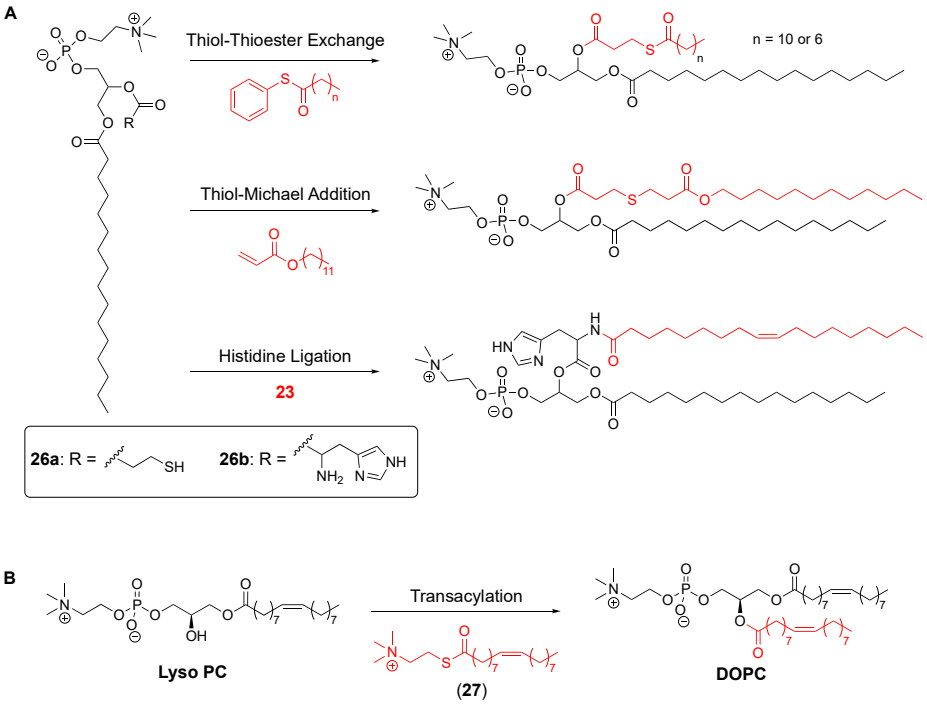


Figure 9. Examples for synthesizing bilayer-forming phospholipids from various reactions, including thiol-thioester exchange, thiol-Michael addition, histidine ligation, or transacylation reaction. The formed lipids are able to spontaneously self-assemble into vesicles.

Although *in situ* membrane synthesis using chemically catalyzed and catalyst-free approaches affords advantages including efficiency, selectivity, and biocompatibility, a primary drawback is that these methods lack the benefits of enzyme-catalyzed systems in terms of enabling genetically controlled synthesis. In the next section, we will discuss that by combining enzymatic catalysis with chemical synthesis, it is possible to develop reactions that are efficient, chemoselective, and genetically controllable.

2.3. Chemoenzymatic methods for membrane synthesis

In their groundbreaking work, the Devaraj group developed the first combination of enzymatic and chemical methods for *in situ* membrane synthesis (Figure 10).[89] This chemoenzymatic approach first entailed expression and purification of the mycobacterial ligase FadD10 from *E. coli*, which was exploited to generate fatty acyl adenylates from fatty acids, magnesium, and ATP precursors. The fatty acyl adenylates were then chemically reacted with amine-functionalized lysolipids to form phospholipids, leading to the growth of vesicles. A turbid solution was observed after 20 min of precursor mixing, as a result of formation of a large population of vesicles. This chemoenzymatic combination resulted in the *de novo* formation and growth of phospholipid membranes, but the enzyme expression and lipid formation reaction were not performed simultaneously.

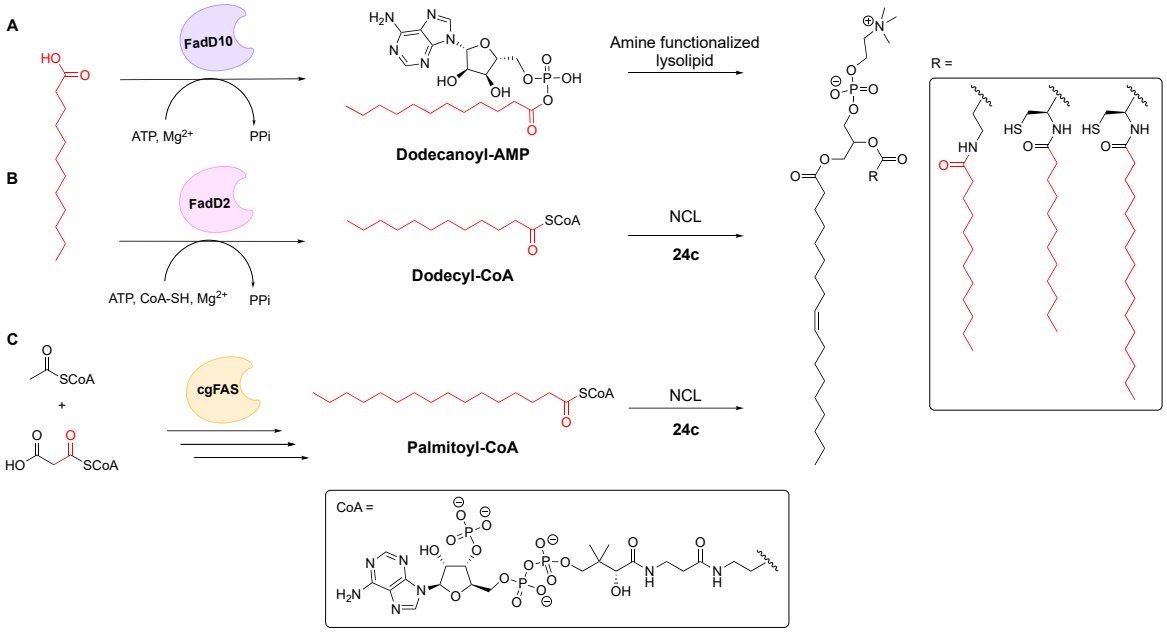


Figure 10. Chemoenzymatic approach for *in situ* building of phospholipid membranes from simple precursors by combining fatty acid synthases with chemical modification.

More recently, a method was developed in which enzyme expression as well as chemical and enzymatic reactions occurred simultaneously in a one pot reaction to generate membrane-forming lipids.[90] DNA encoding a fatty acyl-CoA ligase (FACL) (specifically FAD2) was used to produce fatty acyl-CoA, which immediately coupled with cysteine-modified lysolipids via NCL. This chemoselective and rapid strategy was the first of its kind to synthesize proteins and chemoenzymatically generate lipid membranes in a one-pot reaction. The Devaraj group developed another chemoenzymatic strategy for lipid membrane generation directly from water soluble acetyl-CoA and malonyl-CoA by combining a soluble bacterial fatty acid synthase (cgFAS I) and native chemical ligation.[91] In the enzymatic phase, the type I FAS B from *Corynebacterium glutamicum* (cgFAS I) was used to synthesize palmitoyl-CoA *in situ* from acetyl-CoA and

malonyl-CoA. Then, in the chemical phase, palmitoyl-CoA was coupled with cysteine-modified lysophospholipid **24c** by NCL. All lysolipid **24c** was consumed within 4 hours. The resulting phospholipids then spontaneously self-assembled into lipid vesicles. Small vesicular structures could be observed using fluorescence microscopy after 30 minutes, which grew into large vesicles of 1-2 μm after overnight incubation.

These enzymatic, chemical, and chemoenzymatic methods have been rapidly evolving for *in situ* liposome generation in ways that are controllable, rapid, selective, and robust. Future studies should continue the trend of combining the best qualities of enzymatic and chemical methods to further the field of chemoenzymatic control.

3. Combined liposome demolition and rebuilding

While programmed membrane assembly and release have primarily been explored independently, recent work has begun to demonstrate that these two approaches can be combined for sequential membrane decomposition and regeneration. For example, light irradiation has been exploited for both *in situ* assembly of liposomes and subsequent triggering of content release. Here, Zhang and co-workers utilized CuAAC to drive the *in situ* assembly through the coupling of alkynyl-PC analog **15** and azido-lipid **28** containing a photocleavable *o*-nitro benzyl moiety.[92,93] Following assembly, light irradiation was used to decompose the resulting phospholipid into a new product that permeabilizes the membrane. This approach was extended when Konetski and co-workers exploited photocleavage to induce pinocytosis-like behavior upon irradiation.[94] Such systems can not only enhance payload release but also provide control over the self-assembly process. Prins and co-workers recently reported several dissipative self-assembly systems through

binding interactions between surfactants and nucleotides or ATP.[95-97] Mixing amphiphiles and reagents resulted in the templated formation of self-assembled vesicles, the stability of which could be controlled by the enzymatic hydrolysis of the templating nucleotides/ATP. In one example, the reversible nature of the process was demonstrated by performing up to seven cycles with the same sample; the signal returned to the starting value when adding new batches of ATP each time.

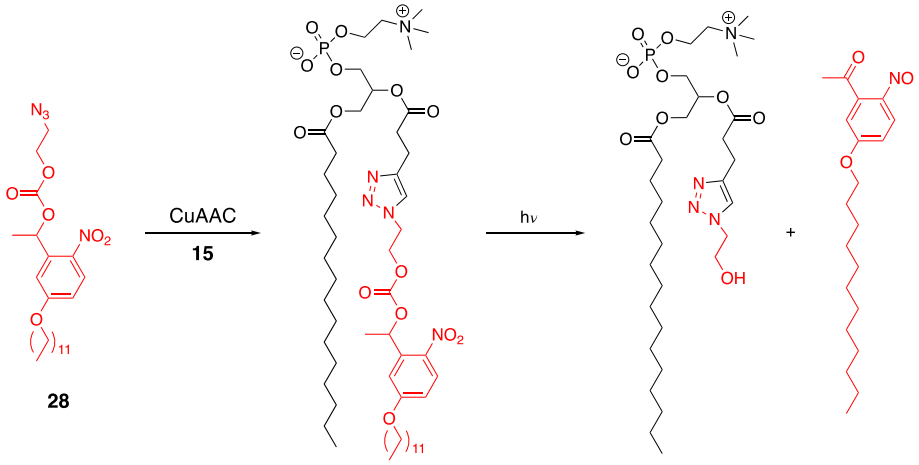


Figure 11. Combination of CuAAC and light irradiation reactions allowed *in situ* membrane formation and subsequent demolition.

Conclusions

The work described in this perspective article shows that a number of clever strategies have been firmly established in a short amount of time for both liposome triggered release and *in situ* membrane synthesis. These approaches both take advantage of our nuanced understanding of lipid self-assembly properties to carefully manipulate nanostructures. However, due to the bold and ambitious applications that are being pursued using this technology, these pioneering examples

open the door for many future studies that are needed. In this manner, complex challenges must be overcome to manipulate membranes within the environments of living organisms.

While numerous creative liposome release strategies have been successfully developed, the bottom line is that these approaches are yet to be included in clinically approved therapeutic liposome formulations. This can be attributed to several obstacles pertaining to the fact that clinical liposomes must be sufficiently robust to survive circulation while remaining effective for triggered release. This includes the need for lipid switches to retain activity in the context of functionalized liposomes decorated with polymer shielding groups and cell-targeting moieties. In addition, the heterogeneity of disease microenvironments poses further impediments towards applicability of smart delivery systems, and thus multi-functional systems that combine different release strategies may be beneficial. Overall, lipid switches that are effective at low percentages and tolerate a wide range of commonly used clinical liposome formulations would be ideal. For these reasons, despite the potential and *in vitro* success of triggered release formulations, further research is needed to translate triggered release protocols into clinical practice.

Rapid advancements toward *in situ* membrane assembly have been achieved in recent years that have rendered this process more robust, controllable, and biocompatible, while also enabling their initiation from simple and accessible precursors. However, the primary goal of this work in generating true artificial cells is a tall order, and one that will require sophisticated aspects of structure and process to be ingrained with in synthetic membranes. The reports discussed herein have begun to meet these challenges through the incorporation of artificial metabolic networks consisting of both enzymatic and catalytic reactions into spontaneously assembled membranes. Future work can focus on increasing the sophistication of processes that can be programmed within

synthetic cells. In this way, while much work needs to be done, exciting recent work has paved the way for fulfilling these grand ambitions.

AUTHOR INFORMATION

Corresponding Author

*Michael D. Best - *Department of Chemistry, University of Tennessee, Knoxville, Tennessee 37996, United States; orcid.org/0000-0001-8737-5910; Email: mdbest@utk.edu*

Author Contributions

‡These authors contributed equally.

The manuscript was written through contributions of all authors.

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Notes

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Author Biographies

Megan L. Qualls received her BS in Nutrition from The University of Tennessee, Knoxville in 2018. Under the supervision of Prof. Brian K. Long, she conducted polymer chemistry research focusing the synthesis of organometallic olefin polymerization catalysts. Also, she worked with Prof. Dallas Donohoe, conducting cellular and molecular nutrition research to investigate the link between microbial-derived metabolites and colorectal cancer. She then began her doctoral studies at The University of Tennessee, Knoxville in Prof. Michael D. Best's laboratory in 2018. Her current research focuses on developing targeted liposome platforms for therapeutic drug delivery applications.

Ruhani Sagar finished her Bachelor's and Master's degrees in Chemistry from Panjab University, India in 2018. During this, she conducted supramolecular chemistry research under the supervision of Dr. Subash Chandra Sahoo focusing on the synthesis and characterization of coordination complexes using succinic acid derived multidentate ligands. She then began her doctoral studies at The University of Tennessee, Knoxville in Prof. Michael D. Best's laboratory in 2018. Her current research focuses on developing triggered release liposome platforms for therapeutic drug delivery applications.

Jinchao Lou is a graduate student at the University of Tennessee at Knoxville, department of Chemistry. She is currently working toward a Ph.D. in Organic Chemistry with Prof. Michael Best. She received her B.S in Chemistry (2016) from Northwest Normal University, Lanzhou, China. Her projects mainly focus on developing stimuli-responsive liposomal platforms for drug delivery applications.

Dr. Michael Best grew up in Rochester, New York. He received his B.S. in Chemistry from Boston College in 1997 working with Prof. Lawrence T. Scott, his Ph.D. Chemistry in 2002 from the University of Texas at Austin with Prof. Eric V. Anslyn, and then performed post-doctoral research with Prof. Chi-Huey Wong at The Scripps Research Institute. In 2005, Dr. Best joined the faculty at the University of Tennessee, where he is currently a Paul and Wilma Ziegler Professor of

Chemistry. Dr. Best's group generally studies the chemical biology of lipids, including the development of stimuli-responsive liposomes using synthetic lipid switches as well as chemical probes for analyzing signaling lipids involved in important biological pathways.

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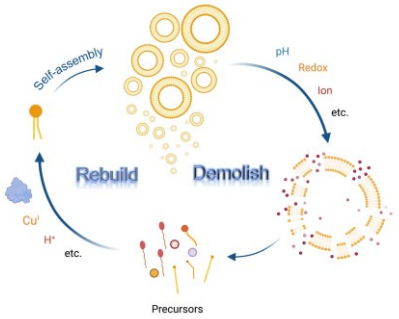
TOC Graphic:


Self-assembly
pH
Redox
Ion
etc.
Rebuild
Demolish
Cu
H
etc.
Precursors