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Communication

Chemoenzymatic Generation of Phospholipid Membranes Mediated by Type I Fatty Acid Synthase

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ABSTRACT: The *de novo* formation of lipid membranes from minimal reactive precursors is a major goal in synthetic cell research. In nature, the synthesis of membrane phospholipids is orchestrated by numerous enzymes, including fatty acid synthases and membrane-bound acyltransferases. However, these enzymatic pathways are difficult to fully reproduce *in vitro*. As such, the reconstitution of phospholipid membrane synthesis from simple metabolic building blocks remains a challenge. Here, we describe a chemoenzymatic strategy for lipid membrane generation that utilizes a soluble bacterial fatty acid synthase (cgFAS I) to synthesize palmitoyl-CoA *in situ* from acetyl-CoA and malonyl-CoA. The fatty acid derivative spontaneously reacts with a cysteine-modified lysophospholipid by native chemical ligation (NCL), affording a noncanonical amidophospholipid that self-assembles into micron-sized membrane-bound vesicles. To our knowledge, this is the first example of reconstituting phospholipid membrane formation directly from acetyl-CoA and malonyl-CoA precursors. Our results demonstrate that combining the specificity and efficiency of a type I fatty acid synthase with a highly selective bioconjugation reaction provides a biomimetic route for the *de novo* formation of membrane-bound vesicles.

All living organisms use phospholipid membranes to control the exchange of materials with the extracellular matrix, isolate and protect sensitive chemical reactions, and maintain homeostasis inside cells.[1] Furthermore, cells require phospholipid membranes for energy production, membrane protein synthesis, and cell signaling.[2,3] Phospholipids are enzymatically generated by membrane-bound acyl-transferases as part of the Kennedy lipid synthesis pathway.[4,5] The fatty acids needed for these biochemical reactions are in turn made by fatty acid synthases (FASs). FASs are ubiquitous proteins that catalyze the synthesis of fatty acids in a highly efficient manner through modularization of enzymatic functions and the use of carrier-mediated substrate shuttling.[6−9]

Drawing inspiration from the biochemical pathways for phospholipid synthesis, various research groups have devised strategies for the bottom-up construction of phospholipid membranes.[10−12] Although recent studies have shown that phospholipids can be generated abiotically using chemical coupling reactions,[13,14] the alkyl chains are typically synthesized in advance and provided externally. Currently, there are no known biomimetic strategies that synthesize phospholipids *de novo* by coupling *in situ* formed fatty acid tails with single-chain amphiphiles. Instead of externally adding alkyl species, we sought to harness a FAS for the formation of fatty acyl-CoAs from simple metabolic building blocks such as acetyl-CoA and malonyl-CoA. FASs can be rationally engineered to modulate the type of fatty acid species made in live organisms.[15] However, we wanted to reconstitute FAS function outside of cells to produce fatty acyl-CoAs *in situ*. Subsequently, the fatty acyl-CoAs could chemically react with lipid precursors to form phospholipid membranes. Such a chemoenzymatic scheme would better mimic biological phospholipid synthesis compared to previous synthetic strategies and would enable the use of medium-chain acyl-CoAs to drive membrane formation. Here, we employ a bacterial type I FAS (cgFAS I), in combination with native chemical ligation (NCL), to spontaneously generate membrane-forming synthetic phospholipids from simple water-soluble fatty acid precursors (Figure 1). Our chemoenzymatic approach enables *de novo* membrane formation, that is, membrane formation in the absence of preexisting membranes. Chemoenzymatic phospholipid formation may provide simpler strategies to generate membrane compartments in synthetic cells,[10,13,16,17] support the advancement of methods for reconstituting membrane proteins,[18,19] and facilitate the synthesis of natural and noncanonical lipids.[20]

We first identified an appropriate FAS for the *in situ* formation of activated fatty acids. While type II FASs are comprised of multiple enzymes working in a coordinated fashion, type I FASs consist of a single multienzyme complex with catalytic domains that interact with each other in a cooperative manner to form fatty acids.[6] Through iterative cycles, a type I FAS utilizes acetyl-CoA and malonyl-CoA to produce medium-chain fatty acids in a stoichiometric fashion (Figure 2A). We considered that a type I FAS would be an ideal enzyme for our system as it would require reconstituting a single multidomain protein. Additionally, some type I FASs, such as yeast and bacterial FASs, produce fatty acyl-CoA as

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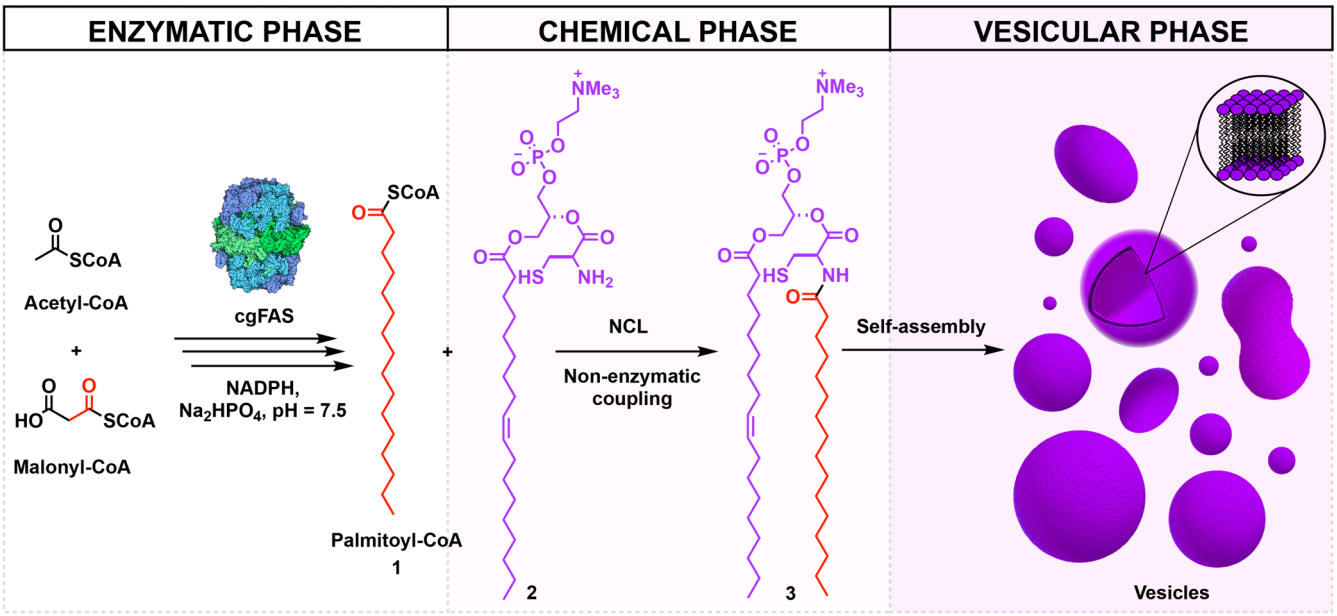

Figure 1. Schematic representation of FAS-mediated chemoenzymatic phospholipid synthesis. A bacterial type I FAS synthesizes palmitoyl-CoA **1** *in situ* using acetyl-CoA, malonyl-CoA, and NADPH. **1** subsequently reacts with cysteine-modified lysophospholipid **2** via native chemical ligation (NCL) to form phospholipid **3**, which spontaneously self-assembles into membrane-bound vesicles.

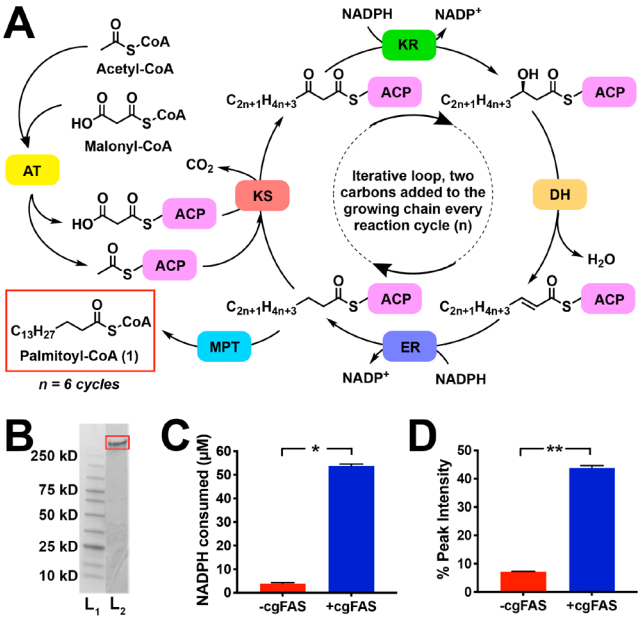

Figure 2. *In situ* synthesis of palmitoyl-CoA **1** mediated by bacterial type I FAS (cgFAS). (A) Schematic representation of the iterative fatty acid elongation cycle. Malonyl-palmitoyltransferase (MPT) transfers the final palmitoyl moiety to a CoA molecule to form **1** [AT: acyl transferase; ACP: acyl carrier protein; KS: ketosynthase; KR: ketoreductase; DH: dehydratase; ER: enoyl reductase]. (B) SDS-PAGE analysis of the His-tagged cgFAS after FPLC purification. Lane 1 (L_1): ladder; Lane 2 (L_2): purified His-tagged cgFAS (325 kDa). (C) NADPH consumption assay analysis, verifying cgFAS activity. Significance was determined using an unpaired *t* test. $^*P < 0.0005$. D) GC-MS FAME analysis of the cgFAS-catalyzed formation of **1** over 1 h. Significance was determined using an unpaired *t* test. $^{**}P < 0.001$. Error bars represent standard deviations (SD) ($n = 3$).

their final product.[7] As coenzyme A is a good leaving group, we reasoned that the bacterial type I FAS would be an appropriate enzyme for generating fatty acyl-CoAs that could be efficiently coupled with appropriate thioester-reactive lysophospholipids to form noncanonical phospholipids *in situ*. We chose to work with type I FAS B from *Corynebacterium glutamicum* (cgFAS I) as it has been shown to primarily produce palmitoyl-CoA[21] (Figure 2A) and has been efficiently expressed in *E. coli*.[22]

N-terminal His_6-tagged type I cgFAS was expressed in *E. coli* and purified by adapting a previously published procedure.[8] The integrity of the protein, as well as its oligomeric state, were verified using size exclusion chromatography (SEC) on a fast protein liquid chromatography (FPLC) column (Figure 2B). The fractions were then subjected to a nicotinamide adenine dinucleotide phosphate (NADPH) consumption assay to verify cgFAS activity (Figure 2C).[8] First, we treated cgFAS I (100 nM) with acetyl-CoA (100 μM), malonyl-CoA (700 μM), and NADPH (1 mM) in 1 mM phosphate (Na_2HPO_4/NaH_2PO_4) buffer, pH 7.4 containing 1 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) at 37 °C. Subsequently, we monitored NADPH oxidation to $NADP^+$ over time by the decrease in fluorescence at 470 nm. The amount of NADPH consumed was verified by making a calibration curve using commercially available NADPH (Figure S2B). In agreement with previous reports, we observed that palmitoyl-CoA **1** was the major product of the cgFAS I-catalyzed reaction.[21,22] Using gas chromatography–mass spectrometry (GC-MS)[8,15] after fatty acid methyl ester (FAME) formation (Figure 2D, Figure S3), we observed that **1** comprised 93% of the total fatty acid species formed. Moreover, using the NADPH consumption assay together with high performance liquid chromatography–mass spectrometry (HPLC-MS), we observed that 41 μM of **1** was produced by the cgFAS I-mediated reaction, corresponding to a 40.6% yield.

We next proceeded to select an appropriate thioester-reactive lysophospholipid for chemical coupling with cgFAS I-synthesized palmitoyl-CoA. We had previously prepared a novel class of cysteine-modified lysophospholipids that can undergo spontaneous acylation by NCL reaction with long-chain thioesters.[10,17,23] We therefore hypothesized that

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cysteine-modified lysolipids would react by NCL with palmitoyl-CoA **1** generated *in situ* by cgFAS I. As a test, we synthesized cysteine-modified lysophospholipid **2** (Scheme S1A) and demonstrated NCL coupling with commercially available palmitoyl-CoA, forming phospholipid **3** (Figure S4B). Briefly, we treated lysophospholipid **2** (1 mM) with palmitoyl-CoA (1 mM) in 10 mM phosphate (Na_2HPO_4/NaH_2PO_4) buffer, pH 7.4 containing 10 mM TCEP at 37 °C. Phospholipid formation was followed using HPLC-MS combined with evaporative light-scattering detection (ELSD) and corroborated by chemically characterized standards (Scheme S1B, Figure S4). Using calibration curves, we determined that 820 μM of phospholipid **3** was formed after 3 h, corresponding to a yield of 82%.

In previous work, we have observed that amphiphilic species are preferentially acylated by amphiphilic reactants, likely promoted by coassembly in micelles or membranes.[13,16,23] To better understand the role self-assembly plays in the formation of the phospholipid product, we investigated the reactivity of lysophospholipid **2** with nonamphiphilic small-chain thioesters. Malonyl- and acetyl-CoA were selected as reactive thioester partners with **2**. Although both substrates contain a reactive thioester moiety that can react by NCL with cysteine-modified lysophospholipid **2**, the absence of a long-chain hydrophobic tail precludes assembly into structures such as micelles. Therefore, we anticipated a difference in their reactivity with **2** in comparison to the previously tested palmitoyl-CoA **1**. As expected, when we attempted to react **2** with malonyl- or acetyl-CoA under our standard NCL reaction conditions, we were unable to detect product formation (Figure S5).

To determine the ability of noncanonical phospholipid **3** to form membrane-bound vesicles, microscopy studies were performed. Neither palmitoyl-CoA **1** nor lysophospholipid **2** formed membranes in aqueous solution. Phospholipid **3** readily formed membrane-bound assemblies when hydrated (Figure 3). Lipid vesicles were initially identified by phase-contrast (Figure 3A) and fluorescence microscopy using the membrane-staining dye BODIPY-FL DHPE (Figure 3B, Figure S6A). Under these conditions, vesicles of 1−10 μm diameter were observed after hydration and tumbling of **3** in phosphate buffer, pH 7.4 at 37 °C for 1 h. Transmission electron microscopy (TEM) also corroborated the formation of vesicular structures (Figure 3C). The encapsulation ability of the phospholipid vesicles was demonstrated by hydrating a thin lipid film of **3** in the presence of 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS), a highly polar fluorescent dye, followed by removal of excess dye by spin-filtration and vesicle characterization using fluorescence microscopy (Figure 3D, Figure S6B).

Having characterized the individual enzymatic and chemical reactions, we next explored combining enzymatic palmitoyl-CoA **1** synthesis with chemical phospholipid **3** synthesis in a one-pot reaction (Figure 4). Briefly, we added lysophospholipid **2** (400 μM) to 10 mM phosphate (Na_2HPO_4/NaH_2PO_4) buffer, pH 7.4 containing cgFAS I (1 μM), acetyl-CoA (1 mM), malonyl-CoA (1 mM), and NADPH (10 mM) along with TCEP (10 mM) at 37 °C. Phospholipid formation was followed using HPLC-MS-ELSD measurements. Optimization of the reaction conditions enabled rapid coupling between cgFAS I generated **1** and **2**. The one-pot reaction afforded the corresponding phospholipid **3** as the prominent product within 30 min (Figure 4A). All of lysophospholipid **2** was consumed in less than 4 h (Figure 4 B) to afford 367 μM of phospholipid

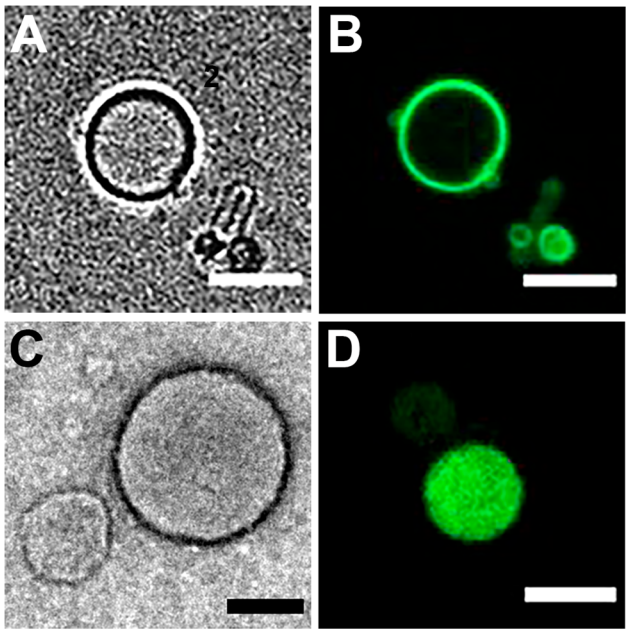

Figure 3. Characterization of phospholipid **3** vesicular structures. (A) Phase-contrast microscopy image of membrane-bound vesicles resulting from the self-assembly of **3**. Scale bar denotes 5 μm. (B) Fluorescence microscopy image of vesicles formed by hydration of a thin film of **3**. Membranes were stained with 0.1 mol % BODIPY-FL DHPE. Scale bar denotes 5 μm. (C) TEM image of negatively stained vesicles of **3**. Scale bar denotes 100 nm. (D) Fluorescence microscope image demonstrating the encapsulation of HPTS in vesicles of **3**. Scale bar denotes 5 μm.

3. After 30 min of reaction, small vesicular structures were detected by fluorescence microscopy using BODIPY-FL DHPE (Figure S7A). After leaving the reaction tumbling overnight at 37 °C, we observed larger vesicles in the range of 1−2 μm in diameter (Figure 4C, Figure S7B).

We next investigated the one-pot chemoenzymatic formation of membranes in the presence of biologically relevant cell membrane components, including cholesterol,[24] ionic small molecules such as guanidine hydrochloride (GuHCl),[25−27] and short-chain alkanols such as decanol.[28] Natural cell membranes are heterogeneous bilayers composed of multiple phospholipids, as well as other lipid species such as cholesterol.[29,30] Since the lipid profile of our vesicles is homogeneous, we wanted to explore the effect of incorporating biologically relevant additives into our system. Therefore, we added cholesterol (400 μM), GuHCl (400 μM) and 1-decanol (400 μM) to the one-pot *in situ* chemoenzymatic reaction forming phospholipid **3**. It has been suggested that stoichiometric addition of cholesterol, GuHCl, and decanol leads to the curvature stabilization and fusion of fatty acid vesicles.[27] We expected similar interactions of such additives with our phospholipid vesicles. We observed that the additives did not perturb the formation of phospholipid **3** membranes and led to the formation of larger, more stable vesicles. Vesicles were stable over 48 h at 37 °C, as observed by fluorescence microscopy using BODIPY-FL DHPE (Figure 4D).

In summary, we have developed a chemoenzymatic route to synthesize noncanonical phospholipids from water-soluble precursors. Given our approach, there should be flexibility to diversify the lipid species generated in the reaction. Even though we utilized cgFAS I to selectively produce palmitoyl-

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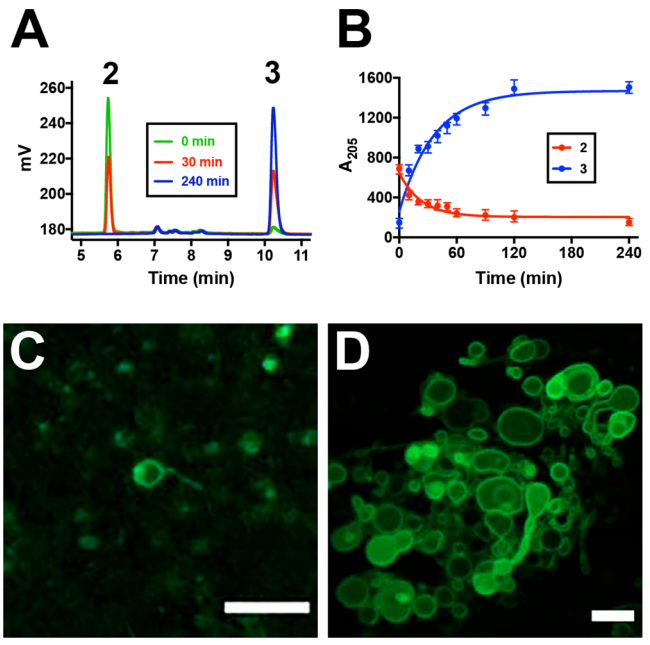

Figure 4. One-pot chemoenzymatic formation and self-assembly of phospholipid **3**. (A) HPLC/ELSD traces corresponding to the NCL-based synthesis of phospholipid **3** from enzymatically generated **1** and lysophospholipid **2**. (B) Kinetic plots of lysophospholipid **2** consumption and phospholipid **3** formation during one-pot synthesis. Absorption at 205 nm was monitored and the area under the peak for compounds **2** and **3** were plotted over time. Data was collected in triplicates. (C) Fluorescence microscopy image of phospholipid **3** vesicles after 4 h of chemoenzymatic reaction. (D) Fluorescence microscopy image of chemoenzymatically formed vesicles of **3** in the presence of GuHCl, decanol and cholesterol. Membranes were stained with 0.1 mol % BODIPY-FL DHPE. Scale bar denotes 5 μm.

CoA, the use of fatty acid synthases from other organisms could enable the formation of a diverse array of fatty acyl-CoA species, which could be subsequently coupled to reactive lysophospholipids to give several noncanonical lipid species. For instance, many bacterial FASs are known to synthesize terminally branched iso-, anteiso-, or omega-alicyclic fatty acids from branched, short-chain carboxylic acid precursors such as methylmalonyl-CoA.[7,31,32] We plan on utilizing the *in situ* synthesis of diverse phospholipid species to facilitate investigations of how lipid membrane composition affects vesicle assembly, growth, and division.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c02121.

Detailed procedures, spectral data, and Figures S1−S7 (PDF)

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Notes

The authors declare no competing financial interest.

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