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In Situ Vesicle Formation by Native Chemical Ligation

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Abstract

Phospholipid vesicles are of intense fundamental and practical interest, yet methods for their *de novo* generation from reactive precursors are limited. A non-enzymatic and chemoselective method to spontaneously generate phospholipid membranes from water-soluble starting materials would be a powerful tool for generating vesicles and studying lipid membranes. Here we describe the use of native chemical ligation (NCL) to rapidly prepare phospholipids spontaneously from thioesters. While NCL is one of the most popular tools for synthesizing proteins and nucleic acids, to our knowledge this is the first example of using NCL to generate phospholipids *de novo*. The lipids are capable of *in situ* synthesis and self-assembly into vesicles that can grow to several microns in diameter. The selectivity of the NCL reaction enables compatibility of *in situ* membrane formation with biological materials such as proteins. This work expands the application of NCL to the formation of phospholipid membranes.

Keywords

native chemical ligation; phospholipid; self-assembly; artificial cell; synthetic biology

Phospholipid membranes are routinely employed in several practical applications such as the study of protein-membrane interactions,^[1] drug-delivery,^[2] origin-of-life studies,^[3] bottom-up synthetic biology,^[4] and synthetic reactors.^[5] While the capability of phospholipids to self-assemble into membranes is well studied,^[6] the *de novo* synthesis and assembly of membranes from simple non-membrane forming reactive precursors is poorly understood. Living organisms are capable of *in situ* synthesis of lipid membranes by utilizing membrane bound acyltransferases and reactive thioester precursors.^[7] Given the well-documented challenges associated with reconstituting lipid synthesizing membrane proteins into vesicles,^[8] several groups have explored methods to generate lipid membranes *de novo* from reactive amphiphilic precursors.^{[9],[10]} However, these methods generally suffer from a range of issues, including the necessity of catalysts, lack of soluble starting materials, use of biologically incompatible reactive precursors and the formation of lipids that have limited structural similarity to natural phospholipids.^[9] Phospholipid membranes are often

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advantageous in complex environments due to their stability and biocompatibility.^[11] Simpler and more robust methods for phospholipid synthesis could also find use as drivers for the growth and division of primitive protocells.^{[11],[12]} Therefore, it would be exciting to develop a catalyst-free method to generate and grow phospholipid membrane vesicles from reactive soluble precursors. Furthermore, it would be interesting to utilize water-soluble thioester precursors, analogous to living cells.^{[7],[13]} Here we demonstrate that the chemoselective native chemical ligation (NCL)^[14] is capable of *in situ* synthesis of phospholipid vesicles from long-chain thioesters. The lipids are capable of *in situ* self-assembly into vesicles that can grow to several microns in diameter. Moreover, the chemoselectivity of the NCL reactions enables compatibility with biological materials such as proteins,^[15] and we demonstrate this by performing an *in situ* encapsulation of green fluorescent protein (GFP).

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The native chemical ligation is one of the most popular tools for the synthesis of large peptides and small proteins.^[16] The mechanism of NCL involves a two-step process consisting of a thiol exchange step between a C-terminal peptide thioester and the sulfhydryl moiety of a N-terminal cysteine residue in another peptide, which prompts an intramolecular nucleophilic attack by the α -amino group of the cysteine (S $\rightarrow$ N acyl rearrangement) to form the final amide bond (Figure 1). Further acylation of the resulting sulfhydryl ("reloading") is also possible under specific conditions.^[17] NCL is extensively used for the synthesis of native proteins, because it efficiently and non-enzymatically connects two peptides to generate a protein with an amide linkage at the reaction site.^[18] Due to its exquisite chemoselectivity, the applications of NCL extend well beyond the scope of protein synthesis.^[19] Here, we demonstrate the use of NCL as a method to couple long-chain acyl thioesters to cysteine-functionalized lysolipids in a highly specific and chemoselective way to form the corresponding phospholipids.

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We initially designed two substrates to mimic the native precursors of the common phospholipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC): a cysteine-functionalized analogue of the lysolipid 1-palmitoyl-*sn*-glycero-3-phosphocholine **1b** and a sodium 2-mercaptoethanesulfonate (MESNA) oleoyl thioester **2** in lieu of oleoyl-CoA (Figure 1, see Supporting Information for full experimental details). Precursors **1b** and **2** are both water-soluble amphiphiles, forming micelles of approximately 5.3 and 3.8 nm in diameter respectively, with critical micelle concentrations (cmc's) below 100 μ M (for **1b**) and 10 μ M (for **2**) (Figures S1 and S2).^[20] The high water solubility of both precursors facilitated NCL at mild conditions in the millimolar range. Under typical NCL conditions (HEPES buffer pH 7.0 containing TCEP as reducing agent), unprotected segments **1b** and **2** coupled to afford amidophospholipid **3**, a novel class of phospholipid that resembles POPC, with the exception of a cysteine-amido linker (Figure 1). Alternatively, addition of precursors in imidazole buffer at neutral pH allowed the NCL ligation and subsequent MESNA thioester reloading,^[17] leading to the formation of amidophospholipid **4**, albeit in relatively lower yield, possibly due to the requirement of multiple reaction steps (Figure 1). Such phospholipid incorporates a second oleoyl chain in the phospholipid architecture, represents an outstanding example of a novel membrane forming phospholipid containing

three alkyl chains (see Supporting Information for additional data), and is reminiscent of unique multichain phospholipids such as 3-*O*-acyl-*D*-erythro-sphingomyelin.^[21]

Phospholipid synthesis was analyzed over time using combined liquid chromatography (LC), mass spectrometry (MS), and evaporate light scattering detection (ELSD) measurements. Addition of MESNA thioester to the cysteine-based lysolipid in the appropriate buffer immediately led to amidophospholipid formation, and this process progressed to near completion over a period of 30 min using millimolar concentrations of reactants (Figure S3).

As expected, neither the cysteine-modified lysolipid **1b** nor the MESNA thioester **2** formed membranes in aqueous solution. However, the purified amidophospholipid products **3** and **4**, when hydrated, readily formed membrane-bound vesicles. Lipid vesicular structures were initially identified by fluorescence microscopy using the membrane staining dye Texas Red[®] DHPE (Figure 2a, Figure S4). Confirmation that the resulting structures were membrane compartments was also achieved by transmission electron microscopy (TEM) (Figure 2b, Figure S5). In this case, aliquots of the hydrated and sonicated phospholipid samples were collected over 400 mesh Cu/Rh grids, which were then negatively stained with uranyl acetate. Under these conditions, electron microscopy revealed the presence of several populations of spherical compartments that were between 50–900 nm wide, consistent with the vesicle architecture. The efficient encapsulation ability of the vesicles was determined by inclusion of a polar fluorophore, 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS), in the hydration media, followed by vesicle characterization using fluorescence microscopy (Figure 2c, Figure S6). Finally, we determined the phase transition temperature of the lipid chains by performing steady-state anisotropy measurements as a function of temperature with the membrane fluidity probe 1,6-diphenyl-1,3,5-hexatriene (DPH).^[22] The measurements indicate that the amidophospholipid membranes composed from **3** and **4** are well-ordered, with fluidity and chain melting temperatures comparable to those of native POPC membranes ($T_c = 270$ K) (Figure 2d, Figures S8 and S9).^[23]

We next explored *de novo* lipid vesicle formation in aqueous solvent. Remarkably, we found that the NCL coupling reaction is capable of driving the highly efficient *in situ* self-assembly of phospholipid membranes into vesicular structures (Figure 3a). When we combined an aqueous solution of **1b** with thioester **2** in the presence of TCEP, we observed the formation of large vesicular structures, both spherical and tubular, by fluorescence microscopy (Figure 3b). One advantage of NCL driven lipid formation compared to previous techniques used for the *de novo* assembly of vesicles, is the chemoselectivity of the reaction and the ability to have selective reaction even in the presence of common biologically relevant functional groups. The selectivity of the reaction should enable compatibility with biological molecules such as proteins. To check for orthogonality and biocompatibility of the lipid forming NCL reaction, as well as the functional stability of the vesicles, GFP was *in situ* encapsulated in the vesicle interior volume. A 220 μ M solution of GFP was diluted in a small volume of HEPES buffer containing lysolipid **1b** and TCEP, and thioester **2** was subsequently added. After 30 min of reaction, non-encapsulated GFP was then removed by spin filtration. When the lipid-containing solution was examined by fluorescence microscopy, stable vesicles containing GFP were observed (Figure 3c, Figure

S7). The compatibility of the lipid formation with biological molecules could lead to applications involving the packaging of therapeutic proteins or the use of these vesicles as compartments for enzymatic reactions.

The morphological transformations of the vesicle assemblies were also monitored by time-lapse phase contrast microscopy at room temperature (Figure 4, Movie S1). No observable aggregates were found immediately after combining **1b** and **2** (1 mM for each) in the presence of TCEP (20 mM). Approximately 5 min after mixing, small granular aggregates began to appear, and then tubular vesicles grew, followed by the conversion to spherical vesicles. This morphological conversion is analogous to the spontaneous transformation of some amphiphilic molecules from micelles to giant vesicles in aqueous dispersions.^[10] We also observed that the NCL between **1b** and **2** frequently sustained the growth of vesicles present in the reaction medium (Figure 4, Movie S2). As a prototypical example, we specifically checked a population of two vesicles of 18 and 15 μm in diameter. We found that the size of the vesicles increased steadily, up to a final value after 15 min of 37 and 24 μm in diameter, respectively. This result corresponds to an approximate quadrupling of the surface area and is possibly due to continued formation of phospholipid **3** within the bilayer of the *in situ* formed vesicles. Our observations of *in situ* vesicle formation correlate well with our LC/MS characterization, which showed that the NCL reaction takes place in a fast, chemoselective and highly specific fashion at neutral pH.

In summary, we have explored the suitability of NCL for the *de novo* synthesis of phospholipid membranes. This highly specific and chemoselective approach has allowed the preparation and full characterization of a new class of amidophospholipids, which *in situ* self-assemble to obtain membrane-bound vesicles. Such amidophospholipids could also be utilized in applications involving protocells. Thus, the NCL reaction can be efficiently used as a non-enzymatic method to drive the *de novo* self-assembly of phospholipid membranes. Importantly, this protocol uses thioester precursors, analogous to enzymatically driven lipid synthesis. Moreover, the orthogonality, the high reaction speed and the biocompatibility of this approach are key features that make it a powerful option for the efficient encapsulation of relevant biomolecules, such as proteins. We foresee future applications of the NCL membrane assembly in advanced synthetic cell studies utilizing phospholipid vesicles as well as in the construction of liposomal drug delivery systems and bioreactors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Synthesis of phospholipids by NCL reaction of a cysteine-functionalized lysolipid and MESNA oleoyl thioester. Framed: the mechanism of the NCL and the possible substrate reloading.

Figure 2.

Characterization of the amidophospholipid vesicular structure. **a)** Fluorescence microscopy image of membrane-containing vesicles formed by hydration of a thin film of phospholipid **3**. Membranes were stained using 1 μM Texas Red® DHPE dye solution. **b)** TEM image of negatively stained self-assembled vesicular structures formed by the amidophospholipid **3**. **c)** Fluorescence microscopy image demonstrating the encapsulation of HPTS in phospholipid **3** membrane vesicles. **d)** Steady-state anisotropy of DPH in membranes formed from amidophospholipids **3** and **4** compared with those from native phosphatidylcholines with the indicated acyl chains. The unitless anisotropy ratio (R) is a measure of the acyl packing of the bilayer, with higher values indicating a more ordered membrane.

Figure 3.

In situ phospholipid membrane assembly driven by a non-enzymatic reaction. **a)** Model of spontaneous vesicle assembly induced by NCL-based amidophospholipid synthesis. **b)** Fluorescence microscopy image of the membrane-containing vesicles formed by spontaneous assembly directed by NCL-based phospholipid **3** synthesis. Membranes were stained using 1 μM Texas Red® DHPE dye solution. **c)** Fluorescence microscopy image demonstrating the encapsulation of GFP in vesicles driven by the *in situ* self-assembly of phospholipid **3** membranes.

Figure 4.

In situ vesicle formation (*top*) and growth (*bottom*). An aqueous buffer solution of cysteine-functionalized lysolipid **1b** (1 mM) and MESNA oleoyl thioester **2** (1 mM) in the presence of TCEP.HCl (20 mM) imaged at different times after initial mixing. The upper panels show phase-contrast images corresponding to the vesicle formation. Initially, phospholipid membranes were not present. However, shortly after mixing of both precursors, the spontaneous formation and growth of vesicle and tubular structures was observed. After 30 min, starting materials were consumed and replaced with large fields of vesicles. The bottom panels are phase contrast micrographs of vesicles growing over a period of 10 minutes in the presence of reactive lipid precursors.