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# Spontaneous Reconstitution of Functional Transmembrane Proteins During Bioorthogonal Phospholipid Membrane Synthesis

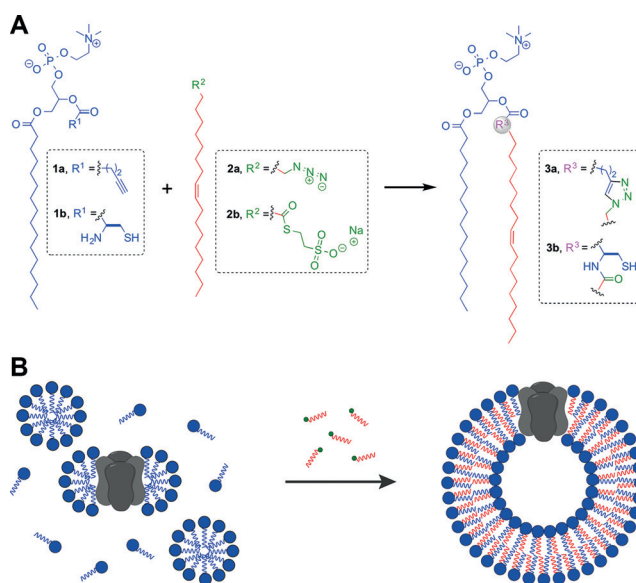
Christian M. Cole, Roberto J. Brea, Young Hun Kim, Michael D. Hardy, Jerry Yang, and Neal K. Devaraj\*

**Abstract:** Transmembrane proteins are critical for signaling, transport, and metabolism, yet their reconstitution in synthetic membranes is often challenging. Non-enzymatic and chemoselective methods to generate phospholipid membranes *in situ* would be powerful tools for the incorporation of membrane proteins. Herein, the spontaneous reconstitution of functional integral membrane proteins during the *de novo* synthesis of biomimetic phospholipid bilayers is described. The approach takes advantage of bioorthogonal coupling reactions to generate proteoliposomes from micelle-solubilized proteins. This method was successfully used to reconstitute three different transmembrane proteins into synthetic membranes. This is the first example of the use of non-enzymatic chemical synthesis of phospholipids to prepare proteoliposomes.

Membrane proteins are key regulators of communication between cellular compartments, functioning primarily as receptors and transporters.<sup>[1]</sup> They also determine the distinctive architecture and adhesion properties of cells.<sup>[2]</sup> Membrane proteins are targeted by the majority of approved pharmaceuticals;<sup>[3]</sup> loss of their function can lead to numerous disease states, including metabolic dysfunction,<sup>[4]</sup> neurodegenerative disorders,<sup>[5]</sup> and cardiovascular malfunctions.<sup>[6]</sup> To study their structure, dynamics, and function, membrane proteins are often reconstituted into lipid bilayers to create proteoliposomes.<sup>[7]</sup> The conventional reconstitution of membrane proteins into proteoliposomes is achieved by using organic extraction, mechanical fragmentation, or detergent solubilization to remove the proteins from native bilayers.<sup>[7]</sup> The ensuing membrane proteins are subsequently introduced into a liposome. This often requires high concentrations of detergent to solubilize proteins from the native bilayer, followed by depletion of the detergent in the presence of phospholipids.<sup>[8]</sup> However, while effective for select membrane proteins, this method is generally time-consuming, and remnants of detergent often remain absorbed on the proteoliposome after detergent removal.<sup>[9]</sup> Therefore, it would be

exciting to develop a strategy to spontaneously reconstitute membrane proteins into synthetic lipid bilayers in a highly specific and chemoselective manner.

In searching for methods to spontaneously form proteoliposomes, we were attracted to the possibility of harnessing bioorthogonal coupling reactions.<sup>[10]</sup> We previously demonstrated the *de novo* synthesis of non-natural phospholipid membranes from bioorthogonally reactive precursors (Figure 1 A).<sup>[11,12]</sup> Synthetic membranes capable of forming vesicles and entrapping polar molecules are rapidly generated by exploiting chemoselective reactions, such as copper catalyzed azide-alkyne cycloaddition (CuAAC)<sup>[11,13]</sup> or native chemical ligation (NCL; Figure 1 A).<sup>[12,14]</sup> However, it was unknown whether integral membrane proteins could incorporate or remain functional in artificial membranes formed through these bioorthogonal coupling reactions. Herein, we describe the spontaneous reconstitution of a variety of membrane



**Figure 1.** *De novo* synthesis of phospholipid membranes and concurrent *in situ* incorporation of proteins. A) Two unique bioorthogonal routes to produce synthetic analogues of POPC. Phospholipid **3a** is formed by CuAAC between alkyne lysolipid **1a** and alkyl azide **2a**. Phospholipid **3b** is formed via NCL between cysteine lysolipid **1b** and thioester **2b**. B) Model for spontaneous reconstitution of transmembrane proteins during the non-enzymatic formation of phospholipid membrane. Protein is solubilized with synthetic mimetics of lysophosphatidylcholine, which act as the detergent to form micelle-solubilized protein complexes. Addition of the reactive alkyl precursor and subsequent coupling results in the spontaneous generation of the corresponding proteoliposomes.

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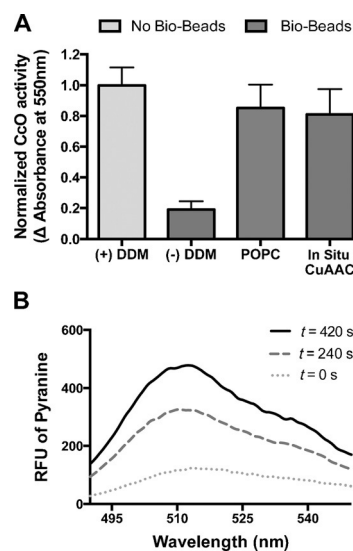
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proteins during bioorthogonal lipid membrane synthesis, with retention of functionality (Figure 1B). Bioorthogonal proteoliposome formation could be a powerful method for the study of membrane proteins and/or their incorporation into synthetic cells.

We initially designed analogues of palmitoyl lysophosphatidylcholine that were conveniently functionalized at the sn2 position with a bioorthogonal reactive group (**1a** or **1b**; Figure 1A). The corresponding reactive lysolipid detergents can be used to solubilize membrane proteins. Upon addition of an alkyl chain bearing a mutually reactive functional group (**2a** or **2b**), a chemoselective coupling reaction transforms the non-membrane-forming precursors into a synthetic mimetic (**3a** or **3b**) of the common phospholipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), which is capable of spontaneously self-assembling to form bilayer membranes. As previously mentioned, CuAAC between an alkyne-modified lysolipid (**1a**) and an oleyl azide (**2a**) can be employed to efficiently construct a triazole-containing phospholipid (**3a**; Figure 1A and Figure S1 in the Supporting Information).<sup>[11]</sup> Alternatively, the NCL approach can be also utilized to rapidly form phospholipids from a cysteine-modified lysolipid (**1b**) and a thioester derivative of oleic acid (**2b**) to produce an amide-containing phospholipid (**3b**; Figure 1A, Figure S2).<sup>[12]</sup> Both non-natural phospholipids (**3a** and **3b**) share many characteristics with native POPC,<sup>[11,12]</sup> including their structure, fluidity, and ability to encapsulate small molecules, thus leading us to hypothesize that the reconstitution of membrane proteins would be possible.

The feasibility of incorporating transmembrane proteins during lipid preparation was demonstrated by synthesis of the triazole phospholipid **3a** in the presence of commercially purified cytochrome *c* oxidase (CcO) from bovine heart.<sup>[15]</sup> The active CcO dimer is an approximately 200 kDa transmembrane protein complex found in bacteria and mitochondria.<sup>[15]</sup> CcO is the terminal protein in aerobic respiration, coupling the reduction of oxygen to proton pumping.<sup>[16]</sup> Despite its large size, CcO can be reconstituted in natural lipid membranes with minimal loss of activity,<sup>[17]</sup> thus making it an appealing choice for initial proof-of-concept studies.

We first measured the activity of CcO by monitoring the decrease in absorbance of its substrate, cytochrome *c*, as it undergoes CcO catalyzed oxidation (Figure S3).<sup>[18]</sup> CcO was initially solubilized with *n*-dodecyl  $\beta$ -D-maltoside (DDM).<sup>[19]</sup> This detergent possesses stabilizing properties that can maintain 100% of the CcO as a highly active monodisperse dimer.<sup>[19]</sup> Measurement of the activity of CcO in DDM was used as a positive control (Figure 2A, (+)DDM). We next removed the DDM with Bio-Beads SM-2<sup>[20]</sup> and overnight dialysis, which led to a significant drop in activity (Figure 2A, (-)DDM). CcO could be reconstituted into POPC liposomes by introducing POPC into the DDM-protein complexes (POPC/CcO molar ratio of 1400:1). The excess DDM detergent was then removed with Bio-Beads SM-2 and overnight dialysis to create functional proteoliposomes containing highly active CcO (Figure 2A, POPC). In situ triazole reconstitution was accomplished by first exchanging the DDM with the lysolipid **1a**. The bioorthogonal CuAAC coupling reaction was carried out with an equimolar concen-



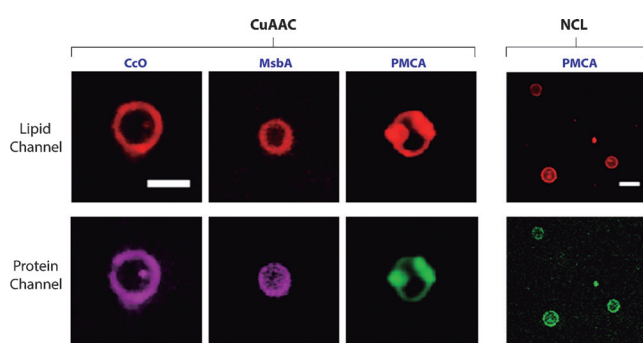
**Figure 2.** Characterization of CcO activity. A) Normalized activity of CcO determined by measuring the absorption peak of cytochrome *c* at 550 nm. (+) DDM: CcO activity as measured in *n*-dodecyl  $\beta$ -D-maltoside (DDM) [positive control]. (-) DDM: CcO activity in DDM following detergent removal using Bio-Beads SM-2 and dialysis [negative control]. POPC: CcO activity after reconstitution in POPC and the removal of detergent with Bio-Beads SM-2 and dialysis. In situ CuAAC: CcO activity after spontaneous reconstitution in the triazole-containing phospholipid membranes. As a precaution, membranes were treated with Bio-Beads SM-2 and dialyzed. B) Fluorescence in relative fluorescence units (RFUs) of the pH sensitive dye (pyranine) encapsulated in CcO proteoliposomes formed in situ by the CuAAC approach. Upon adding cytochrome *c*, there is a gain in fluorescence at 515 nm, thus indicating that pyranine is being deprotonated as a result of the shuttling of protons across the membrane.

tration of the oleyl azide **2a**, 1 mM CuSO<sub>4</sub>, and 2 mM hydroquinone. After 2 h, the copper-catalyzed process resulted in the formation of compound **3a** and complete disappearance of **1a**, as judged using combined liquid chromatography (LC), mass spectrometry (MS), and evaporative light scattering detection (ELSD) measurements (Figure S1). To further ensure that the observed CcO activity was not due to residual lysophospholipid detergent, the vesicles were treated with Bio-Beads SM-2 and dialyzed overnight. Remarkably, the activity of the CcO that was spontaneously reconstituted during triazole lipid synthesis closely mirrors the activity of CcO reconstituted in POPC by using the more traditional methods (Figure 2A, in situ CuAAC; Figure S4). This demonstrates that the bioorthogonal triazole reaction can spontaneously reconstitute active CcO into a bilayer. Solubilizing detergent is consumed during phospholipid formation and is thus eliminated. We estimated the yield of reconstituted CcO using the CuAAC methodology to be 3.0  $\mu$ g, which corresponds to 60% incorporation. CcO reconstitution using traditional methods and POPC gave us yields closer to 3.4  $\mu$ g, which corresponds to 70% incorporation.

One of the advantages of studying proteins in well-defined membranes is the ability to evaluate functions that rely on the selective permeability of phospholipid membranes. Although we demonstrated that CcO retained its ability to oxidize

cytochrome *c*, we were interested in analyzing whether the triazole membranes could support the proton-pumping function of CcO. Adapting a previously published procedure,<sup>[21]</sup> we employed the pH-sensitive dye pyranine on the inside of the in situ generated vesicles to test the proton-pumping activity of the transmembrane protein complex (Figure 2B and Figure S5). While monitoring fluorescence at 515 nm, cytochrome *c* was added. We observed an increase in fluorescence with time, which is indicative of protons being pumped across the membrane. This experiment demonstrates that CcO proton pumping remains functional and that the membranes formed via CuAAC coupling can maintain a proton gradient.

We next investigated the morphology and type of membrane structures formed during in situ bioorthogonal proteoliposome synthesis by using spinning-disk confocal microscopy (Figure 3, CcO; Figure S6). CcO was labeled



**Figure 3.** Spinning-disk confocal fluorescence microscopy images of spontaneously reconstituted proteoliposomes produced by using either CuAAC (left) or NCL (right). The lipid channel shows the location of the lipid membrane staining dye Texas Red DHPE. The protein channel shows the location of fluorescent transmembrane proteins [Proteins modified with AlexaFluor488 NHS ester (CcO and MsbA) or EGFP (PMCA2)]. CuAAC scale bar: 5  $\mu\text{m}$ . NCL scale bar: 10  $\mu\text{m}$ .

using 10 equivalents of the AlexaFluor488 N-hydroxysuccinimide (NHS) ester before the maltoside detergent was exchanged with **1a**. The fluorophore-modified CcO was then used for the in situ CuAAC coupling as previously described. After proteoliposome formation, the lipid-staining dye Texas Red DHPE was added at 1.0 mol%. As expected for a reconstituted membrane protein, we observed strong colocalization of CcO with Texas Red DHPE, thus indicating that CcO is primarily localized to the triazole-containing phospholipid membranes (Figure 3, CcO; Figure S6).

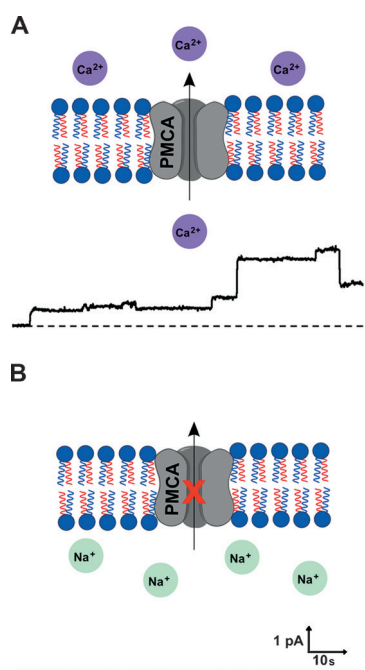
Having shown that purified proteins could be spontaneously reconstituted, we next determined whether it would be possible to directly reconstitute membrane proteins overexpressed in bacteria into artificial membranes. To answer this question, we synthesized the membrane protein MsbA,<sup>[22]</sup> which is a member of a class of ABC transporters responsible for multiple-drug resistance, in *E. coli* cells. After protein production, cells were lysed and proteins were solubilized with 500  $\mu\text{M}$  of reactive lysolipid **1a**. Subsequent affinity purification allowed us to obtain the pure protein, which was

then modified by using 10 equivalents of the AlexaFluor488 NHS ester. The labeled protein/alkyne-lysolipid micelles were reacted by CuAAC with an equimolar concentration of oleyl azide **2a**. Fluorescently modified MsbA showed excellent colocalization with the synthetic membrane, thus indicating that the protein can be reconstituted successfully into proteoliposomes directly from cell lysate (Figure 3, MsbA).

As a final test, we reconstituted a mammalian plasma membrane calcium ATPase fusion protein (PMCA2-EGFP) into proteoliposomes (Figure 3, PMCA).<sup>[23]</sup> Calcium ATPases are key components in the regulation of cellular calcium ion homeostasis and signaling.<sup>[24]</sup> Owing to its ten membrane-spanning segments, PMCA2 can be a challenging protein to reconstitute into membranes.<sup>[25]</sup> Furthermore, expression is typically performed in mammalian cells, which often results in unsatisfactory protein yields.<sup>[26]</sup> We utilized a recombinant PMCA2 fused to EGFP to aid in visualizing membrane incorporation.<sup>[27]</sup> HeLa cells expressing the protein were physically lysed, and high-speed centrifugation was used to collect the insoluble membrane fraction, which was solubilized with 600  $\mu\text{M}$  of alkyne lysolipid **1b**. The His-tagged protein was then purified using Ni-NTA resin. The elution buffer contained 300 mM imidazole in order to elute the PMCA2-EGFP. However, we found that the protein required extensive washing to remove free imidazole, since millimolar concentrations of imidazole can coordinate the copper catalyst and inhibit the CuAAC reaction. The incompatibility of CuAAC with high concentrations of imidazole is a limitation when working with His-tagged proteins. However, after the removal of excess imidazole, CuAAC was successfully used to reconstitute the mammalian protein (Figure 3, CuAAC PMCA).

Since it would be useful to enable spontaneous proteoliposome formation in the presence of imidazole, we explored the reconstitution of PMCA2 into synthetic membranes produced through NCL. NCL does not require metal catalysts and is compatible with high concentrations of imidazole.<sup>[28]</sup> Reconstitution of PMCA2 by using NCL was performed in a similar manner to that described above, except that 600  $\mu\text{M}$  of compound **1b** was used for solubilization. After elution from the Ni-NTA resin, the NCL reaction was initiated by addition of equimolar amounts of oleoyl thioester **2b** in the presence of 10 mM DTT. In situ vesicle formation took place and PMCA2-EGFP incorporation was verified by fluorescence microscopy (Figure 3, NCL PMCA).

PMCA2 is a complex transmembrane protein that has been previously shown to mediate transient current fluctuations of calcium ions across membranes.<sup>[23]</sup> To determine whether PMCA2-EGFP was active within membranes composed of amide-containing phospholipid **3b**, we monitored calcium ion transport by using a standard black lipid membrane (BLM) setup (Figure 4 and Figure S7).<sup>[29]</sup> A bilayer of phospholipid **3b** in *n*-decane was painted onto a Teflon pore separating two aqueous chambers as described previously.<sup>[30]</sup> The painted membranes formed from phospholipid **3b** were stable for at least 2 h and exhibited a constant membrane capacitance of approximately 60 pF. This membrane capacitance value provides an estimate for the mem-



**Figure 4.** Monitoring the activity of the selective calcium ion transport protein PMCA2 by measuring the change in conductance across a planar lipid bilayer. A) The flux of  $\text{Ca}^{2+}$  through PMCA2 was observed in a black lipid membrane (BLM) system with synthetic phospholipid **3b**.  $\text{Ca}^{2+}$  ion flux across the membrane mediated by PMCA2 was measured under an applied potential of 80 mV. B) In comparison, no ion current events were observed when  $\text{Ca}^{2+}$  ions were replaced by  $\text{Na}^{+}$  ions in the recording medium. The dashed line in **A** corresponds to the current trace in **B**.

brane thickness of approximately 3 nm,<sup>[31]</sup> which suggests that **3b** forms a single bilayer rather than a multilayered structure in these BLM experiments. The BLM studies also demonstrate that the membrane characteristics of synthetic phospholipid **3b** are comparable to those of other typical phospholipids in terms of the formation of stable suspended lipid bilayers.<sup>[32]</sup> After determining that the suspended membranes formed from **3b** are stable, PMCA2-EGFP proteoliposomes were then fused with the bilayer. To verify that PMCA2 could mediate a flux of calcium ions across the membrane, we monitored the current versus time across the membrane in the presence of an 80 mV applied potential and observed transient ion current fluctuations that resembled previously reported ion current events from purified PMCA2 in proteoliposomes (Figure 4A and Figure S7).<sup>[23]</sup> Measurement of the current at different applied potentials revealed at least two conductance states for PMCA2-EGFP under our recording conditions, with estimated conductance values of 10 and 31 pS (Figure S7). These conductance values are similar to previously reported divalent conductance states of PMCA2 as estimated from patch-clamp recordings.<sup>[33]</sup> On the other hand, when  $\text{Na}^{+}$  ions were introduced to the recording media instead of  $\text{Ca}^{2+}$  ions, no ion-current events were observed under the same recording conditions (Figure 4B and Figure S7). These BLM recording experiments demonstrate that PMCA2-EGFP retains its ability to mediate the transport of

calcium (and not sodium) ions when embedded in synthetic membranes formed through NCL.

In summary, we have explored the suitability of bioorthogonal coupling reactions for driving the in situ formation phospholipid membranes and concomitant spontaneous reconstitution of functional membrane proteins. This method should be compatible with alternative reactive lipids to create specific compositions of lipid membranes. Additionally, lysolipid derivatives could be used to solubilize and incorporate natural lipids. We are currently exploring the in situ synthesis of more-complex lipid membranes, as well as developing a small library of lysolipid and thioester detergents that can be used to optimize the reconstitution of alternative membrane proteins. We foresee biotechnological applications that make use of the spontaneous reconstitution of transport proteins to control the composition inside synthetic cells, which could be used as chemical microreactors or biosensors.

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