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Enzyme-Triggered Cascade Reactions and Assembly of Abiotic Block Copolymers into Micellar Nanostructures

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Supporting Information

ABSTRACT: Catalytic action of an enzyme is shown to transform a non-assembling block copolymer, composed of a completely non-natural repeat unit structure, into a self-assembling polymer building block. To achieve this, poly(styrene) is combined with an enzyme-sensitive methacrylate-based polymer segment carrying carefully designed azobenzene side chains. Once exposed to the enzyme azoreductase, in the presence of coenzyme NADPH, the azobenzene linkages undergo a bond scission reaction. This triggers a spontaneous 1,6-self-elimination cascade process and transforms the initially hydrophobic methacrylate polymer segment into a hydrophilic hydroxyethyl methacrylate structure. This change in chemical polarity of one of the polymer blocks confers an amphiphilic character to the diblock copolymer and permits it to self-assemble into a micellar nanostructure in water.

Developing strategies to gain control over formation or disruption of polymeric nanostructures is a significant goal of nanotechnology. This research goal has implications in the areas of drug delivery, biological sensing and imaging, and tissue engineering applications, among others.[1] Therefore, a large effort is devoted to the construction of nanosized polymer particles, such as micelles, that can respond to an external stimulus.[2] The nature of the stimuli employed, however, is most often limited to changes in pH, temperature, radiation, or electric field.[3] Enzymes represent an attractive alternative to the aforementioned stimuli due to their high selectivity, substrate specificity, and mild operating conditions.[4,5] In this regard, various examples are reported in which an assembled micellar nanostructure undergoes a disassembly process through an enzymatic action.[6] The reverse approach, in which an enzyme triggers assembly of a synthetic polymer into a micellar structure, however, remains limited to two examples.[7] In addition, these examples are based exclusively on a natural motif (i.e., hydrolysis or formation of phosphate esters). With a goal to diversify the available toolbox (i.e., enzyme/substrate pair) for the preparation of polymer nanostructures and enlarge the repertoire of the enzymatically activated self-assembling systems, here, we describe the development of a new strategy (Figure 1). In this approach, a self-assembly precursor is designed to have two polymeric segments composed of completely non-natural repeat units (Scheme 1). One segment is composed of poly(styrene), which is hydrophobic and insensitive to the presence of an enzyme. The other segment, also hydrophobic in nature, carries azobenzene moieties attached to a hydroxyl methacrylate-based polymer repeat unit through a benzyl−carbonate linkage. Once brought in contact with the enzyme azoreductase,[8] in the presence of

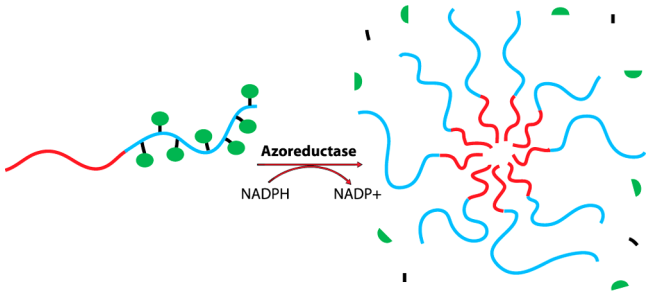
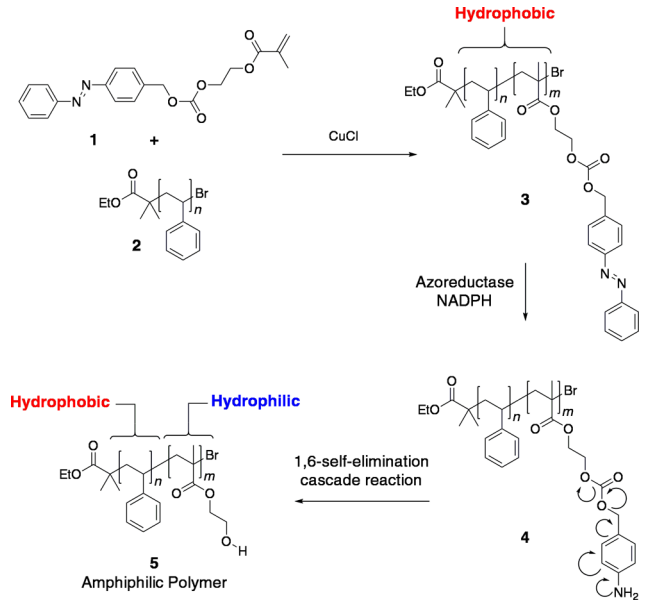


Figure 1. Schematic representation of a polymer building block and its assembly into a micellar nanostructure upon enzymatic activation in water.

Scheme 1. Synthesis of Block Copolymer 3, Cleavage of Azobenzene Linkages upon Enzymatic Action, and Subsequent 1,6-Self-Elimination Reaction To Yield Amphiphilic Assembly Precursor 5



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coenzyme NADPH, the azobenzene linkage undergoes a cleavage reaction,[8,9] which triggers a spontaneous 1,6-self-elimination cascade process (Scheme 1).[10] This transforms the initially hydrophobic segment into a hydrophilic hydroxyethyl methacrylate structure. The alteration in chemical polarity of one of the copolymer blocks imparts an amphiphilic nature to the diblock copolymer and allows it to assemble into a micellar nanostructure in water.

The enzyme azoreductase is produced by the microbial flora of human intestine. Therefore, azoreductase-sensitive systems are of relevance to colon-related therapeutic applications.[11] For example, a small-molecule drug such as sulfasalazine, in which cleavage of the azobenzene bond releases the therapeutically active 5-aminosalicylic acid moiety, is used in inflammatory bowel disease treatment. Moreover, a variety of azobenzene-based polymers have been investigated for their colon-specific delivery applications.[9]

To establish the aforementioned concept, a careful design of the enzyme-responsive polymer segment was critical. This was achieved by placing a carbonate moiety, as a leaving group, at the benzylic position of the azobenzene functionality in the hydroxyethyl methacrylate-based monomer **1** (Scheme 1). It was envisaged that enzymatic cleavage of the azobenzene group would result in the formation of an aniline group at each polymer repeat unit.[8,9] Such electron-rich aromatic species, with a leaving group placed at the benzylic position, are known to undergo an azaquinone methide-mediated spontaneous 1,6-self-elimination process.[10] The driving force for such reactions comes from an increase in the entropy of the system coupled with the irreversible formation of the thermodynamically stable CO_2 molecule. In this way, the enzyme-triggered cascade elimination reaction would transform the hydrophobic azobenzene methacrylate polymer into a hydroxyl-carrying hydrophilic polymer segment.

To test the feasibility of the proposed approach, monomer **1** was polymerized through an atom-transfer radical polymerization (ATRP)[12] protocol using poly(styrene) macroinitiator **2** ($M_{n(NMR)}$ = 10400, $M_{n(GPC)}$ 8500, $PDI_{(GPC)}$ = 1.1). This gave rise to the targeted block copolymer **3** ($M_{n(NMR)}$ = 28800, $M_{n(GPC)}$ = 47500, $PDI_{(GPC)}$ = 1.1). The block copolymerization was evidenced by a shift to lower retention time of the copolymer **3**, as compared to the macroinitiator **2**, in gel permeation chromatography (GPC) analysis (Figure 2). In ^{1}H NMR spectroscopy, copolymer **3** displayed aromatic resonances of poly(styrene) (6.3−7.2 ppm) as well as azobenzene side chains (7.4−8.0 ppm) (Figure S2). The benzylic group could be observed at 5.1 ppm, and the methylene units located adjacent to the carbonate and the ester moieties could be detected in the range of 4−4.5 ppm.

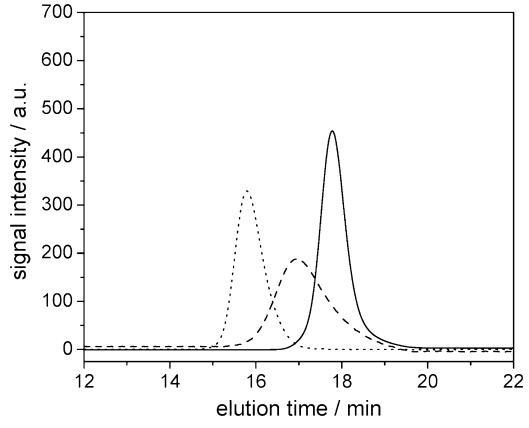

Figure 2. GPC traces of macroinitiator **2** (solid line), copolymer **3** (dotted line), and copolymer after cleavage of the azo linkages and subsequent self-elimination reaction (dashed line).

To test the enzyme sensitivity of the prepared polymeric substrate, block copolymer **3** was suspended in water and then exposed to a mixture of the enzyme azoreductase and coenzyme NADPH. The mixture was incubated at 37 °C for a period of 12 h. Since the polymer was suspended in pure water that did not contain any cosolvents (typically used in micellar preparation), elevating the temperature assisted the micellization process.[13] Finally, to remove the cofactor and its byproducts, the aqueous solution was dialyzed against water. Examination of the purified aqueous solution by UV−vis spectroscopy confirmed that the azobenzene linkages were cleaved, as no absorption signal could be observed at 450 nm after the enzymatic treatment of copolymer **3** (Figure 3). This aqueous solution was spin-coated onto a silicon substrate for observation with the help of atomic force microscopy (AFM). These experiments indicated formation of spherical structures in the size range of 30−60 nm (Figures 4 and S3−S5). Transmission electron microscopy (TEM) experiments were

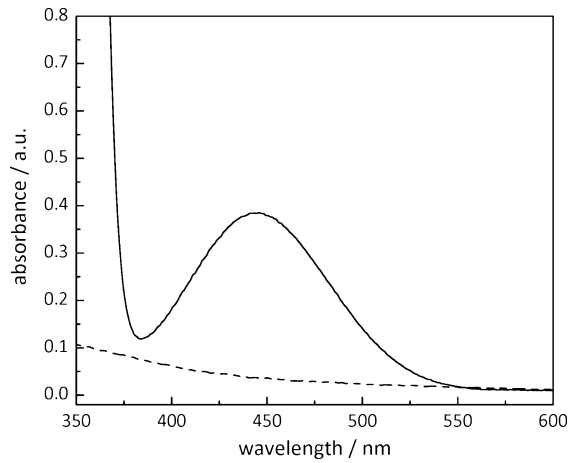

Figure 3. UV−vis spectra of polymer **3** in chloroform (solid line) and in an aqueous solution after the enzymatic reaction and purification (dashed line).

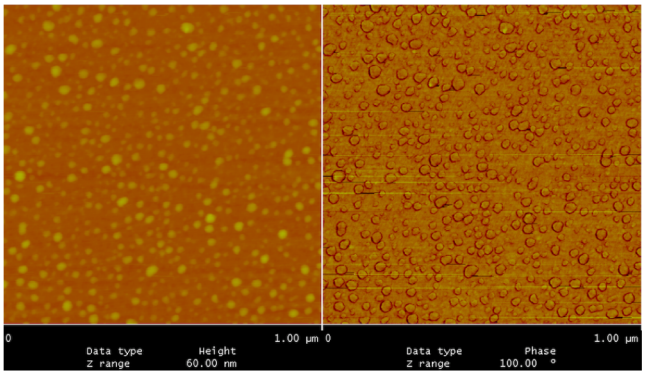

Figure 4. AFM height (left) and phase (right) images (1×1 μm^2) obtained upon spin-coating the aqueous solution after the enzymatic reaction.

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carried out by mounting an aqueous drop (~15 μL) of the micellar solution onto a carbon-coated copper grid and staining it with ruthenium tetraoxide vapors. In TEM micrographs, the periphery of the spherical structures appeared more intensely stained than the interior (Figures 5, S6, and S7). This suggested

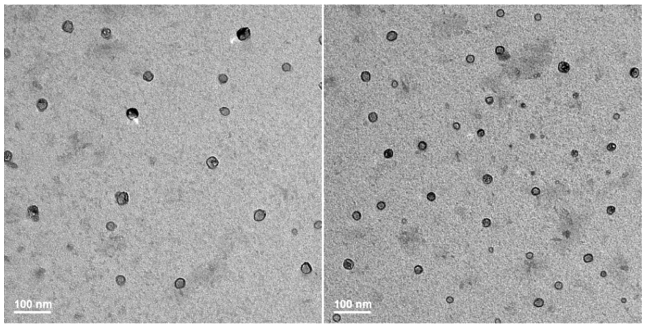

Figure 5. TEM micrographs of the aqueous micellar solution (see Figures S4 and S5 for large area images).

that the micellar shell was composed of the hydrophilic poly(hydroxyethyl methacrylate) segment known for its high affinity to ruthenium.[14] Dynamic light scattering (DLS) studies further corroborated that the aqueous solution was indeed made up of polymer nanoparticles ranging in size from 40 to 90 nm (Figure 6). The difference in the observed size range may

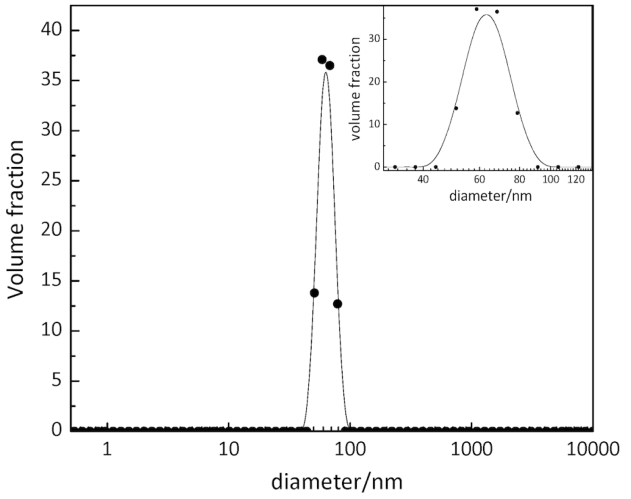

Figure 6. DLS data obtained from the aqueous solution after the enzymatic reaction.

come from the fact that AFM and TEM measurements examined the dry state while DLS measurements reflected on the solvated state of the material.

To examine the molecular structure of the polymer after the enzymatic reaction, the purified micellar solution was extracted with an organic solvent and studied with the help of ^{1}H NMR spectroscopy. This study suggested that the assembling polymer building block was indeed composed of poly(styrene) and poly(hydroxyethyl methacrylate) segments, as each proton resonance signal could be properly identified and the assignments were in good agreement with the literature reports (Figure S8).[15] As expected, this material appeared colorless due to the removal of the azobenzene chromophores from the molecular structure (Figure 7).

Figure 7. Digital photographs of bulk polymer material before (left) and after (right) the enzymatic reaction.

Finally, the purified assembling polymer was subjected to GPC analysis (Figure 2). This examination revealed that the hydrodynamic volume of the polymer decreased after the enzymatic reaction. This is most likely due to the fact that precursor polymer **3** carried carbonate-linked azobenzene side chains. These bulky side groups were removed from the second segment of the diblock copolymer upon enzymatic action. This change in molecular structure of the precursor polymer, **3**, resulted in a decrease in the hydrodynamic volume of the assembling polymer, **5**.

To summarize, the enzyme azoreductase could be employed as a trigger to assemble a carefully designed polymer with a completely non-natural chemical composition into a nanostructured morphology in water. In essence, this work diversifies the available enzyme/polymer-substrate toolbox for the preparation of polymer nanostructures and broadens the range of enzymatically triggered self-assembling systems. Furthermore, the azoreductase activation capability of the present concept may present some possibilities to create colon-related delivery/imaging systems.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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

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Notes

The authors declare no competing financial interest.

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