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Tapping a Bacterial Enzymatic Pathway for the Preparation and Manipulation of Synthetic Nanomaterials

Ti-Hsuan Ku, Swagat Sahu, Nicolas M. Kosa, Kevin M. Pham, Michael D. Burkart, and Nathan C. Gianneschi*

Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093, United States

Supporting Information

ABSTRACT: We present a spherical micelle generated in a three-step sequence in which a farnesyl-pantetheine conjugate is phosphorylated, adenylated, and phosphorylated once more to generate a farnesyl-CoA amphiphile that self-assembles into spherical micelles. A sphere-to-fibril morphological switch is achieved by enzymatically transferring the farnesyl group of the farnesyl-CoA micelle onto a peptide via phosphopantetheinyl transferase to generate a peptide amphiphile. Each step in the sequence is followed with characterization by HPLC, MS, TEM, and DLS. This system offers an entry into cofactor-mediated peptide decoration by extending the principles of bioresponsive polymeric materials to sequential enzyme cascades.

Enzymes are used by natural systems to build complex molecules and structures through selective and often sequential reactions using simple starting materials. Furthermore, there are several examples where enzymes have been utilized to initiate the formation of nanoscale structures, induce morphological switches, and cause gelation.[1−9] Therefore, we were inspired to harness a known, multistep enzymatic biosynthetic pathway to build nanoscale particles and structures from small molecule precursors. The overarching goal of pursuing this type of strategy is ultimately the development of engineered biological systems for the programmed generation of nanostructures, with implications for how such materials are prepared, scaled-up, and evolved for function. As a basic starting point, we chose to utilize nonribosomal peptide and polyketide biosynthesis pathways from bacteria.[10−16] Therefore, we aimed to appropriate the enzymatic pathway that generates *holo*-acyl carrier protein (*holo*-ACP) and *holo*-peptidyl carrier protein (*holo*-PCP) from pantothenic acid[17−22] to construct an amphiphilic molecule from simple building blocks capable of spontaneous assembly into a responsive nanostructure.

Our strategy begins with the synthesis of a small molecule containing a pantetheine moiety and a farnesyl group (Figure 1). The pantetheine moiety will ultimately serve as an adaptor molecule that can facilitate the transfer of the farnesyl group from one polar headgroup to another via phosphopantetheinyl transferase (PPTase), hence the farnesyl functions as a hydrophobic tail. The first enzyme, pantothenate kinase (Kinase 1), transfers a phosphate to the primary alcohol on the pantetheine moiety in preparation for adenylation of the

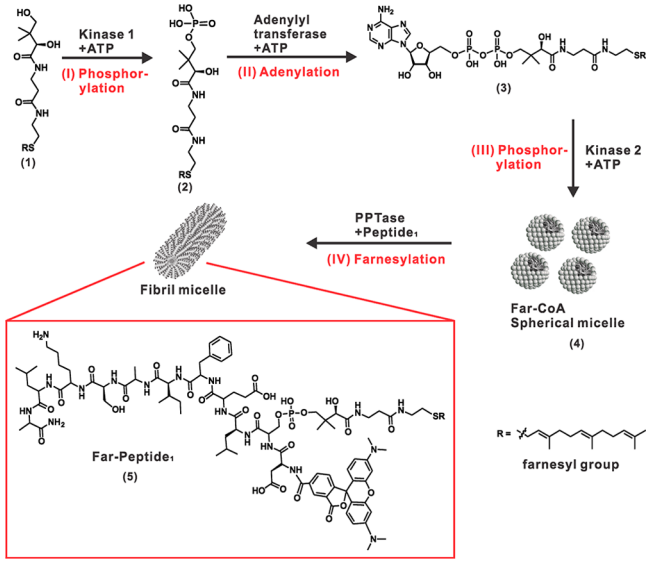

Figure 1. Chemoenzymatic preparation of a **SM** and a **FM**. Here, a farnesyl-pantetheine conjugate (**1**) was used as the starting material. **1** was phosphorylated by Kinase 1 to generate the phosphorylated product (**2**). **2** was adenylated by an adenyl transferase (phosphopantetheine adenylyltransferase) to generate the dephospho-Far-CoA (**3**). Following phosphorylation of **3** with Kinase 2, **4** was formed, which spontaneously self-assembles into a **SM**. This micelle is then transformed into a **FM** by transferring the farnesyl group onto Peptide$_1$, a labeled version of a known, competent 11 amino acid fragment of the natural protein substrate (ACP or PCP) for PPTase enzymes, via recognition of the pantetheine adaptor molecule.

structure by an adenylyl transferase. A complete farnesyl-CoA (Far-CoA) amphiphile is generated following phosphorylation at the adenosine 3′-hydroxyl by dephosphocoenzyme A kinase (Kinase 2). We reasoned that spontaneous aggregation of the amphiphile in water should result in particle formation. We envisioned replacement of the amphiphilic headgroup of the amphiphile with a peptide capable of changing the hydrophobic to hydrophilic ratio sufficiently to induce a morphological change from a spherical micelle (**SM**) to a fibril micelle (**FM**) structure. This idea was supported by the work of Stupp and others[5,23−26] who have shown that a wide range of peptide-amphiphiles of similar design readily form fibrils in solution.

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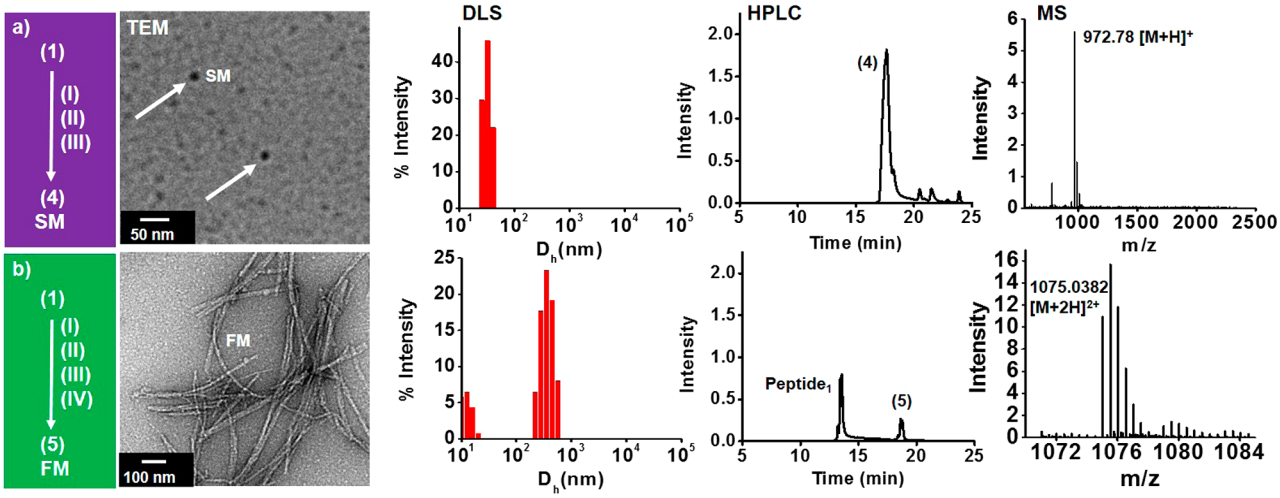

Figure 2. Sequential one-pot chemoenzymatic synthesis of **4** and **5**, giving rise to **SM** and **FM**, respectively, in aqueous, buffered solution. (a) Reaction mixture of **1**, Kinase 1, adenyl transferase, and Kinase 2 with ATP to form **4** and **SM**. (b) Reaction mixture of **1**, Kinase 1, adenyl transferase, Kinase 2, ATP, PPTase, and $Peptide_1$ generates **5** and **FM**. Reaction products were purified by HPLC (20−90% ACN with 0.1% TFA over 25 min), lyophilized, and resuspended in Tris buffer for analysis by TEM, DLS, HPLC, and MS. Analysis of reactions to generate **2** and **3** are shown in Figure 1S. In addition, analysis of the crude, one-pot reaction mixtures are shown in Figure 2S.

A suitable peptide for this endeavor is ybbR, an 11-residue peptide fragment of a peptidyl carrier protein domain that is recognized by SFP, the PPTase from *Bacillus subtilis*.[27,28] Sfp transfers the phosphopantetheine moiety of CoA to the hydroxyl group of a serine residue near the N-terminus of the ybbR peptide. Burkart et al.[29−31] have demonstrated that pantetheine can be conjugated to a variety of small molecules while still being recognized by the PPTase after enzymatic generation of the modified CoA *in situ*. The PPTase-mediated covalent transfer of the modified phosphopantetheine to the ybbR peptide serves as a reliable scaffold for custom label attachment using a wide variety of chemical groups. In this work, we endeavored to test these capabilities in the context of nanomaterial preparation and manipulation.

We designed and synthesized a simple, small molecule building block containing farnesyl conjugated to pantetheine via a thioether linkage (**1**, Figure 1). This compound would serve as the template for construction of the CoA- and peptide-containing amphiphiles. To demonstrate the feasibility of our approach, we followed each step in the proposed enzymatic sequence by high-pressure liquid chromatography (HPLC), monitoring the consumption of each starting material and the appearance of the corresponding product in the sequence. We then characterized the crude reaction mixture of each step alongside the isolated products - which had been separated from the reaction mixture by HPLC, lyophilized, and resuspended/dialyzed into Tris buffer by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), transmission electron microscopy (TEM), and dynamic-light scattering (DLS). To begin, farnesyl-pantetheine (**1**) was treated with Kinase 1 in the presence of ATP to yield the phosphorylated product (**2**) at nearly 50% conversion, as verified by HPLC (Figure 1S). The next two steps were similarly executed with adenylation of (**2**) and phosphorylation of the resulting adenosine product, with both reactions nearing quantitative conversion (Figures 2 and 1S). Importantly, no uniformly aggregated structures were seen by TEM or DLS (Figure 1Sa,b) until addition of the final phosphate group by Kinase 2, which yielded the fully formed Far-CoA amphiphile (**4**, Figure 1). This structure assembled into a spherical micelle (Figure 2). A one-pot mixture was then prepared in which **1** was treated with all four enzymes at once, Kinase 1, adenylyl transferase, Kinase 2, and PPTase, in the presence of ATP and $Peptide_1$ (a rhodamine-labeled ybbR peptide) converting with a yield of 40% to farnesyl-$Peptide_1$ (**5**) (Figure 2b). Similar structures were observed in crude reaction mixtures (Figure 2S).

To verify that the Far-CoA generated by the first three enzymes in the sequence does, indeed, form spherical micelles, we independently synthesized the authentic Far-CoA amphiphile via conjugation of *trans,trans*-farnesyl bromide to CoA (see Supporting Information for synthetic details). After conjugation, the amphiphile was dissolved in HEPES buffer and sonicated for 20 min. This preparation yielded uniformly shaped, spherical micelles approximately 10−15 nm in diameter as characterized by TEM and DLS (Figures 3 and 3S), which is consistent with the morphology and size of the chemo-enzymatically prepared spherical micelles (Figure 2a). To confirm the observation that **5** generated via the one-pot approach forms fibril micelles, we then treated the spherical micelle assemblies with $Peptide_1$ and PPTase for 6 h at 37 °C. A 100-fold increase in hydrodynamic diameter was observed by DLS and resulted in a dramatic change in the morphology of the assembly (Figures 3 and 4S), together with the appearance of fibril structures observed via TEM. HR-MS verified the exchange of the CoA headgroup for $Peptide_1$ (Figure 5S). To further characterize the nature of the peptide within the fibrillar structures, circular dichroism spectra of **5** were obtained and are indicative of β sheet secondary structure (Figure 6S).

To monitor $Peptide_1$ transfer to the farnesyl group and assembly into fibrils, we used a Förster resonance energy transfer (FRET) assay (Figure 4). Critically, $Peptide_1$ and a S2G (serine (S) in the "2" position with respect to the N-terminus replaced with glycine (G)) mutant $Peptide_{1\_S2G}$ were labeled with an N-terminal rhodamine dye. Either peptide was then mixed with a fluorescein-labeled version ($Peptide_2$), **SM** and the PPTase. FRET between fluorescein (the donor) and rhodamine (the acceptor) would only be observed if the two peptides are mixed upon aggregation to form the fibril structure. The scenario in which the control $Peptide_{1\_S2G}$

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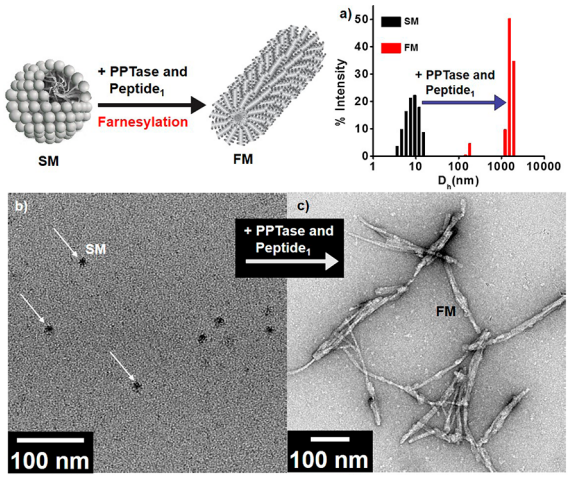

Figure 3. Chemically synthesized and purified **4** was dialyzed from DMSO into buffered water to generate **SM**. TEM and DLS of PPTase-catalyzed Far-Peptide$_1$ formation is shown. 40 μM Peptide$_1$ and 160 μM Far-CoA **SM** (bottom left TEM image) were reacted with 10 μM PPTase at 37 °C for 6 h followed by image with TEM (bottom right TEM image), which **FM** can be observed. Size distributions of Far-CoA **SM** and Far-Peptide$_1$ **FM** from the DLS measurement (top right plot) are consistent with TEM images.

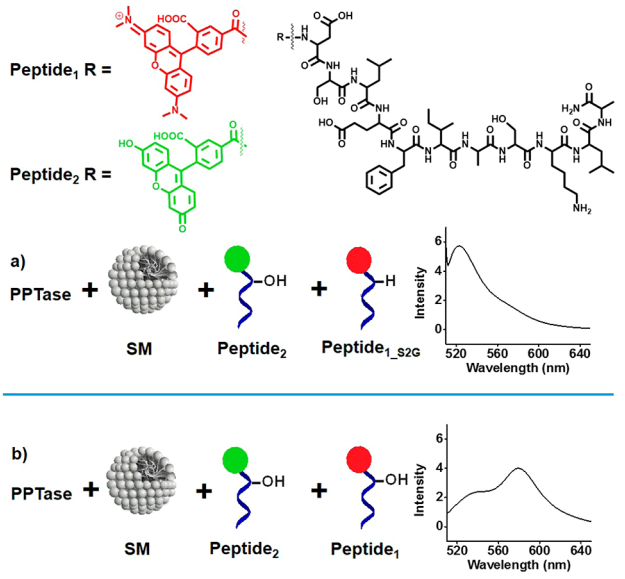

Figure 4. FRET spectra of the PPTase-catalyzed reaction of Peptide$_1$ and its control. Fluorescence emission scans (λ_{ex}: 492 nm) of (a) 10 μM Peptide$_2$ and 100 μM Peptide$_{1_S2G}$ control peptide or (b) 10 μM Peptide$_2$ and 100 μM Peptide$_1$ reacted with 160 μM **SM** and 10 μM PPTase at 37 °C for 6 h are shown in the right panel.

peptide is used as phosphopantatheine acceptor should yield no FRET signal because the mutant is not recognized by the PPTase. A significant FRET peak (580 nm) was clearly observed when a ratio of 1:10 of Peptide$_2$ to Peptide$_1$ was used (Figures 4b and 7S for 1:5 and controls), which was not seen for Peptide$_2$ with control Peptide$_{1_S2G}$ (Figure 4a), confirming that the PPTase was necessary for construction of the peptide-containing amphiphiles and the **FM** structures. This FRET assay was also employed utilizing **1** in the full, four enzyme one-pot reaction with Peptide$_1$, Peptide$_2$, and Peptide$_{1_S2G}$, and similar results were observed (Figure 8S).

The kinetics of PPTase catalyzed **5** formation was further evaluated by monitoring the Peptide$_1$ peak disappearance in the HPLC trace where the identity of the chromatogram peaks were confirmed via HR-MS (Figures 5S and 9S). A time course of the reaction was followed at several concentrations of Peptide$_1$ to determine kinetics of the farnesyl transfer (Figure 9Sb). The measured k_{cat}/K_M (0.04 μM^{-1} min^{-1}) is consistent with previously reported values for the PPTase,[32] suggesting that the affinity for and reactivity of the CoA substrate is not compromised by being part of the spherical micellar assembly.

As an initial exploration of the potential reversibility of the morphology switch we utilized an acyl carrier protein hydrolase (AcpH), recently reported by Burkart et al. to selectively recognize and hydrolyze the phosphopantetheine moiety from the ybbR peptide substrate.[33,34] We monitored this hydrolysis by HPLC and saw clear evidence of regeneration of Peptide$_1$ from **5**, albeit at low yields. We also performed MALDI-TOF MS to further confirm the identity of the regenerated Peptide$_1$ (Figure 5Sc,d). The resulting product was further characterized by TEM and DLS (Figure 10S) to verify the fibril micelle was deformed by AcpH hydrolysis.

In summary, we have demonstrated that a well-characterized enzymatic pathway used in bacterial cells to construct *holo*-ACP and *holo*-PCP can be adopted to build nanoscale architectures from simple chemical building blocks. We envision future studies involving the repurposing of other cellular processes for the preparation of designer nanomaterials *in vivo*.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

ngianneschi@ucsd.edu

Notes

The authors declare no competing financial interest.

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