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Spontaneous Reconstitution of Functional Transmembrane Proteins During Bioorthogonal Phospholipid Membrane Synthesis

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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. Here we describe the spontaneous reconstitution of functional integral membrane proteins during the *de novo* synthesis of biomimetic phospholipid bilayers. The approach takes advantage of bioorthogonal coupling reactions to generate proteoliposomes from micelle-solubilized proteins. Using this methodology, we successfully reconstitute three different transmembrane proteins into synthetic membranes. This is the first example of using non-enzymatic chemical synthesis of phospholipids to prepare proteoliposomes.

Graphical abstract

Reconstitution of integral membrane proteins using biorthogonal methodologies allows for the spontaneous incorporation of active proteins in a bilayer. This chemoselective approach results in a fast and clean reconstitution without the need for dialysis to remove excess detergent. This chemical biology tool is the first example of using non-enzymatic chemical synthesis of phospholipids to prepare proteoliposomes.

Keywords

membrane protein; phospholipid; self-assembly; proteoliposome; synthetic biology

Membrane proteins are key regulators of communication between cellular compartments, functioning primarily as receptors and transporters.¹ They also determine the distinctive architecture and adhesion properties of cells.² Membrane proteins are targeted by the majority of approved pharmaceuticals;³ loss of their function can lead to numerous disease states, including metabolic dysfunction,⁴ neurodegenerative disorders,⁵ and cardiovascular malfunctions.⁶ To study their structure, dynamics, and function, membrane proteins are often reconstituted into lipid bilayers creating proteoliposomes.⁷ 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.⁷ The ensuing membrane proteins are subsequently introduced into a liposome. This often requires employing high concentrations of detergent to solubilize proteins from the native bilayer, followed by the depletion of the detergent in the presence of phospholipids.⁸ 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.⁹ 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.¹⁰ We previously demonstrated the *de novo* synthesis of non-natural phospholipid membranes from bioorthogonal reactive precursors (Figure 1A)^{11,12} Synthetic membranes capable of forming vesicles and entrapping polar molecules are rapidly generated by exploiting chemoselective reactions, such as the copper catalyzed azide alkyne cycloaddition (CuAAC)^{11,13} or the native

chemical ligation (NCL) (Figure 1A).^{12,14} However, it was unknown whether integral membrane proteins could incorporate or be functional in artificial membranes formed through the corresponding bioorthogonal coupling reactions. Here we describe the spontaneous reconstitution of a variety of membrane proteins during bioorthogonal lipid membrane synthesis, with retention of functionality (Figure 1B). Bioorthogonal proteoliposome formation could be a powerful methodology for the study of membrane proteins and/or their incorporation into synthetic cells.

We initially designed analogues of palmitoyl lysophosphatidylcholine, which 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, the 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, Figure S1).¹¹ 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**), producing an amide-containing phospholipid (**3b**) (Figure 1A, Figure S2).¹² Both non-natural phospholipids (**3a** and **3b**) share many characteristics with native POPC,^{11,12} including their structure, fluidity and ability to encapsulate small molecules, leading us to hypothesize that membrane protein reconstitution 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.¹⁵ The active CcO dimer is a ~200 kDa transmembrane protein complex found in bacteria and mitochondria.¹⁵ CcO is the terminal protein in aerobic respiration, coupling the reduction of oxygen to proton pumping activity.¹⁶ Despite its large size, CcO can be reconstituted in natural lipid membranes with minimal loss of activity,¹⁷ 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).¹⁸ CcO was initially solubilized with *n*-dodecyl β -D-maltoside (DDM).¹⁹ This detergent possesses stabilizing properties that can maintain 100% of the CcO as a highly active monodisperse dimer.¹⁹ 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-2²⁰ and overnight dialysis, which led to a significant drop in activity (Figure 2A, (-) *DDM*). CcO could be reconstituted into POPC liposomes by introducing POPC to 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, which contained 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 concentration of the oleyl azide **2a**, 1 mM

CuSO₄, and 2 mM hydroquinone. After 2 hours, 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 CcO that was spontaneously reconstituted during triazole lipid synthesis closely mirrors the activity of CcO reconstituted in POPC using 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 thus eliminated. We estimate the yield of reconstituted CcO using the CuAAC methodology is 3.0 μg, corresponding to 60% incorporation. CcO reconstitution using traditional methods and POPC gave us yields closer to 3.4 μg, corresponding 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,²¹ we employed a pH sensitive dye, pyranine, on the inside of the *in situ* CuAAC vesicles to test the proton pumping activity of the transmembrane protein complex (Figure 2B, Figure S5). While monitoring fluorescence at 515 nm, cytochrome *c* was added. We observed an increase in fluorescence with time, indicative of protons being pumped across the membrane. This experiment demonstrates that CcO proton pumping remains functional, and that 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 using 10 equivalents of Alexa Fluor® 488 NHS-ester before the maltoside detergent was exchanged with **1a**. The fluorescently modified CcO was then used for the *in situ* CuAAC coupling reaction as previously described. After proteoliposome formation, a lipid staining dye, Texas Red® DHPE, was added at 1.0 mol%. As expected for a reconstituted membrane protein, we observed strong co-localization of CcO with Texas Red® DHPE, 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 if it would be possible to directly reconstitute membrane proteins into artificial membranes from bacteria during an overexpression growth. To answer this question, we synthesized the membrane protein MsbA,²² which is a member of a class of multi-drug resistant ABC transporters, in *E. coli* cells. After protein production, cells were lysed and proteins were solubilized with 500 μM of reactive lysolipid **1a**. Subsequent affinity purification allowed us to obtain the pure protein, which was then modified using 10 equivalents of Alexa Fluor® 488 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 co-localization with the synthetic membrane, indicating that the protein can be reconstituted successfully in 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).²³ Calcium ATPases are key components in the regulation of cellular calcium ion homeostasis and signaling.²⁴ Due to its ten membrane-spanning segments, PMCA2 can be a challenging protein to reconstitute into membranes.²⁵ Furthermore, expression is typically performed using mammalian cells, which often results in sub-par protein yields.²⁶ We utilized a recombinant PMCA2 gene fused to EGFP to aid in visualizing membrane incorporation.²⁷ Proteins synthesized in HeLa cells were physically lysed, and high-speed centrifugation was used to collect the insoluble membrane fraction, which was solubilized with 600 μ 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 preventing the CuAAC reaction from ensuing. The incompatibility of CuAAC with high concentrations of imidazole is a limitation when working with His-tagged proteins. However, after removal of excess imidazole, the CuAAC methodology was successful in reconstituting 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 using NCL. NCL does not require metal catalysts and is compatible with high concentrations of imidazole.²⁸ Reconstitution of PMCA2 using NCL was performed similar to as described above, except that 600 μ 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.²³ To determine whether PMCA2-EGFP was active within membranes composed of amide-containing phospholipid **3b**, we monitored calcium ion transport using a standard black lipid membrane (BLM) setup (Figure 4, Figure S7).²⁹ A bilayer of phospholipid **3b** in *n*-decane was painted onto a Teflon® pore separating two aqueous chambers as described previously.³⁰ The painted membranes formed from phospholipid **3b** were stable for at least 2 hours, and exhibited a constant membrane capacitance of $\sim$ 60 pF. This membrane capacitance value provides an estimate for the membrane thickness of $\sim$ 3 nm,³¹ 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 with other typical phospholipids in formation of stable suspended lipid bilayers.³² After determining that the suspended membranes formed from **3b** are stable, PMCA2-EGFP proteoliposomes were then fused with the bilayer. To verify 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 of purified PMCA2 in proteoliposomes (Figure 4A, Figure S7).²³ 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.³³ On the other hand, when Na⁺ ions were introduced to the recording media instead of Ca²⁺ ions, no ion current events were observed under the same recording conditions (Figure 4B, Figure S7). These BLM recording experiments demonstrate that PMCA2-EGFP retains its ability to mediate calcium (and not sodium) ion transport when embedded in synthetic membranes formed by NCL.

In summary, we have explored the suitability of bioorthogonal coupling reactions for driving *in situ* phospholipid membrane formation and concomitant spontaneous reconstitution of functional membrane proteins. This methodology should be compatible with alternative reactive lipids to create specific lipid membrane compositions. 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 using the spontaneous reconstitution of transport proteins to control the composition inside synthetic cells, which could be used as chemical microreactors or biosensors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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 non-enzymatic phospholipid membrane formation. Protein is solubilized with synthetic mimetics of lysophosphatidylcholine, acting as the detergent to form micelle-solubilized protein complexes. Addition of the reactive alkyl precursor and subsequent coupling reaction results in the spontaneous generation of the corresponding proteoliposomes.

Figure 2.

Characterization of CcO activity. A) Normalized activity of CcO determined by measuring the absorption peak of cytochrome *c* at 550nm. (+) *DDM*: CcO activity as measured in *n*-dodecyl β -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 being reconstituted in POPC and the removal of detergent with Bio-Beads® SM-2 and dialysis. *In situ CuAAC*: CcO activity after spontaneously being reconstituted in the triazole-containing phospholipid membranes. As a precaution, membranes were treated with Bio-Beads® SM-2 and dialyzed. B) 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 of fluorescence, as measured at 515 nm, indicating that pyranine is being deprotonated from the shuttling of protons across the membrane.

Figure 3.

Spinning disk confocal fluorescence microscopy images of spontaneously reconstituted proteoliposomes using either the CuAAC (*left*) or NCL (*right*) coupling methodologies. 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 Alexa Fluor® 488 NHS-ester (CcO and MsbA) or EGFP (PMCA2)]. CuAAC scale bar denotes 5 μm. NCL scale bar denotes 10 μm.

Figure 4. Monitoring the activity of the selective calcium ion transport protein PMCA2 by measuring the change in conductance across a planer lipid bilayer. **A)** The flux of Ca^{2+} through PMCA2 was observed in a black lipid membrane (BLM) system using synthetic phospholipid **3b**. 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 Ca^{2+} ions were replaced by Na^{+} ions in the recording medium. Dash line in **A** corresponds to the current trace in **B**.