Spontaneous Phospholipid Membrane Formation by Histidine Ligation

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Abstract A major challenge for the construction of artificial lipid membranes is the development of simple and robust methods for mimicking natural phospholipid membrane generation. Here we describe a nonenzymatic and chemoselective approach that relies on histidine ligation to form phospholipids de novo from water-soluble amphiphilic precursors. The resulting phospholipids can spontaneously self-assemble into micron-sized vesicles and encapsulate biomacromolecules.

Key words self-assembly, phospholipid, membrane, histidine ligation, protocell

Living organisms carry out the de novo synthesis of lipid membranes by enzymatic acyl transfer reactions that couple single-chain amphiphiles to form phospholipids (Figure 1, A).1 Efficient synthetic methodologies mimicking these biochemical coupling steps offer a general route towards artificial lipid membranes.2 While the capability of phospholipids to self-assemble into membranes has been well characterized,3 the de novo synthesis and formation of membranes from simple precursors is less explored, partly due to the lack of model systems. Consequently, one of the major goals of chemical prebiology is the development of novel methodologies for the construction of self-assembling non-natural membranes. The long-term goal is to gain a better understanding of the fundamental structural, dynamical, and biochemical features required for nature to build living systems.4 A recent strategy for membrane construction is the use of catalyst-free methods to couple reactive water-soluble precursors, aiming to control the synthesis and assembly of highly ordered membrane architectures with defined functionality.5 Here we demonstrate that the chemoselective histidine ligation (HL)6,7 reaction can be utilized to drive the de novo synthesis of phospholipids from histidine-functionalized lysolipids and fatty acyl thioesters (Figure 1, B). The corresponding phospholipids can spontaneously self-assemble to generate micron-sized vesicles. The selectivity, high reactivity, and the biocompatibility of

Figure 1 De novo formation of phospholipid membranes. A) Natural synthesis pathway of phospholipids from acyl-CoA and lysolipid. B) De novo synthesis of phospholipids by histidine ligation (HL) reaction of an N-terminal histidine-functionalized lysolipid (1 or 4) and MESNA oleoyl thioester (2) to afford the corresponding amidophospholipid (3 or 5). Chemical structures of all the lipids used in this study are highlighted at the bottom.
the lipid-based HL are key features that make it a powerful tool for the efficient encapsulation of relevant biomolecules, such as proteins.

The HL is one of the most popular strategies for orthogonal peptide ligation. However, ligation methodologies between N-terminal histidine (His) and C-terminal thioester groups are often inefficient. Here we demonstrate the use of HL as an efficient approach to couple histidine-functionalized lysolipids to long-chain acyl thioesters in a highly chemoselective way to form the corresponding phospholipids. The proposed mechanism of the HL involves a two-step process consisting of an acyl-exchange step between a long-chain acyl thioester and the NIm moiety of a histidine residue in a lysolipid, which prompts an intramolecular nucleophilic attack by the α-amino group of the histidine (NIm → Nα acyl rearrangement) to form the final amide bond (Scheme 1).

We initially synthesized two substrates to mimic the native precursors of the common phospholipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC): a histidyl derivative of the lysolipid 1-palmitoyl-sn-glycero-3-phosphocholine (1) and a sodium 2-mercaptoethanesulfonate (MESNA) oleoyl thioester (2) (Figure 1, B, Figure S1). Precursors 1 and 2 are water-soluble amphiphiles, forming micelles of approximately 8.9 and 3.8 nm in average diameter, respectively, with critical micelle concentrations (cmc’s) below 10 μM (Figure S2). The high water solubility of both precursors facilitated de novo phospholipid synthesis by HL in mild conditions and at low millimolar concentration ranges. Under neutral HL conditions (HEPES buffer pH 7.5), precursors 1 and 2 coupled over 24 hours to afford amidophospholipid 3, a novel phospholipid that resembles POPC, with the exception of a histidyl linker (Figure 1, B, Figure S1). Alternatively, analogous HL experiments using the water-soluble precursors lysolipid 4 and oleoyl thioester 2 allow efficient formation of the phospholipid 5, a mimic of the native phospholipid 1-oleoyl-2-oleoyl-sn-glycero-3-phosphocholine (DOPC) (Figure 1, B, Figure S1). For both cases, phospholipid formation was analyzed using combined liquid chromatography (LC), mass spectrometry (MS), and evaporative light-scattering detection (ELSD) measurements (Figure 2).

As expected, neither the histidine-modified lysolipids 1 nor the oleoyl thioester 2 formed membranes in aqueous solution. However, the phospholipids 3 and 5 readily formed membrane-bound vesicles in situ. Lipid vesicular structures were initially identified by fluorescence micros-
copy using the membrane staining dye Texas Red® 1,2-di-hexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (Texas Red® DHPE, Figure 3, A). The encapsulation ability of the phospholipid vesicles was determined by hydration of the corresponding thin lipid films in the presence of a polar fluorescent dye, 8-hydroxy-pyrene-1,3,6-trisulfonic acid (HPTS), followed by removal of nonencapsulated dye by spin-filtration and vesicle characterization using fluorescence microscopy (Figure 3, B).

We next explored de novo vesicle formation in an aqueous medium, observing that the HL coupling reaction is capable of driving the highly efficient in situ self-assembly of phospholipid membranes into vesicular structures. Combination of an aqueous solution of the histidine-lysolipid or with the oleoyl thioester resulted in the formation of large vesicular structures, both spherical and tubular, which were readily observed by fluorescence microscopy (Figure S3).

The morphological transformations of the vesicle assemblies were also monitored by phase-contrast microscopy (Figure 4). No observable aggregates were found immediately after the combination of both precursors. Approximately 10 minutes after mixing, small granular aggregates began to appear, which were transformed into spherical vesicles. We also observed that the HL sustained the growth of vesicles present in the reaction medium, which is possibly due to continued formation of phospholipid within the bilayer of the in situ formed vesicles.

One advantage of the HL-driven lipid formation is the chemoselectivity of the reaction, even in the presence of relevant biological molecules such as proteins. To check for orthogonality and biocompatibility of the lipid-forming HL reaction, EGFP was spontaneously encapsulated in situ by addition of the corresponding precursors to a solution containing EGFP. After 24 hours of reaction and removal of nonencapsulated EGFP by spin filtration, the lipid-containing solution was immediately (less than 5 min) examined by fluorescence microscopy, and stable vesicles containing EGFP were observed (Figure 5). The compatibility of lipid formation with biological molecules could lead to applications involving the use of these vesicles as bioreactors.
In summary, we have developed a highly efficient and chemoselective approach based on HL reaction to readily prepare a new class of amidophospholipids, which self-assemble in situ to form synthetic membranes. The selectivity, high reactivity, and the biocompatibility of this methodology are key features that make it a powerful tool for the efficient encapsulation of relevant biomolecules for bottom-up synthetic biology applications.

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Supporting Information
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References and Notes
(8) General Procedure for the Synthesis of Histidine-Functionalized Lysolipids: 1-Palmitoyl-2-(His)-(sn-glycero-3-phosphocholine) (1)
A solution of 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (47.5 mg, 95.8 μmol), N-Boc-L-His(Trt)-OH (119.2 mg, 239.5 μmol), DMAP (70.2 mg, 574.8 μmol), and Et3N (46.7 μL) was stirred at r.t. for 10 min. Then, DMAP (7.4 mg, 60.9 μmol) and Et3N (46.7 μL) were successively added. After 10 min stirring at r.t., the mixture was extracted with H2O (2 × 3 mL), 1H NMR (500.13 MHz, CD3OD): δ = 7.89–7.61 (1 H, 1 × CH3), 7.20–6.90 (1 H, 1 × CH2), 5.42–5.15 (1 m, 1 × CH), 4.54–3.85 (1 m, 1 × CH + 1 × CH2), 3.71–3.51 (1 m, 1 × CH + 1 × CH2), 3.47–2.20 (2 H, 1 × CH2), 1.71–1.48 (2 H, 1 × CH2), 1.44–1.16 (1 m, 24 H, 12 × CH)), 0.90 (t, J = 6.6 Hz, 3 H, 1 × CH3). 13C NMR (125.77 MHz, CD3OD): δ = 174.9, 168.1, 136.9, 134.0, 117.2, 74.2, 67.8, 64.9, 63.1, 54.7, 54.5, 34.9, 34.7, 33.1, 30.8, 30.8, 30.7, 30.7, 30.6, 30.5, 30.4, 30.2, 29.7, 29.6, 25.9, 23.7, 14.4. ESI-MS (TOF): m/z (%) = 633 (100) [MH]+. ESI-HRMS (TOF): m/z calc for C54H80N4O10P [MH]+: 976.5307; found: 976.5306.
(10) MESNA Oleyl Thiosteer (2)
A solution of oleic acid (199.2 mg, 670.0 μmol) in CH2Cl2 (5 mL) was stirred at 0 °C for 10 min, and then DMAP (7.4 mg, 60.9 μmol) and EDC-HCl (128.4 mg, 670.0 μmol) were successively added. After 10 min stirring at 0 °C, sodium 2-mercaptopethanesulfonate (MESNA, 100.0 mg, 609.1 μmol) was added. After 5 h stirring at r.t., the mixture was extracted with H2O (2 × 3 mL), and the combined aqueous phases were washed with EoAc (3 mL). After evaporation of H2O under reduced pressure, the residue was washed with MeCN (5 mL), and then filtered to yield 194.7 mg of 1-Palmitoyl-2-[N-Boc-L-His(Trt)]-sn-glycero-3-phosphocholine (2) as a white solid [75%]. 1H NMR (500.13 MHz, CDCl3): δ = 5.36–5.27 (m, 2 H, 2 × CH3), 3.05–2.99 (m, 2 H, 1 × CH2), 2.60–2.51 (m, 4 H, 2 × CH2), 2.02–1.92 (m, 4 H, 2 × CH2); 1.58–1.49 (m, 2 H, 1 × CH2), 1.34–1.18 (m, 20 H, 10 × CH2). 85 (t, J = 6.9 Hz, 3 H, 1 × CH3). 13C NMR (125.77 MHz, CDCl3): δ = 198.7, 129.8, 129.7, 51.0, 43.4, 31.4, 29.2, 29.1, 28.9, 28.8, 28.6, 28.5, 28.3, 26.7, 26.6, 25.1, 24.4, 22.2, 14.1. ESI-MS (TOF): m/z (%) = 429 (100) [MH]+. ESI-HRMS (TOF): m/z calc for C54H80N4O10P [MH]+: 976.5398; found: 976.5396.
(12) General Procedure for the Synthesis of Histidine-Functionalized Phospholipids: 1-Palmitoyl-2-[His-(oleoyl)]-sn-glycero-3-phosphocholine (3)
1-Palmitoyl-2-[His]-sn-glycero-3-phosphocholine (1, 2.00 mg, 3.16 μmol) and MESNA oleyl thiosteer (2, 1.36 mg, 3.16 μmol) were dissolved in 633 μL of 100 mM HEPES buffer pH 7.5 and...
stirred under N₂ at r.t. After 24 h, the corresponding mixture was filtered using a 0.2 μm syringe-driven filter, and the crude solution was purified by HPLC, affording 1.2 mg of the phospholipid 3 as a colorless film [43%, tᵣ = 8.2 min (Zorbax SB-C18 semipreparative column, 100% Phase B, 15.5 min)]. ESI-MS (TOF): m/z (%) = 898 (100) [MH⁺]. ESI-HRMS (TOF): m/z calcld for C₄₈H₉₀N₄O₉P [MH⁺]: 897.6440; found. 897.6437.

(13) Monitoring the Progress of Phospholipid Formation
12.5 μL (10 mM stock solution) of lysolipid 1 and 15 μL (10 mM stock solution) of thioester 2 were added to 22.5 μL of HEPES buffer (100 mM, pH 7.5) at r.t. Then, 7.5 μL of the reaction mixture were taken out, diluted with 92.5 μL MeOH and injected into HPLC at various time points (0, 2, 4, 8, 12, and 24 h). Method used: 50–95% Phase A in Phase B (8 min), and then 95% Phase A in Phase B (4 min). The progress of the reaction was monitored from the disappearance of peak corresponding to lysolipid 1 and appearance of peak corresponding to the phospholipid 3.

(14) Membrane Staining with Texas Red® DHPE
5 μL of a 10 mM solution of phospholipid 3 in MeOH–CHCl₃ (1:1) and 0.5 μL of a 0.1 mM solution of Texas Red® DHPE in EtOH were added to a glass vial, placed under a steady flow of N₂, and dried for 10 min to prepare a lipid film. Then, 100 μL of H₂O were added to the lipid film and briefly vortexed. The solution was tumbled at r.t. for 30 min. Afterward, the resulting cloudy solution was diluted with an additional 200 μL of H₂O and transferred to a 100 kDa molecular weight cut-off (MWCO) centrifugal membrane filter and centrifuged for 3 min at 10,000 rcf (Eppendorf 5415C). The solution was similarly washed for additional 5× to remove any nonencapsulated dye. Then, 5 μL of the vesicle solution were placed on a clean glass slide, secured by a greased cover slip, and imaged on a spinning disc confocal microscope (488 nm laser) to observe encapsulation of HPTS.

(15) Encapsulation of HPTS
10 μL of a 10 mM solution of phospholipid 3 in MeOH–CHCl₃ (1:1) were added to a glass vial, placed under a steady flow of N₂, and dried for 10 min to prepare a lipid film. Then, 100 μL of 0.1 mM HPTS aqueous solution were added to the lipid film and briefly vortexed. The solution was tumbled at r.t. for 30 min. Afterward, the resulting cloudy solution was diluted with an additional 200 μL of H₂O and transferred to a 100 kDa molecular weight cut-off (MWCO) centrifugal membrane filter and centrifuged for 3 min at 10,000 rcf (Eppendorf 5415C). The solution was similarly washed for additional 5× to remove any nonencapsulated dye. Then, 5 μL of the vesicle solution were placed on a clean glass slide, secured by a greased cover slip, and imaged on a spinning disc confocal microscope (488 nm laser) to observe encapsulation of EGFP in the in situ formed vesicles.