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# Controlling and Switching the Morphology of Micellar Nanoparticles with Enzymes

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Supporting Information

**ABSTRACT:** Micelles were prepared from polymer-peptide block copolymer amphiphiles containing substrates for protein kinase A, protein phosphatase-1, and matrix metalloproteinases 2 and 9. We examine reversible switching of the morphology of these micelles through a phosphorylation−dephosphorylation cycle and study peptide-sequence directed changes in morphology in response to proteolysis. Furthermore, the exceptional uniformity of these polymer-peptide particles makes them amenable to cryo-TEM reconstruction techniques lending insight into their internal structure.

In biology, stimuli-responsive multisubunit assemblies are ubiquitous, and mimicking these systems via synthetic approaches is of increasing interest. Interfacing such synthetic materials with biological systems is particularly promising for a range of biomedical applications including targeted drug delivery and molecular diagnostics.[1] Within this class of materials are particles capable of changing morphology in response to stimuli. Enzymes are attractive and unique stimuli with great potential in this regard, as they propagate an amplified response via catalytic reactions,[2] can be highly substrate specific, and have expression patterns sometimes associated with disease states.[3] Nanoscale assemblies of block copolymer amphiphiles are well-suited for the development of functional, stimuli-responsive systems because changes in the chemical or physical nature of the amphiphile[4] can lead to formation, destruction, or morphological transformations.[5] However, while there are examples of enzyme-responsive formation and destruction of such materials,[6] there are no examples of enzymatic switches of micellar morphology.[7] This is despite the tremendous interest in enzymes as stimuli for responsive materials in general,[8] and the power of tunable amphiphilicity for switching the shape and size of nanoscale particles, as demonstrated for a range of other stimuli.[7]

To develop nanoparticles capable of enzyme-directed morphological transformations, we hypothesized that peptides, as enzyme substrates, could be utilized as hydrophilic head groups in polymeric amphiphiles (Figure 1).[9,10] When properly designed, these polymer-peptide amphiphiles would aggregate to generate enzymatically responsive micelles. To validate this hypothesis, we explored enzymatic modulation of particle morphology via common post-translational modification processes utilized to manipulate biomolecular assemblies in natural systems. Furthermore, cryoelectron microscopy

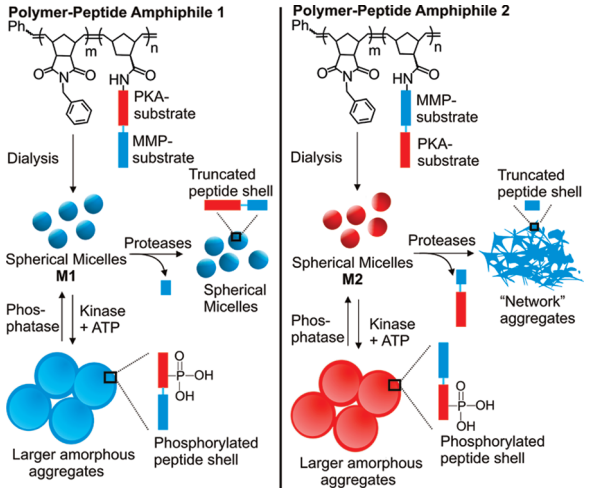


**Figure 1.** Peptide-substrate polymeric amphiphiles assemble into spherical micelles. The peptide substrates within the micelle corona interact with enzymes to generate a variety of morphologies of polymeric amphiphile aggregates depending on the design of the peptide substrate and enzymes added.

(cryo-TEM) and three-dimensional (3D) image reconstruction were used to confirm the spherical micellar morphology and uniformity of the particles and to determine their radial density profile.[11]

Amphiphilic polymer-peptides were designed, containing substrates for four different cancer-associated enzymes: protein kinase A (PKA),[12] protein phosphatase-1 (PP1),[13] and matrix-metalloproteinases MMP-2 and MMP-9.[3b−d] By incorporating these enzyme substrates into the polar head groups of the copolymers, the micelle morphology and aggregation behavior of the materials can be modified using the following mechanisms: (1) phosphorylation by PKA at serine residues, (2) dephosphorylation by PP1 at serine residues, (3) peptide cleavage by MMPs at Gly-Leu peptide bonds. We reasoned that enzymatic reactions occurring within the shell of the particles would facilitate changes in the steric bulk, and electrostatic properties of the amphiphiles, and would result in changes to the overall architecture via the establishment of new equilibria for surfactant aggregation.[4] Also, we expected enzyme-directed responses to be influenced by the design of the peptide substrate. To test this hypothesis, spherical micelles (**M1**, **M2**) were prepared from amphiphilic peptide-brush copolymers that differed only in the relative

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**Table 1. Peptide-Shell Polymeric Amphiphiles and Resulting Parameters for Micelles M1 and M2**

| | Peptide substrate[a] | m[b] | n[b] | Polymer $M_n$, g/mol[c] | $M_w/M_n$[c] | $D_h$[d] | PDI[d] |
|---|---|---|---|---|---|---|---|
| **M1** | H N- LRRASLGKGPLGLAG | 34 | 6 | 19470 | 1.01 | 24 | 0.27 |
| **M2** | H N- KKPLGLAGLRRASLG | 34 | 5 | 19430 | 1.17 | 33 | 0.19 |

[a] PKA/PP1 and MMP substrates are shown in red and blue, respectively, with phosphorylation or cleavage sites boldfaced and underlined. Peptides are conjugated to the polymer through the amino termini. [b] Block size of $m$ ("phenyl block") was determined by SEC-MALS ($M_n$ = 8553 g/mol), and $n$ was estimated via SEC-MALS and UV−vis as described in the Supporting Information. [c] Polymer $M_n$ and $M_w/M_n$ determined by SEC-MALS. [d] Hydrodynamic diameter and micelle PDI (polydispersity) were determined by DLS.

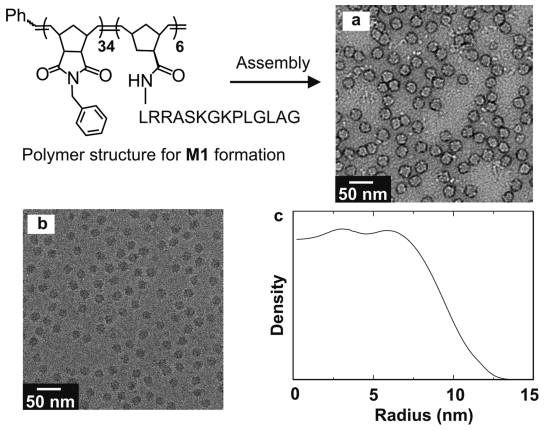


**Figure 2.** TEM characterization of **M1**. (a) Micrograph of **M1** sample, stained with 1% uranyl acetate. (b) Micrograph of unstained vitrified **M1** sample. (c) Reconstructed radial density plot from cryo-TEM data (SI, Figure 2S).

ordering of the peptide substrates (Table 1). Ring-opening metathesis polymerization (ROMP)[1n] was used to synthesize a block copolymer of hydrophobic side chains (phenyl groups) and *N*-hydroxysuccinimide side chains[14] for subsequent conjugation with peptides. To prepare **M1** and **M2**, the block copolymer amphiphiles were dissolved in DMSO/DMF (1:1) and dialyzed against buffered water for 24 h (Table 1, Supporting Information (SI), Figure 1S).

The diameters of the spherical particles, as confirmed by transmission electron microscopy (TEM), scanning electron microscopy (SEM), and dynamic light scattering (DLS: hydrodynamic diameter, $D_h$), were between 24 and 33 nm (Table 1). We further examined **M1** using cryo-TEM followed by single-particle, 3D image reconstruction to characterize and define the particles in a native hydrated state (Figure 2).[15] Intriguingly, the radial density profile for the spherically averaged reconstruction (Figure 2c) has a similar shape to that simulated and measured for other copolymer and surfactant-based micelles as determined by alternative techniques.[16] In particular, the materials show low density at the central core and a region of higher density at the edge of the core and in the surrounding shell. This profile is consistent with a hydrophobic core radius of 5 to 7 nm. From individual particle images we assigned radii to the micelles, defined as the distance from the center at which the minimum density occurs (SI, Figure 2S). The results indicate that the particles are spherical micellar architectures, 24 nm in average diameter with a maximum variation of 3 nm.

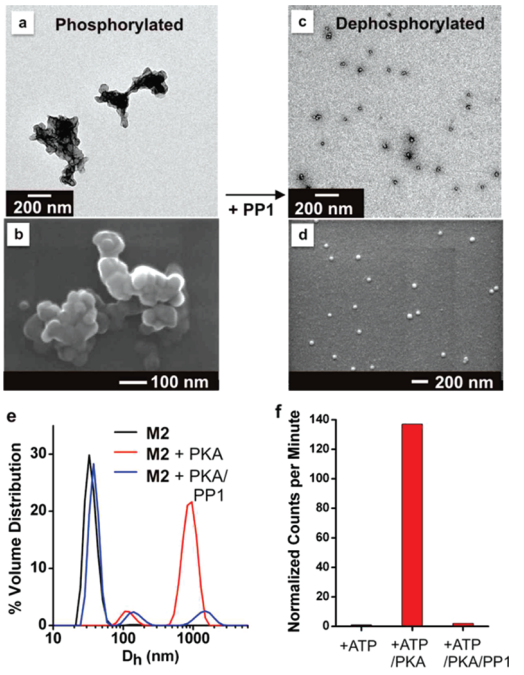


**Figure 3.** Response of **M2** to sequential additions of PKA and PP1. (a) TEM and (b) SEM: **M2** (20 $\mu$M with respect to polymer-peptide amphiphile) treated with PKA (2500 U) plus ATP (2 mM) and incubated at 30 °C for 24 h followed by dialysis. (c) TEM and (d) SEM: Phosphorylated particles subjected to either dialysis or heat denaturation of PKA (20 min, 65 °C) prior to treatment with PP1 (2.5 U) at 30 °C for 24 h. (e) DLS confirms increase and decrease of aggregate size in solution via phosphorylation and dephosphorylation respectively. (f) Phosphorylation and dephosphorylation were confirmed by radiolabeling the particles using [$\gamma$-$^{32}$P] ATP. Heat denaturation and extended dialysis had no effect on **M2** micelles alone. No change is observed with ATP (2 mM) without addition of PKA.

To establish that micelle morphology can be reversibly altered through enzymatic reactions, the micelles were subjected to phosphorylation by PKA and subsequent dephosphorylation by PP1 (Figure 3 for **M2** data and SI Figure 3S for **M1** data). When treated with PKA and ATP (2 mM) for 24 h at 30 °C, the initially spherical **M2** (at 20 $\mu$M, with respect to polymer-peptide amphiphile) changed dramatically in morphology (Figure 3a,b). A 50-fold increase in hydrodynamic diameter was observed (Figure 3e) together with the appearance of amorphous structures in TEM images. The phase transition occurs as phosphate group introduction into the shell of the micellar aggregates produces a significant change in structure and charge of the polymer-peptide amphiphiles.[17] However, rather than an increase in hydrophilicity causing an increase in surface curvature resulting in smaller micelles, we observe aggregation into larger structures. Therefore, it is plausible that aggregation is the result of salt bridge formation between phosphorylated particle shells, or that particles aggregate as a result of dipole-induced-dipole interparticle interactions.

Subsequent treatment of the phosphorylated micelles with PP1 for 24 h at 30 °C, following heat denaturation at 65 °C, resulted in a reversion to the original size and morphology of particle (Figure 3c−e). Radiolabeling was conducted to confirm that phosphorylation and dephosphorylation occurred through the cycling process (Figure 3f). In this experiment, **M2** was treated with PKA and radioactively labeled ATP. Following removal of excess ATP by dialysis, phosphorylation was observed with a


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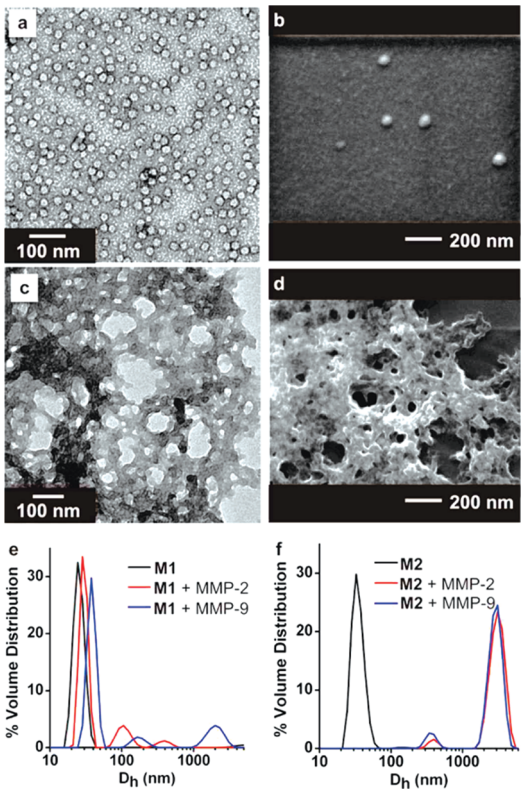


**Figure 4.** Response of micelles **M1** and **M2** to treatment with MMPs. (a) TEM **M1** + MMP-2. (b) SEM **M1** + MMP-2. (c) TEM **M2** + MMP-2. (d) SEM **M2** + MMP-2. (e−f) DLS for particles indicating changes in particle size upon MMP treatment. Micelles at 20 $\mu$M (with respect to polymer-peptide amphiphile) were incubated with MMPs (100 $\mu$U) at 37 °C for 24 h (Tris-HCl, 50 mM, pH 7.4).

scintillation counter. Results show that PKA successfully phosphorylated the particles, with the extent of phosphorylation by PKA estimated to be greater than 95% for **M2** (see SI). Subsequent treatment with PP1 (again followed by dialysis) resulted in removal of the phosphate group. To establish that this process is indeed reversible, three cycles of phosphorylation/dephosphorylation were successfully performed and analyzed by radiolabeling and via DLS (SI, Figures 4S and 5S). Together, these enzymatically driven processes demonstrate the power of this approach to switching the morphology of a micellar particle via a selective biochemical reaction, not a change in bulk solution properties such as pH or temperature.

To examine the role of site-specific, proteolytic cleavage on micelle morphology, **M1** and **M2** were treated with two cancer-associated proteases, MMP-2 and MMP-9, which were expected to have similar effects as they share a cleavage site. (Figure 4 and SI, Figure 6S). Reactions were performed on 20 $\mu$M solutions of micelles, (concentration is with respect to polymer-peptide amphiphile), for 24 h at 37 °C. TEM and SEM data showed no change in **M1** morphology (Figure 4a,b), but DLS measurements indicated the formation of some larger aggregates in solution (Figure 4e). By contrast, a dramatic change in morphology (Figure 4c,d) and hydrodynamic diameter (Figure 4f) was observed upon treating **M2** with MMP. SEM and TEM images both show evidence of the formation of an amorphous network upon peptide cleavage. The cleavage efficiency is estimated by HPLC analysis of the product to be approximately 21% (SI, Figure 7S), with product identity confirmed by MALDI-MS (SI, Figure 8S). No visible precipitate was formed in solution during this process. We infer from these results that the position of the cleavage site in the amphiphile plays a critical role in how the micelle responds to proteolysis and that complete shell cleavage is not necessary for phase transition. In particular, cleavage at sites more proximal to the polymer backbone leads to more dramatic morphological changes because of a larger change in peptide shell structure. Indeed, it is likely that the difference in the behavior of **M1** and **M2** was further accentuated by the fact that cleavage of the **M1** peptide removes three hydrophobic residues, while cleavage of the **M2** peptide leads to the loss of all hydrophilic residues. Importantly, these results indicate a tunable relationship between peptide-sequence design and enzymatically directed morphology changes.

These studies demonstrate the feasibility of designing enzymatically switchable micellar particle morphology. This was achieved by incorporating peptides as the hydrophilic block of a polymeric amphiphile. In general biomolecules including proteins,[9,18] peptides,[10,19] and nucleic acids[11,20] are attractive synthons for the development of supramolecular biomaterials[21] because they are selective as substrates for enzymes, have inherently specific recognition properties, and consist of well-defined structural elements. It is anticipated that multienzyme responsive systems like those described here will provide a route toward materials capable of signaling specific patterns of multiple biochemical stimuli. Also, the ability to program the nature of particle responses to disease-associated enzymes has broad implications for *in vivo* delivery and detection strategies where surface chemistry and morphology have critical roles in determining the targeting and pharmacokinetics of materials.

## ■ ASSOCIATED CONTENT

**Supporting Information.** Experimental details including synthetic methods, materials characterization, enzymatic conditions, and procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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