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In Situ Synthesis of Phospholipid Membranes

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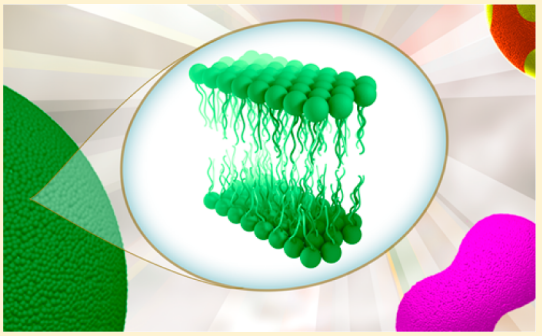
ABSTRACT: Cells produce lipid membranes de novo through a complex sequence of enzymatic reactions that are difficult to reconstitute in a minimal system. We set out to take a different approach and mimic the synthesis of phospholipids using abiotic but highly selective bioconjugation reactions. Here, I outline several of our group's recent advances in exploring chemoselective reactions for stitching together lipid fragments to form membrane-forming lipids from non-membrane-forming precursors. Rapid chemical reactions can be harnessed to achieve facile de novo synthesis of lipid membranes, and spontaneous membrane formation can be applied for the reconstitution of membrane proteins, encapsulation and concentration of nanomaterials, and the study of lipid membrane remodeling. I conclude by briefly outlining future challenges and opportunities.



All living organisms possess lipid membranes, barrier structures that are principally composed of phospholipids. Phospholipid vesicles are used as model systems for mimicking living cell membranes and have found wide application in bottom-up synthetic biology,[1,2] drug delivery,[3] microreactor design,[4] and origin of life studies.[5,6] It has been hypothesized that the coupling of catalytic activity with phospholipid synthesis was a crucial and early step in the evolution of life on Earth,[7,8] and over the last few decades, there has been significant interest in reconstituting natural biochemical pathways to drive lipid membrane synthesis in minimal synthetic cells.[9−12] Previous studies using reconstituted purified enzymes have demonstrated the synthesis of additional lipid components.[13] However, lipid synthesis yields were extremely low (<10%), and as a consequence, enzyme-driven de novo vesicle formation or vesicle division was not feasible.[13] The poor efficiency of phospholipid synthesis using lipid-synthesizing enzymes is likely due to the fact that natural lipid synthesis pathways are complex, relying on multiple enzyme-catalyzed steps and involving several spatially sequestered precursors and cofactors. Furthermore, several key lipid synthesizing enzymes, such as lipid acyltransferases, are integral membrane proteins that are difficult to reconstitute with high yield and activity.[13] These inefficiencies limit rapid generation of new membranes, which would facilitate propagation of vesicles as synthetic "cells" as well as practical applications where membrane proteins or materials need to be quickly reconstituted. To circumvent these problems, we sought to develop simplified nonenzymatic approaches to generate phospholipid vesicles quickly and in high yield from reactive precursors.

There are two major pathways of lipid synthesis in eukaryotic cells.[14] Phospholipids are initially synthesized by the formation of acyl chains, which are then coupled to glycerol based head groups. This de novo, or Kennedy, pathway of lipid synthesis is quite complex and involves multiple enzymatic reactions. Mimicking the Kennedy pathway nonenzymatically would be quite challenging. An alternative lipid synthesis pathway is the remodeling pathway or Lands cycle. In this pathway, a lipid formed via the de novo pathway is acted on by an enzyme such as phospholipase A_2, leading to the removal of one of the acyl chains. The resulting lysophospholipid is then combined with a thioester derivative of a fatty acid through the action of a lysophospholipid acyltransferase, forming a new lipid with different acyl chain composition (Figure 1A). We were inspired by the Land's cycle, since a single enzymatic step leads to the formation of a membrane forming phospholipid from two non-membrane-forming single-chain precursors, in this case a lysophospholipid and a thioester derivative of a fatty acid. Indeed, previous work from Deamer and co-workers had shown that crude microsomes with acyltransferase activity were capable of forming phospholipid membranes in the presence of appropriate lipid precursors.[9,15] We therefore sought to mimic the function of the acyltransferase by exploring whether organic chemical reactions could be used to couple together lipid fragments, namely molecules that closely resembled single-chain lipid precursors such as a lysophospholipid and fatty acid. The use of organic reactions to form phospholipids would potentially enable nonenzymatic de novo synthesis of lipid membranes.

One method to form phospholipids is through coupling of lysophospholipid with an activated ester of a fatty acid, for instance, an *N*-hydroxysuccinimide ester. Unfortunately, the use of such reactions is not compatible with biological functional groups such as amines or thiols and may not be expected to proceed efficiently in aqueous media due to hydrolysis. For these reasons, we searched for more chemoselective reactions to achieve lipid coupling and were immediately attracted to the

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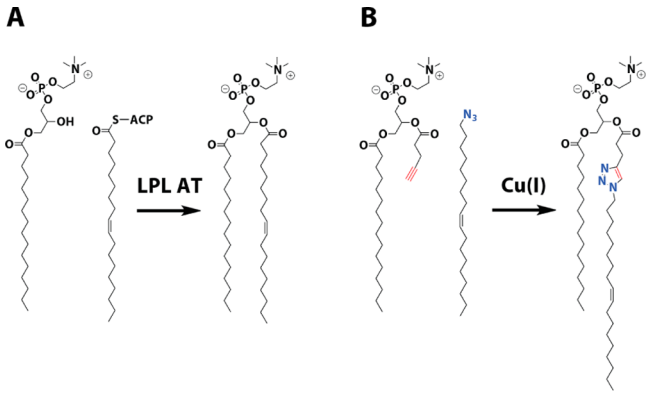

Figure 1. Synthesis of phospholipid membranes using bioorthogonal chemistry. (A) Native phospholipid synthesis uses acyl-transfer reactions between lysophospholipids and thioester derivatives of fatty acids, which are catalyzed by membrane-bound enzymes. (B) Mimicking the enzymatic process using copper-catalyzed cycloaddition between an alkyne derivative of a lysophospholipid and oleyl azide. The resulting triazole phospholipid is a close analogue of a natural phospholipid. LPL AT, lysophospholipid acyltransferase, ACP, acyl carrier protein. Reproduced from ref 18. Copyright 2012 American Chemical Society.

idea of using bioorthogonal reactions.[16] Bioorthogonal coupling reactions are now commonly utilized and proceed efficiently in water and in the presence of a vast array of biologically relevant functional groups. One of the most commonly used bioorthogonal conjugation reactions is the copper-catalzyed azide−alkyne cycloaddition (CuAAC).[17] Thus, in our first demonstration of in situ phospholipid synthesis, we decided to utilize CuAAC "click" chemistry to couple together lipid fragments.[18] We decided to modify a lysophospholipid with an alkyne and use a long-chain azide in lieu of a fatty acid. In the presence of copper catalysts, these two fragments couple together forming a close analogue of a natural phospholipid with the exception of a triazole linkage (Figure 1B). Surprisingly, the presence of the triazole linkage appears to have little effect on the membrane-forming properties of the phospholipid, and, somewhat remarkably, the physical characteristics of the resulting membranes (e.g., melting temperature, fluidity) are quite similar to membranes formed from analogous native phospholipids.

If the azide and alkyne precursors are mixed together in water in the absence of copper, an emulsion forms but no membranes can be visualized with a membrane-staining fluorescent dye. Once the Cu^{1+} catalyst is supplied, within minutes large tubules and vesicles can be visualized, with many appearing to bud from the azide oil droplets (Figure 2). After many hours, the reactants are nearly consumed, and numerous vesicles are observed. In parallel, monitoring the reaction by liquid chromatography mass spectrometry validates that the triazole forming reaction has taken place. Bolstered by time-lapse videos, we believe that the alkyne lysolipid, which is dispersed as micelles, is able to react at the interface of the azide oil droplets, creating layers of phospholipid that eventually hydrate and bud off forming tubules and vesicles.

In this initial work, we used hydrated copper ions as the catalyst and were able to achieve de novo membrane synthesis in aqueous systems. Soon after, we began experimenting with copper-binding heterocyclic ligands, which were known to accelerate the CuAAC reaction.[19] Indeed, when heterocyclic ligands were added, the copper reducing agent concentrations could be dramatically reduced and the reaction progressed even in the presence of mild copper chelators such as TES buffer. In such conditions, in the absence of ligand, triazole formation between lipid fragments does not take place. One interesting class of heterocyclic ligands that are well-known to accelerate the CuAAC reaction are oligotriazoles such as tris-(benzyltriazolylmethyl)amine (TBTA).[19] A unique feature of oligotriazole-assisted triazole synthesis is that the ligands themselves can be synthesized by the very reaction they help catalyze (Figure 3A). Thus, under appropriate conditions, oligotriazole synthesis displays the features of an autocatalytic

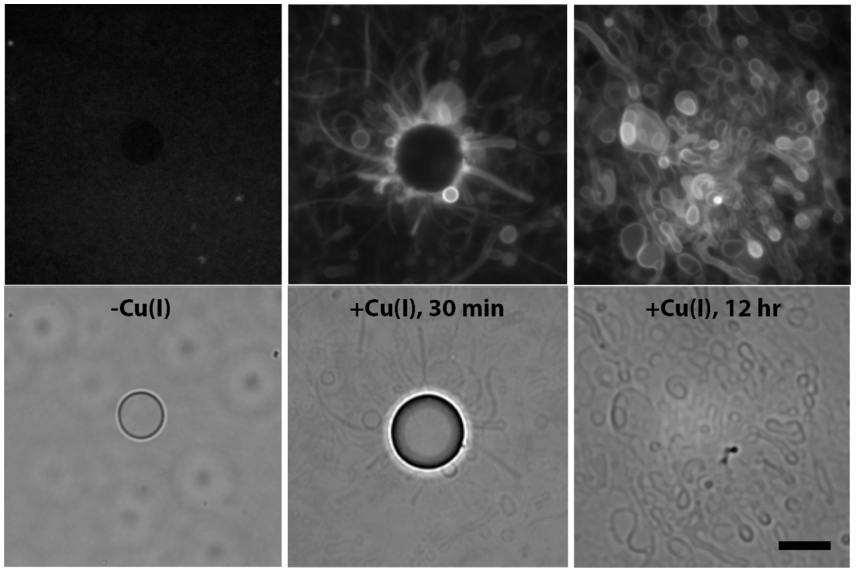

Figure 2. Spontaneous vesicle assembly driven by triazole-based phospholipid synthesis. An aqueous emulsion of oleyl azide (5 mM) and alkyne lysolipid (5 mM) imaged without copper catalyst (left), 30 min after addition of catalyst (0.25 mM) (middle), or 12 h after addition of catalyst. Top panels are fluorescence images of vesicles using a membrane dye (Rh-DHPE, 2 μM). Bottom row shows the corresponding phase contrast images. See text for discussion. Reproduced from ref 18. Copyright 2012 American Chemical Society.

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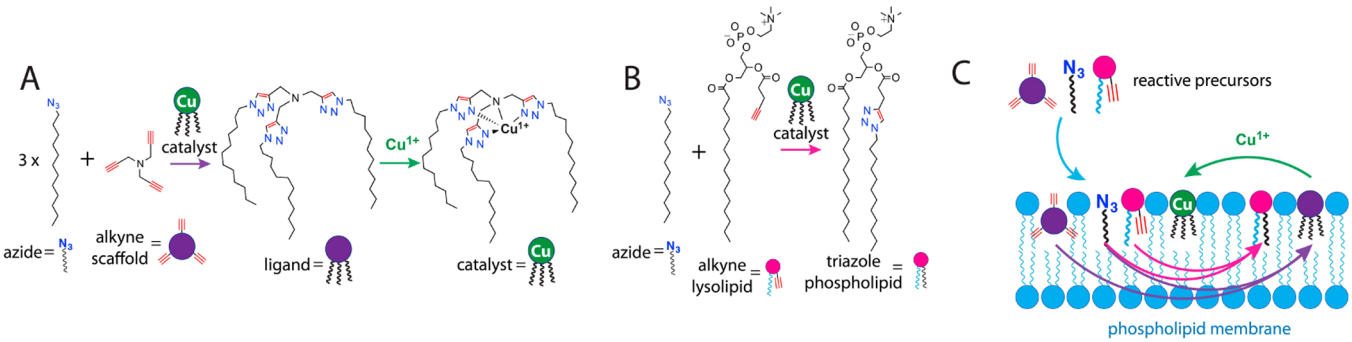

Figure 3. Self-reproducing catalyst drives phospholipid synthesis. (A) Oligotriazole (ligand) is capable of binding Cu^{1+} ions to form a catalytic complex (catalyst). The complex catalyzes the synthesis of additional ligand from 1-azidododecane (azide) and tripropargylamine (alkyne scaffold), which, upon metalation with copper, produces additional catalytic molecules. (B) The copper complex also catalyzes the formation of triazole phospholipid from azide and an alkyne derivative of a lysolipid. (C) Membrane embedded catalysts act on azide and alkyne precursors, synthesizing more phospholipid and oligotriazole ligands, which, upon metalation, become new catalysts. Reproduced with permission from ref 6. Copyright 2015 National Academy of Sciences.

reaction. Utilizing oligotriazole self-reproduction, we demonstrated an autocatalytic system for achieving the continual growth and reproduction of lipid membranes.[6] Cellular membranes of living organisms are dynamic structures, and there have been numerous constructs that model phospholipid membranes. However, unlike natural membranes, biomimetic systems cannot sustain growth owing to an inability to replenish phospholipid-synthesizing catalysts. Oligotriazole autocatalysis enabled the design and synthesis of artificial membranes embedded with synthetic, self-reproducing catalysts capable of perpetuating phospholipid bilayer formation.

In our work, we replaced the complex biochemical pathways used in nature with an autocatalyst that also drives lipid synthesis (Figure 3B), leading to the continual formation of triazole phospholipids[18] and membrane-bound oligotriazole catalysts from simpler starting materials. The synthesis of triazoles by copper-catalyzed azide−alkyne cycloaddition[17] is highly selective, has a high thermodynamic driving force, and works well in water.[20] The oligotriazole catalyst was synthesized by coupling tripropargylamine with 1-azidododecane, forming a tris(lauryl triazole)amine (TLTA) ligand. The TLTA trimer acts as a catalyst for further TLTA ligand synthesis in the presence of Cu^{1+} and TES buffer, completing an autocatalytic cycle. The background reaction is inhibited by the presence of TES buffer (140 mM), a buffer that can coordinate free copper.[21]

Lipid vesicles containing TLTA were capable of generating molecular catalysts and phospholipids in the presence of catalyst and phospholipid azide and alkyne precursors (Figure 3C). Therefore, synthetic vesicles can continually catalyze lipid synthesis. The growth of vesicles was monitored using time-lapse fluorescence microscopy and a vesicle-tracking algorithm that monitors vesicle size and location.[22] When catalytic triazole membranes from a serial transfer experiment were added to solutions containing reactive precursors, vesicular structures appeared that grew slowly in size over a period of hours (Figure 4). We also demonstrated that the catalytic membranes are able of remodel their physical composition in response to changes in the environment, in this case by demonstrating a preferential incorporation of alkyl azide precursors.

One advantage of using non-biological reactions to trigger phospholipid synthesis is the possibility of using unconventional strategies to control the formation of lipid membranes. Several investigators have shown that the CuAAC reaction can

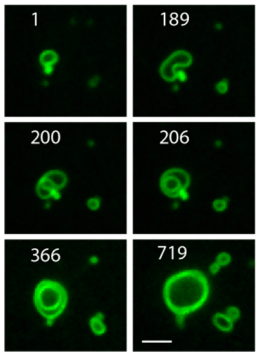

Figure 4. Lipid synthesis results in an increase in vesicle surface area and volume. Numbers are elapsed time, in minutes, from start of fluorescence imaging. Scale bar represents 3 μm. Reproduced with permission from ref 6. Copyright 2015 National Academy of Sciences.

be controlled by light, typically through a mechanism by which light triggers the reduction Cu^{2+} and thus formation of the active Cu^{1+} catalyst.[23,24] In collaboration with the Bowman laboratory, we demonstrated that ruthenium photoredox reactions can be used to trigger phospholipid membrane formation in response to visible light.[25] Subsequent work has shown that alternative mechanisms can also be used for light-triggered lipid formation, even enabling spatiotemporal control of membrane synthesis.[26] It will be interesting to see if additional advances enable the use of light to spatiotemporally control lipid synthesis within single vesicles, possibly allowing localized curvature events and stimulation of membrane division or fusion.

During our work with the azide−alkyne cycloaddition, we also became interested in exploring whether alkynes could react with other functional groups to create phospholipid analogues in a selective manner. It is well-known that alkynes can react with thiols via the thiol−yne "click" reaction, forming dithioethers.[27,28] We synthesized various polar head groups bearing alkynes and reacted them with long-chain alkyl thiols via photoinitiated thiol−yne click chemistry, which results in the sequential conjugation of two alkyl chains and the formation of various phospholipids and glycolipids.[29] Interestingly, the structure of the resulting dithioether lipids resembles that of naturally found ether lipids. The synthesis can be done in one pot and is straightforward and modular, with the ability to easily change the alkyl chain length and the polar headgroup.

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A unique property of thioether lipids, compared to standard diacyl phospholipids, is their expected ability to withstand hydrolysis in the presence of phospholipases. We exploited this property by creating large vesicles with mixtures of natural and thioether phospholipids, and exposing them to phospholipase. The enzyme rapidly breaks down the natural phospholipid, and shrinkage of the vesicles is observed. Encapsulated small molecules or proteins leaked out during the reaction, despite the vesicle maintaining its overall structure and the inability to visualize defects by light microscopy (Figure 5A). We believe it

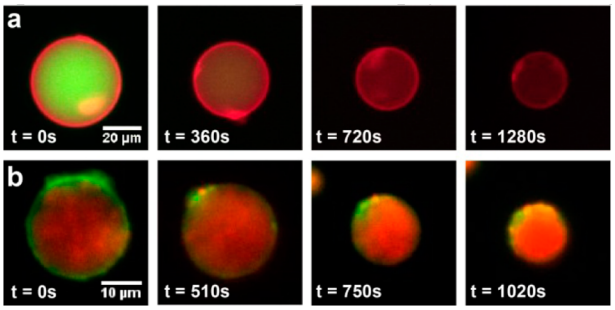

Figure 5. Time-lapse microscopy images of shrinking vesicles. (A) GFP (green) leaks from vesicles upon phospholipase induced shrinkage. The GUV membrane was stained with Texas Red DHPE. (B) 100 nm polystyrene nanoparticles (red) were contained and concentrated in response to phospholipase induced shrinkage. The membrane was stained with NBD-PE. Reproduced with permission from ref 29. Copyright 2015 Royal Society of Chemistry.

is likely that small nanopores are being formed during lipid hydrolysis. Indeed, if larger 100 nm fluorescent beads are encapsulated within the vesicles, the beads do not escape and appear to be concentrated after the reaction (Figure 5B). These "shrink-wrap" vesicles could find application as a novel way to concentrate materials in liposomes such as nanoparticle payloads and larger macromolecular complexes.

Although our initial work with azides and alkyne precursors demonstrated that nonenzymatic in situ phospholipid membrane synthesis was feasible, we were aware of several shortcomings. Some of the precursors (e.g., alkyl azides) were insoluble in water, and the reactants typically formed a two-phase emulsion. We envisioned that a monophasic dispersion might be desirable in particular applications. The triazole-forming reaction used to link the lipid fragments together requires Cu^{1+} ions as catalysts. Such redox-active metal ions might prove to be problematic in the presence of biological molecules or living systems, given the ability of copper to damage biomolecules and be cytotoxic.[30,31] Finally, the azide and alkyne precursors are quite exotic functional groups which, although contributing to the "bioorthogonal" nature of the coupling, also meant that our system was very far removed from biochemically analogous reactions that use thioester derivatives of fatty acids. To overcome these issues, we decided to pursue developing lipid synthesis reactions that could occur spontaneously in the absence of catalysis and used water-soluble thioester derivatives as precursors.

In considering a chemoselective coupling reaction that uses thioesters as reactants, we were immediately drawn to the native chemical ligation.[32] The native chemical ligation is a powerful tool that is widely used by protein chemists to conjugate peptide fragments together.[33] An N-terminal cysteine on one peptide reacts with a thioester on another peptide by trans-thioesterification. Subsequently, an irreversible S−N acyl shift occurs, resulting in the formation of an amide and coupling of the two peptides. We hypothesized that the native chemical ligation could be adapted for the synthesis of phospholipids (Figure 6). We synthesized a single-chain phospholipid fragment bearing a cysteine modification and also created a thioester derivative of a fatty acid bearing a charged headgroup. Both of these lipid fragments were readily dispersed in water and form micelles. When combined, native chemical ligation spontaneously and rapidly takes place, leading to the generation of membranes composed of phospholipid analogues.[1] The membranes were capable of forming sealed vesicles and had physical properties similar to natural phospholipid membranes. Furthermore, the formation of membranes could be monitored by light microscopy (Figure 7). Images taken upon initial mixing showed no aggregates, as expected since the precursors only form submicroscopic micelles. However, over time we observed the appearance of wormlike micelles and aggregates, which eventually collapse to form more spherical vesicles. The rapid speed of the reaction was striking, with full formation of product occurring within 1 h. We believe the favorable reaction kinetics are due to the formation of organized mixed micelles, forcing high local concentration of the reactants. An interesting future question will be whether or not higher resolution techniques such as cryo-electron microscopy will be able to capture the intermediate structures between micelles and vesicles.

Adapting a peptide ligation technique for phospholipid synthesis led us to consider whether alternative peptide ligation strategies could also be exploited for conjugating lipid fragments together. Furthermore, the use of thiol containing cysteines requires that adequate reducing agents are present. We recently had success in adapting the histidine ligation technique to couple single-chain lipids bearing a histidine residue to thioester derivatives of fatty acids.[34] This technique might prove useful in situations where the presence of reducing agent or thiols is not desirable.

One important aspect of biological phospholipids is their amazing diversity of head groups and acyl chains. Some estimates indicate that there are over 100 different phospholipid species in mammalian cells.[35] An important mechanism that contributes to this diversity of phospholipids is lipid remodeling.[36] Lipid remodeling uses enzymes such as phospholipases, acyltransferases, or transacylases to modify the structure of already synthesized phospholipids. Phospholipid remodeling is important for lipid signaling, is responsible for creating disaturated phospholipids needed for pulmonary surfactant, and is a requirement for cardiolipin, an important mitochondrial specific phospholipid. To illustrate the importance of phospholipid remodeling, consider that a genetic defect in the gene tafazzin, a transacylase that remodels the acyl chains of cardiolipin, leads to a debilitating condition known as Barth's syndrome.[37,38] Our initial forays into phospholipid synthesis utilized irreversible chemoselective reactions to stitch together lipid fragments. While these reactions crudely mimicked the formation of phospholipids, we were unable to recapitulate phospholipid remodeling after synthesis due to the irreversible nature of the chemistry.

To mimic lipid remodeling, we required a selective coupling reaction that could also be reversible. However, it is important that the coupling product remains stable enough in water to preserve membrane function and enable typical vesicle properties such as the ability to encapsulate small molecules.

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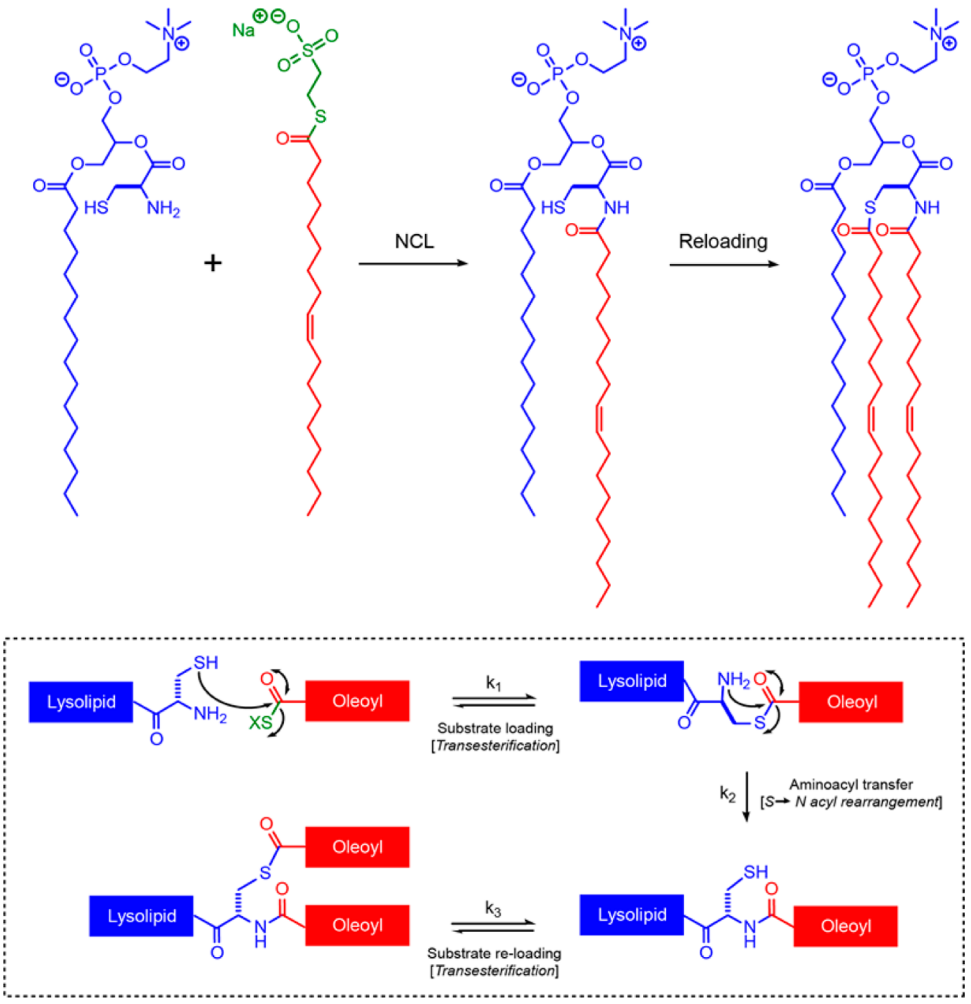

Figure 6. Synthesis of phospholipids by the native chemical ligation reaction of a cysteine-functionalized lysolipid and a thioester derivative of a fatty acid. Framed: the mechanism of the native chemical ligation and possible substrate reloading. Reproduced with permission from ref 1. Copyright 2014 Wiley.

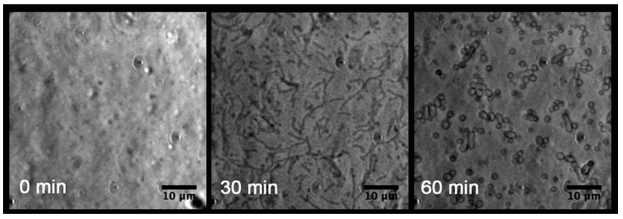

Figure 7. In situ vesicle formation. An aqueous buffer solution of cysteine-functionalized lysolipid and a thioester derivative of oleic acid was imaged at different times after initial mixing. Initially, phospholipid membranes were absent. However, shortly after mixing of both precursors, the de novo formation and growth of vesicle and tubular structures was observed. Reproduced with permission from ref 1. Copyright 2014 Wiley.

Given our previous adaptation of the native chemical ligation to lipid synthesis, we were excited to read a report in the literature describing a reversible native chemical ligation for peptides.[39] The method utilizes *N*-methylcysteines that reversibly form tertiary amides, which can readily undergo a trans−cis isomerization followed by an N−S acyl shift reaction. We explored the application of the reversible native chemical ligation to lipid membranes and were able to show that the tertiary amide containing phospholipids formed were stable enough to form liposomes and could be remodeled by exposure to new *N*-methylcysteine-modified single-chain phospholipid precursors.[40] The overall reaction, where an acyl chain is transferred from one phospholipid to another, is analogous to the remodeling reaction catalyzed by transacylase enzymes. To our knowledge, this was the first example of nonenzymatically remodeling the acyl chains of artificial phospholipid membranes. While exposure to new *N*-methylcysteine-containing lipid fragments leads to reversible remodeling, exposure to cysteine containing lipid fragments leads to irreversible remodeling as the product no longer is expected to undergo N−S acyl shift. We decided to exploit irreversible remodeling to trigger compositional and organizational changes in giant unilamellar vesicles (GUVs). It was well-known from previous studies that GUVs containing mixtures of saturated and unsaturated phospholipids, along with cholesterol, can spontaneously form microdomains of disordered and ordered regions.[41] We decided to form a GUV from unsaturated phospholipids that could be remodeled by native chemical ligation (Figure 8). Addition of a saturated cysteine-containing phospholipid fragment triggered irreversible remodeling and the formation of a mixture of saturated and unsaturated phospholipids. Using a membrane dye that associates with less ordered regions, the process of reorganization could be

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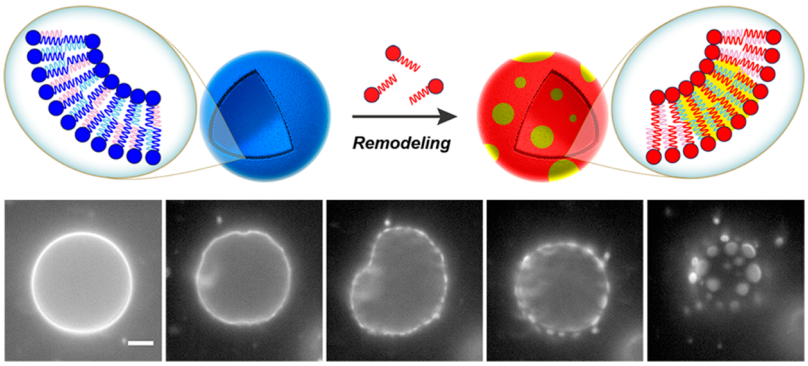

Figure 8. Vesicle microdomain formation induced by nonenzymatic lipid remodeling. (Top) Representation of microdomain formation directed by membrane remodeling of phospholipid vesicles. GUVs composed of unsaturated lipids are remodeled by exchange reactions with a saturated cysteine-functionalized lysolipid, leading to the formation of a new class of GUVs composed of a mixture of unsaturated and saturated lipids. (Bottom) Fluorescence microscopy images showing membrane remodeling and subsequent microdomain formation driven by the nonenzymatic remodeling reactions. An aqueous buffer solution of GUVs composed of two unsaturated *N*-methylcysteine phospholipids (18:1−18:1 and 16:0−18:1) and cholesterol (400 μM total lipid concentration) is combined with a saturated (16:0) cysteine-functionalized lysolipid (400 μM) in the presence of DTT (2 mM) and imaged at different times after initial mixing (times from left to right, 0, 76, 80, 88, and 104 s). Initially, lipid microdomains are not present. However, shortly after the species are mixed, lipid remodeling takes place and microdomain formation is observed, as indicated by the partitioning of a fluorescent dye into discrete circular regions of the membrane. Scale bar denotes 10 μm. Reproduced with permission from ref 40. Copyright 2016 National Academy of Sciences.

visualized by fluorescence microscopy. Initially the dye is homogeneously distributed in the GUV. Addition of the saturated acyl chain causes morphological changes to the membrane. However, the vesicle maintains its overall integrity, and within minutes, bright and dark domains of micron size are observed, indicating that spontaneous microdomain formation has been triggered. It is interesting that the addition of a single reagent and a spontaneous reaction can lead to such dramatic micron scale changes in vesicle organization. We hope to be able to utilize this technique to study how such changes in membrane organization can affect the function of embedded membrane proteins (vide infra). Additionally, it is important to point out that head-groups could also be exchanged using reversible covalent chemistry. By adding phospholipid fragments with a negatively charged headgroup, remodeling changes the overall charge of the membranes and leads to the electrostatic recruitment of proteins that can affect membrane curvature. It is likely that additional headgroup modifications will become feasible, and therefore this technique could offer a precise methodology to change lipid head groups.

Our interest in using organic chemistry to trigger in situ phospholipid synthesis stemmed from a basic science question of whether or not such reactions were feasible and an interest in the growth and possible division of lipid assemblies. However, as we began to develop strategies to couple lipid fragments together, we started to explore possible biological applications. In particular, we felt our technology could be relevant to the reconstitution of integral membrane proteins. Integral membrane proteins make up a large fraction of all proteins, and many, such as G protein-coupled receptors (GPCRs), are important drug targets.[42,43] Thus, there is intense interest in the study of the structure and function of membrane proteins. Integral membrane proteins have hydrophobic transmembrane regions that are stable within lipid bilayers and often require lipid bilayer reconstitution to have proper structure and function. It is not trivial to isolate expressed membrane proteins, and protocols typically require large amounts of detergent for solubilization. Strategies often require the use of specific detergents to prevent possible loss of protein structure and function followed by slow and time-consuming removal of detergent.[44] Detergent removal may not be completely successful and can lead to loss of function.

We recognized that our lipid precursors, being single-chain-charged amphiphiles, are capable of acting as reactive detergents. We thus hypothesized that we might be able to solubilize integral membrane proteins using a cysteine-modified single-chain phospholipid as a reactive detergent and then subsequently react the detergent with a thioester via native chemical ligation, forming phospholipid membranes. The integral membrane proteins might find themselves spontaneously embedded in the resulting membranes. The benefit of this approach is that detergent removal is achieved by a rapid chemical reaction that forms phospholipid, and thus sluggish techniques like dialysis are not needed. Furthermore, the ability to react away (and therefore remove) the detergent with near quantitative yield would possibly improve reconstituted protein function.

In our initial study of in situ lipid synthesis for protein reconstitution technology (INSYRT), we decided to work with the well-studied integral membrane protein cytochrome c oxidase (CcO).[45] By tagging the protein with a fluorescent dye, we could observe its location after membrane formation. The protein could be solubilized in a lysolipid analog bearing a cysteine. After addition of an alkyl thioester, native chemical ligation takes place between the cysteine lysolipid and thioester, and membrane bound vesicles were observed. The enzyme was visualized in the membrane, colocalized with a membrane staining dye, indicating that CcO could in fact spontaneously end up in the membrane after the lipid synthesis reaction. While this was interesting, it was not clear from the imaging studies whether the protein remained functional. We verified CcO functionality by demonstrating that the protein could oxidize its native substrate, cytochrome c, and that CcO remained capable of pumping protons across the membrane. Bolstered by this initial success, we quickly tested two additional membrane proteins that also displayed the ability to be spontaneously incorporated into lipid membranes.

G protein coupled receptors (GPCRs) are the largest class of transmembrane proteins in eukaryotic cells and are important drug targets.[46,47] Therefore, there is tremendous interest in

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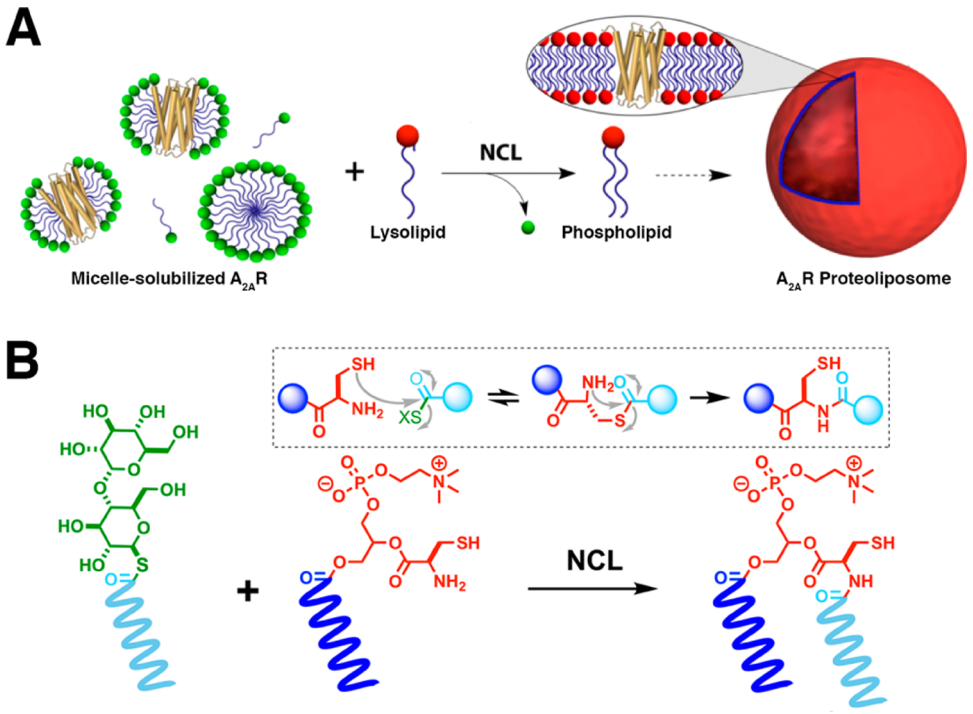

Figure 9. De novo synthesis of lipid membranes and in situ reconstitution of GPCRs. (A) Model for native chemical ligation based phospholipid membrane formation with embedded $A_{2A}R$. (B) Synthesis of phospholipids by native chemical ligation of acyl maltose thioesters and cysteine-functionalized lysophospholipids. Reproduced from ref 50. Copyright 2017 American Chemical Society.

studying GPCR function in reconstituted systems. Unfortunately, GPCRs tend to be challenging to reconstitute, and their function can be easily disrupted during purification and reconstitution. Specialized nonionic detergents such as *n*-dodecyl-*β*-D-maltoside (DDM) have been developed to help aid in their reconstitution.[48] Furthermore, recent years have shown an increased movement toward usage of lipid nanodisks for reconstitution of active membrane proteins as a complementary approach to proteoliposome reconstitution.[49] We decided to explore whether INSYRT could be amenable to reconstituting GPCRs. In collaboration with the Sunahara lab, we sought to reconstitute the adenosine A_{2A} receptor ($A_{2A}R$).[50] Since $A_{2A}R$ is known to be highly sensitive to detergent, we created a thioester detergent that closely resembled the structure of DDM. Following solubilization of $A_{2A}R$ in the thioester DDM analog, a cysteine modified lysophospholipid was added, leading to the spontaneous formation of proteoliposomes (Figure 9). Fluorescently tagged $A_{2A}R$ could be visualized trapped in the lipid membranes. Furthermore, the GPCRs were shown to be capable of binding to well-known agonists and antagonists, with binding constants very similar to those measured using GPCRs entrapped in lipid nanodiscs (Figure 10). The ability to rapidly entrap functional GPCRs in proteoliposomes should have several applications and advantages compared to nanodiscs. For instance, the entrapment in sealed vesicles should enable reconstitution of signal transduction processes across membranes. Furthermore, vesicle isolation will allow studies on the effect of membrane curvature on GPCR function, something that is not feasible with planar nanodiscs. One area for future study is to explore whether in situ lipid synthesis could control the orientation of membrane proteins during reconstitution, as currently we do not have evidence that this is the case. Developing methods to create asymmetric membranes, with differing inner and outer leaflet

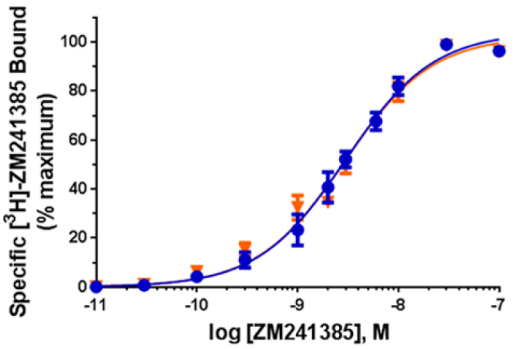

Figure 10. Radiolabeled orthosteric ligand equilibrium experiments with reconstituted $A_{2A}R$ in synthetic liposomes. [^{3}H]-ZM241385 saturation curves with in situ formed $A_{2A}R$ proteoliposomes (blue line) and $A_{2A}R$ reconstituted nanodiscs (orange line). Reproduced from ref 50. Copyright 2017 American Chemical Society.

composition, would better mimic natural cell membranes and may help control the orientation of reconstituted proteins. Use of our previously described nonenzymatic remodeling technique, such that modification only occurs on the extracellular face of liposomes, may enable the creation of such asymmetric membranes, and is a current topic of exploration.

In summary, we have implemented selective chemical reactions to trigger the in situ synthesis of phospholipids. The use of specific chemical reactions enables the de novo formation of lipid membranes, a phenomenon that has not been observed in biology where membranes appear to always originate from preexisting membranes. In situ lipid synthesis has been utilized to achieve lipid self-reproduction, light-triggered lipid synthesis, and the trapping of proteins, either within the aqueous core of formed vesicles for soluble proteins

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or within the membranes themselves for integral membrane proteins. It seems possible that this unique method of synthesizing artificial phospholipids could have future application in the packaging and study of membranes proteins, particularly how proteins respond to changes in membrane reorganization, which would be difficult to study using minimal reconstituted systems. There are several open questions in the synthesis of lipid membranes that our lab is interested in pursuing. The lipid composition of natural membranes is not symmetric, with unique lipid distributions found in the inner leaflet compared to the outer leaflet. Methods to control and maintain specific lipid distributions could enable studies of how lipid asymmetry affects biological properties such as the function of bound integral membrane proteins. While we focused on nonenzymatic methods to generate lipid membranes, future synthetic cells may require methods to link gene expression with membrane expansion, and this might require some novel methods for enzymes to efficiently drive lipid synthesis. We are also exploring if our chemoselective strategies can be used in the presence of living systems to enable synthetic lipid formation in living cells. The synthesis of unique phospholipids in living cells may enable a better understanding of how specific lipid species affect properties such as membrane curvature and signaling in cells. Our group looks forward to these future research directions and applying new chemistries to the formation and study of lipid membranes.

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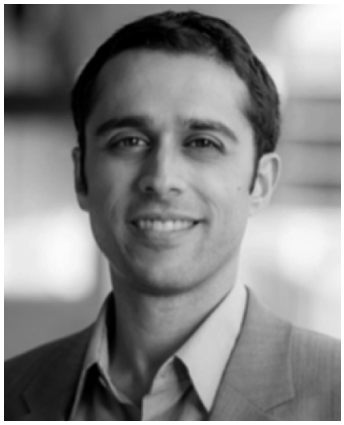
Notes

The author declares no competing financial interest.

Biography



Neal K. Devaraj received a dual B.S. in Chemistry and Biology from the Massachusetts Institute of Technology in 2002 and his Ph.D. in Chemistry from Stanford University under the direction of James Collman and Christopher Chidsey in 2007. After a postdoctoral position in molecular imaging in the lab of Ralph Weissleder at the Harvard Medical School, he joined the faculty of the University of California, San Diego in 2011. His research interests are focused on the design of bioconjugation reactions for addressing problems in bottom-up synthetic biology and molecular imaging. He is the recipient of the 2017 ACS Award in Pure Chemistry sponsored by the Alpha Chi Sigma Fraternity and the Alpha Chi Sigma Educational Foundation and the 2016 National Fresenius Award sponsored by Phi Lambda Upsilon, The National Chemistry Honor Society.

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