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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. 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.[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 to create 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 high concentrations of detergent to solubilize proteins from the native bilayer, followed by 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 bioorthogonally 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 copper catalyzed azide–alkyne cycloaddition (CuAAC)[11,13] or native chemical ligation (NCL; Figure 1A).[12,14] 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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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 mimic (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).

Alternatively, the NCL approach can also be 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). Both non-natural phospholipids (3a and 3b) share many characteristics with native POPC 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. The active CcO dimer is an approximately 200 kDa trans-dimer. The CcO can be reconstituted in natural lipid membranes with minimal loss of activity 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, which underscores CcO catalyzed oxidation (Figure S3). CcO was initially solubilized with n-dodecyl β-D-maltoside (DDM). Despite its large size, CcO can be reconstituted in natural lipid membranes with minimal loss of activity[7] thus making it an appealing choice for initial proof-of-concept studies.

We measured the activity of CcO in DDM by monitoring the decrease in absorbance of its substrate, cytochrome c, which underscores CcO catalyzed oxidation (Figure S3). CcO was initially solubilized with n-dodecyl β-D-maltoside (DDM). This detergent possesses stabilizing properties that can maintain 100% of the CcO as a highly active monodisperse dimer.[8] The active CcO dimer is an approximately 200 kDa trans-dimer. The CcO can be reconstituted in natural lipid membranes with minimal loss of activity thus making it an appealing choice for initial proof-of-concept studies.

We measured the activity of CcO in DDM by monitoring the decrease in absorbance of its substrate, cytochrome c, which underscores CcO catalyzed oxidation (Figure S3). CcO was initially solubilized with n-dodecyl β-D-maltoside (DDM). DDM was used as a positive control (Figure 2A, (+)-DDM). We next removed the DDM with Bio-Beads SM-2 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 absorbance peak of cytochrome c at 550 nm. (+) 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 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. An 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.
cytochrome c, we were interested in analyzing whether the triazole membranes could support the proton-pumping function of CcO. Adapting a previously published procedure, 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-disc confocal microscopy (Figure 3, CcO; Figure S6). CcO was labeled 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 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 μm. NCL scale bar: 10 μm.

![Figure 3. Spinning-disc 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 μm. NCL scale bar: 10 μm.](image-url)

As a final test, we reconstituted a mammalian plasma membrane calcium ATPase fusion protein (PMCA2-EGFP) into proteoliposomes (Figure 3, PMCA). Calcium ATPases are key components in the regulation of cellular calcium ion homeostasis and signaling. Owing to its ten membrane-spanning segments, PMCA2 can be a challenging protein to reconstitute into membranes. Furthermore, expression is typically performed in mammalian cells, which often results in unsatisfactory protein yields. We utilized a recombinant PMCA2 fused to EGFP to aid in visualizing membrane incorporation. 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 μM 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. Reconstitution of PMCA2 by using NCL was performed in a similar manner to that described above, except that 600 μM 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. 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). A bilayer of phospholipid 3b in n-decane was painted onto a Teflon pore separating two aqueous chambers as described previously. 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 membrane.
branched thickness of approximately 3 nm, 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. 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). 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. 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 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 biorthogonal 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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