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

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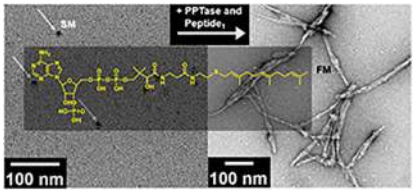
Abstract

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, mass spectrometry, transmission electron microscopy (TEM) and dynamic light scattering (DLS). This system offers an entry into cofactor-mediated peptide decoration by extending the principles of bioresponsive polymeric materials to sequential enzyme cascades.

Graphical Abstract



Enzymes are used by natural systems to build complex molecules and structures through selective and often sequential reactions using simple starting materials. We were inspired to harness a known enzymatic pathway to build nanoscale particles and structures and chose to utilize non-ribosomal peptide and polyketide biosynthesis pathways from bacteria.[1–7] 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[8–13] to construct an amphiphilic molecule from simple building blocks capable of spontaneous assembly into a responsive nanostructure.

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Supporting Information

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

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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 head group to another via phosphopanthentheinyl 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 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 head group 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[14–18] who have shown that a wide range of peptide-amphiphiles of similar design readily form fibrils in solution.

A suitable peptide for this endeavor is ybbR, an 11-residue peptide fragment of a peptidyl carrier protein domain that is recognized by the Sfp PPTase from *Bacillus subtilis*.[19,20] Sfp transfers the phosphopantetheine moiety of CoA to the hydroxyl group of a serine residue near the N-terminus of the ybbR peptide. Burkart and co-workers[21–23] 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 pan-tetheine via a thioether linkage (1, Figure 1). This compound would serve as the template for construction of the CoA-containing and peptide-containing amphiphiles. To demonstrate the feasibility of our approach, we followed each step in the proposed enzymatic sequence by 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 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), as verified by MALDI-TOF MS. The next two steps were similarly executed with adenylation of (2) and phosphorylation of the resulting adenosine product. Importantly, no uniformly aggregated structures were seen by TEM or DLS (Figure 1S_a–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 farnesyl-pantetheine (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

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peptide) (Figure 2b). Similar structures were observed in crude reaction mixtures (Figure 2S).

To verify that the Far-CoA generated by the first 3-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 minutes. This preparation yielded uniformly shaped, spherical micelles approximately 10–15 nm in diameter as characterized by TEM and DLS (Figure 3 and Figure 3S), which is consistent with the morphology and size of the chemoenzymatically prepared spherical micelles (Figure 2a). To confirm the observation that farnesyl-Peptide$_1$ (Far-Peptide$_1$) generated via the one-pot approach forms fibril micelles, we then treated the spherical micelle assemblies with Peptide$_1$ and PPTase for 6 hours at 37°C. A 100-fold increase in hydrodynamic diameter was observed by DLS (Figure 3a) and resulted in a dramatic change in the morphology of the assembly (Figure 3b–c and Figure 4S), together with the appearance of fibril structures observed via TEM. HR-MS verified the ex-change of the CoA head group for Peptide$_1$ (Figure 5S).

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$_{1_S2G}$ peptide is used as phosphopantathein 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 (Figure 4b and Figure 6S 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 farnesyl-pantetheine (1) in the full, four enzyme one-pot reaction with Peptide$_1$, Peptide$_2$, and Peptide$_{1_S2G}$ and similar results were observed (Figure 7S).

The kinetics of PPTase catalyzed Far-Peptide$_1$ (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 (Figure 5S and 8S). A time course of the reaction was followed at several concentrations of Peptide$_1$ to determine kinetics of the farnesyl transfer (Figure 8S_b). The measured k_{cat}/K_M (0.04 $\mu M^{-1}min^{-1}$) is consistent with previously reported values for the PPTase,[24] 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 and co-workers to selectively recognize and hydrolyze the phosphopantetheine moiety from the ybbR peptide substrate.[25,26] We monitored this hydrolysis by HPLC and saw clear evidence of

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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 5S_c–d). The resulting product was further characterized by TEM and DLS (Figure 9S) 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*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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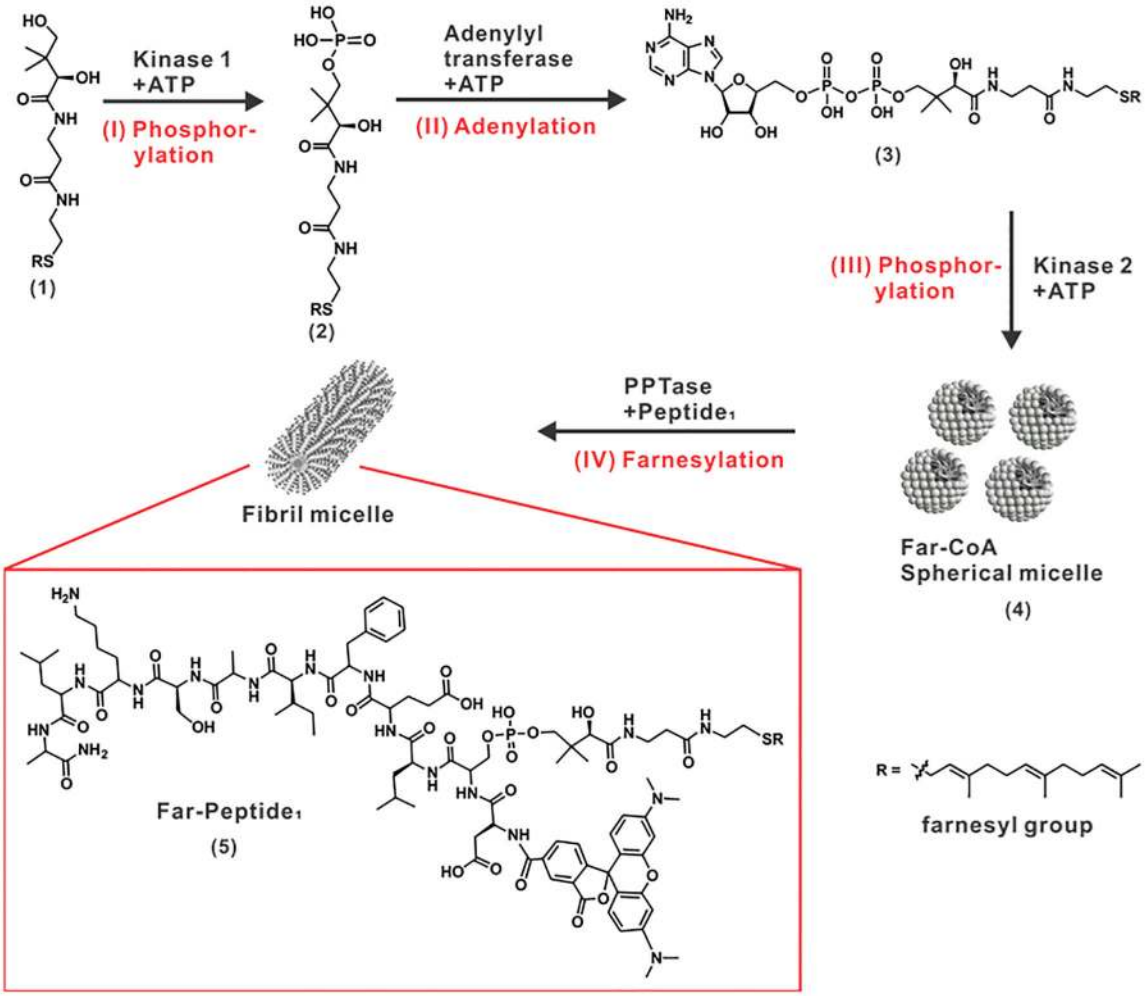

Figure 1.

Chemoenzymatic preparation of a spherical micelle (**SM**) and a fibril micelle (**FM**). Here, a farnesyl-pantetheine conjugate (**1**) was used as the starting material. **1** was phosphorylated by Kinase 1 (pantothenate kinase) 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 (dephosphocoenzyme A kinase), **4** was formed, which spontaneously self-assembles into a spherical micelle (**SM**). This micelle is then transformed into a fibril micelle structure (**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 phosphopantatheinyl transferase (PPTase) enzymes, via recognition of the panthetheine adaptor molecule.

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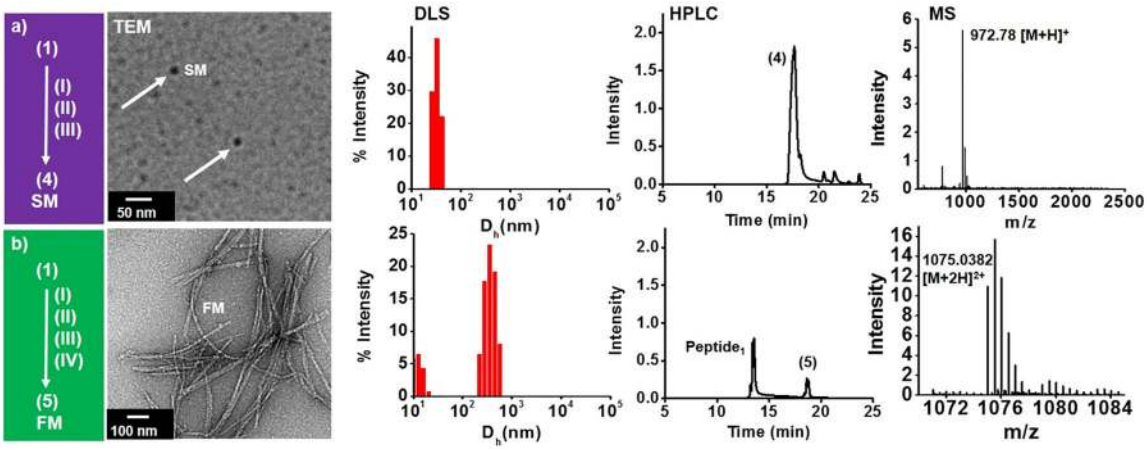

Figure 2.

Sequential one-pot chemoenzymatic synthesis of Far-CoA (**4**) and Far-Peptide$_1$ (**5**), giving rise to spherical micelle (**SM**) and fibrillar micelle (**FM**) respectively in aqueous, buffered solution. a) Reaction mixture of (**1**), Kinase 1, adenyl transferase, and Kinase 2 with ATP to form Far-CoA (**4**) and **SM**. b) Reaction mixture of (**1**), Kinase 1, adenyl transferase, Kinase 2, ATP, PPTase and Peptide$_1$ generates Far-Peptide$_1$ (**5**) and **FM**. Reaction products were purified by HPLC (20 – 90% ACN with 0.1% TFA over 25min), lyophilized and re-suspended in Tris buffer for analysis by TEM, DLS, HPLC and MS. Analysis of reactions to generate **2** and **3** are shown in Supporting Information Figure 1S. In addition, analysis of the crude, one-pot reaction mixtures are shown in Supporting Information Figure 2S

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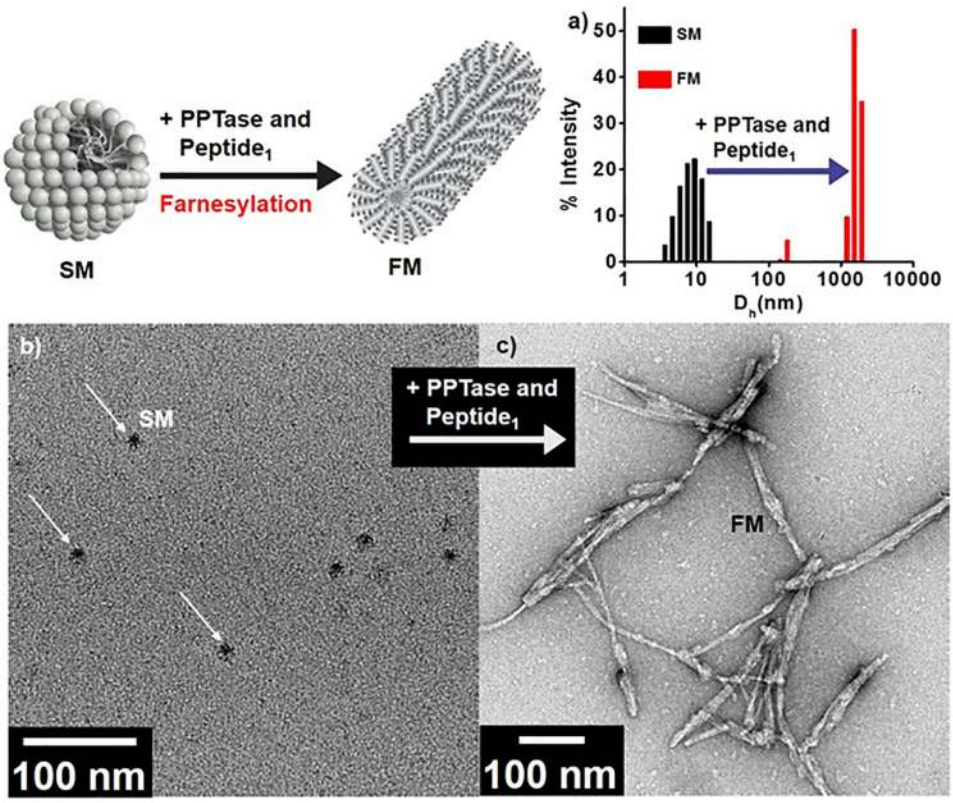

Figure 3.

Chemically synthesized and purified **4** was dialyzed from DMSO into buffered water to generate spherical micelle **(SM)**. Transmission electron microscopy (TEM) and dynamic light scattering (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 hours followed by image with TEM (bottom right TEM image), which fibril micelles (**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.

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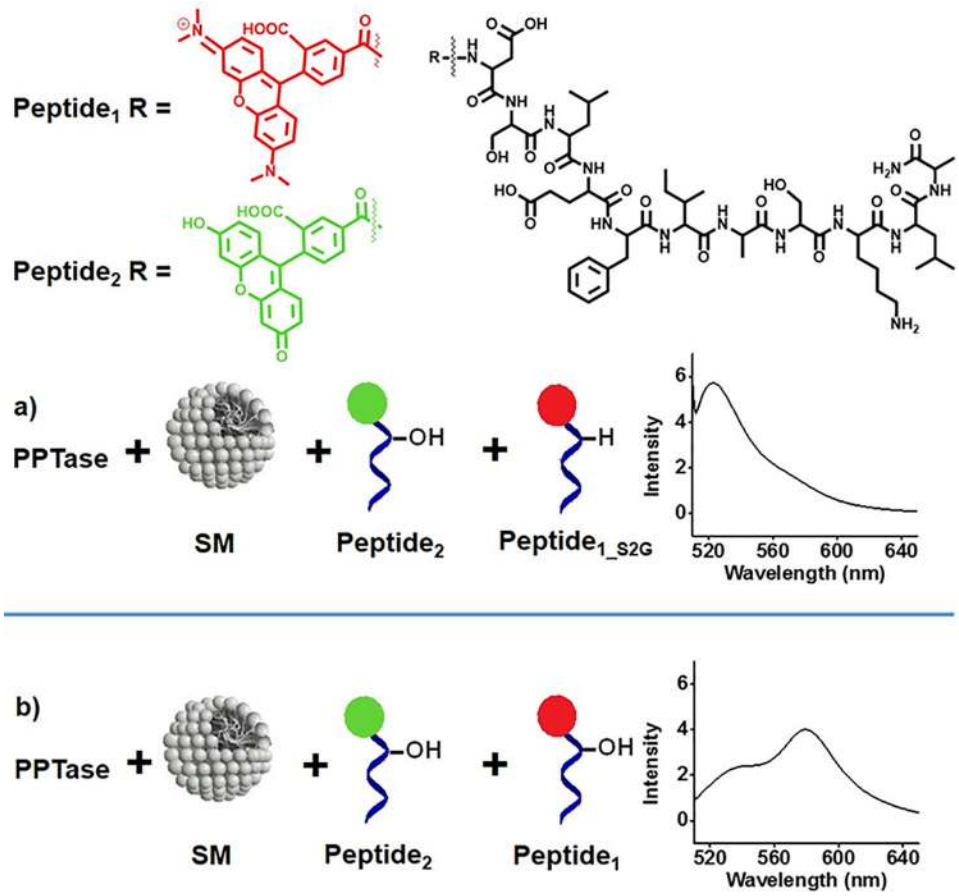

Figure 4.

Förster resonance energy transfer (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 hours are shown in the right panel.