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Enzymatically Triggered Self-Assembly of Block Copolymers

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The increasing interest in nanostructures for biomedical and material science applications has motivated the development of controlled methods for the fabrication and assembly of various molecular systems into functionalized nanostructures. One of the most successful strategies is the assembly of micelles and other supramolecular structures from amphiphilic block copolymers.[1−5]

The next major challenge in this rapidly expanding field is the triggered formation of such nanostructures. Several research groups have recently reported triggering of stimuli-responsive block copolymers to self-assemble into micelles.[5−18] These reports describe the use of copolymers that are generally composed of a hydrophilic block and a stimuli-responsive block and are often regarded as "smart" polymers. Upon an external stimulus the character of the functional groups of the responsive block changes (e.g., from hydrophilic to hydrophobic) and the polymer can be switched from double-hydrophilic to amphiphilic.[6−9] Altering the solubility of such polymers can control the size and shape of the resulting nanostructures. To date, thermal,[10−12] pH dependent,[13−15] photochemical,[16] combinations,[17,18] and most recently chemically responsive[18] activation of the self-assembly process have been reported. While these approaches offer great control over the triggering process, they all lack the high degree of selectivity that can be achieved by enzymatic activation.

In this report we introduce the concept of enzymatically triggered self-assembly of block copolymers under physiological conditions. This approach is based on incorporating vinyl monomers that contain an enzymatic activated substrate, which renders the monomer water-soluble. The polymerization of such monomers with a polyethylene glycol (PEG) macroinitiator yields water-soluble block copolymers. Upon cleavage of the enzymatic substrates by the activating enzyme, the solubilizing moieties are removed from the vinyl polymer backbone to yield a hydrophobic block. Consequently, the polymer becomes amphiphilic and undergoes self-assembly to form colloidal nanostructures *in situ* (Figure 1).

Block copolymers **1a** and **1b**, containing phosphate moieties that can be cleaved by acid phosphatase (APase)[19] were synthesized by nitroxide-mediated polymerization (NMP)[20] of dibenzyl 4-vinylphenyl phosphate monomer with a monomethyl ether 5 kDa PEG macroinitiator **2**[21] to yield the dibenzyl-protected block copolymer **3a** (MW (NMR) = 10 kg/mol) and **3b** (MW (NMR) = 16 kg/mol). Deprotection by trimethylsilyl bromide, in the presence of collidine,[22] then gives the desired polymers **1a** and **1b**, respectively (Scheme 1). Full cleavage of the benzyl groups was confirmed by the disappearance of the corresponding peaks in the ^{1}H NMR and by shifts of ca. 8 ppm to lower field in ^{31}P NMR (see Figures S3 and S4 in the Supporting Information).

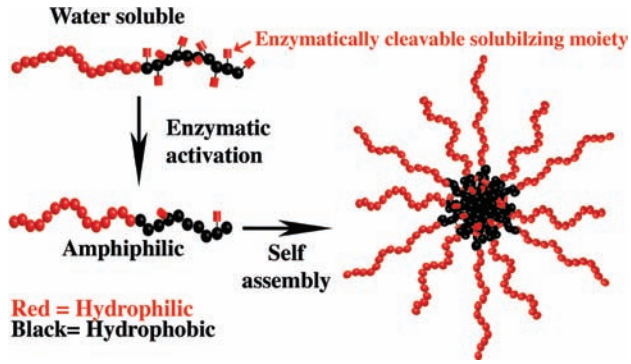
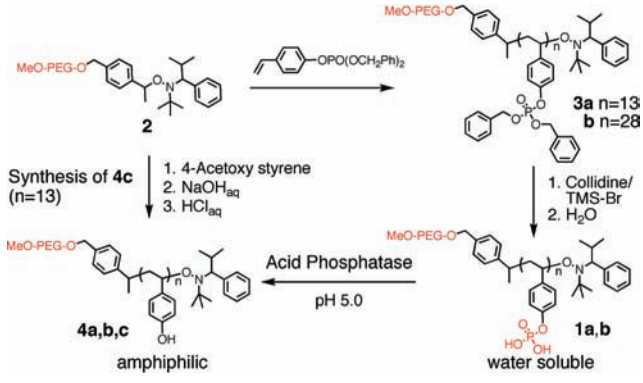


Figure 1. Schematic presentation for enzymatic activation of a water-soluble diblock copolymer to give an amphiphilic diblock copolymer and subsequent self-assembly into colloidal nanostructures (Note: full cleavage of the solubilizing moieties is not required for self-assembly).

Polymers **1a** and **1b** were freeze-dried to give white powders, which were directly dissolved into DI water to yield aqueous stock solutions. These solutions were further diluted into buffer solution at pH 5 (the optimal pH for the activating enzyme).[23] As both block copolymers **1a** and **1b** were found to give similar results, only **1a** is discussed below with comparable data for **1b** available (Supporting Information).

Scheme 1. Synthetic Approach for the Enzymatic and Chemical Preparation of Amphiphilic Diblock Copolymers



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To demonstrate the enzymatic cleavage of the phosphate groups of the polymers, ^{31}P NMR spectra of polymer **1a** (3 mg/mL) in a citrate buffer pH 5 with and without the activating enzyme (0.13 mg/mL) were measured at different time points. Figure 2 shows clear evidence for the cleavage of the phosphate groups by APase which is apparent from both the decrease of the peak corresponding to the phosphate ester attached to the polymer backbone (at −3.5 ppm) and the formation of a new peak that corresponds to the released phosphoric acid (at 1 ppm). No cleavage was observed in the absence of APase (data not shown). The extended reaction times for the NMR experiments are due to the significantly reduced, relative concentration of the enzyme. While an increased concentration of the polymer is required for the NMR study (signal-to-noise ratio), a similar increase in enzyme concentration is not possible due to its limited solubility in aqueous solutions (0.14 mg/mL).

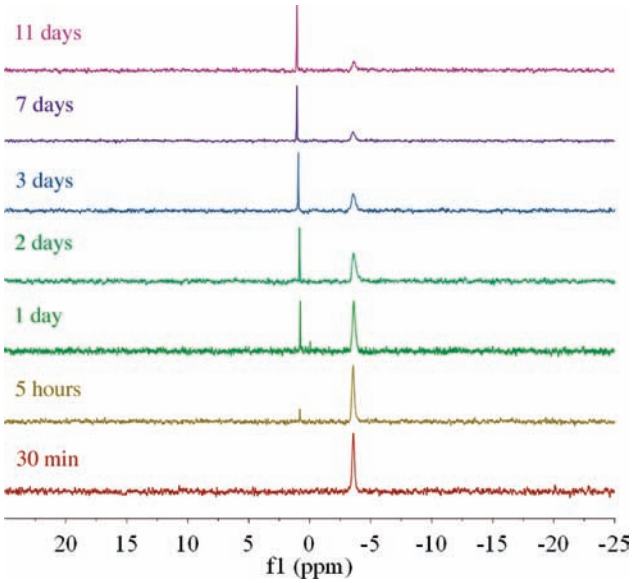

Figure 2. ^{31}P NMR spectra of polymer **1a** with APase in citrate buffer at pH 5. The signals at −3.5 and 1 ppm correspond to the polymer bound phosphate and cleaved phosphoric acid, respectively.

The ^{31}P NMR experiments reveal that some amount of phosphate remains bound to the polymer even after long incubation times (see Supporting Information). We assume that after the enzyme starts cleaving the phosphate groups, the polymer then becomes amphiphilic and self-assembles before all phosphate moieties have been cleaved. Encapsulation of unreacted phosphates inside the core of the colloidal nanostructures leads to steric isolation, and the residual phosphates become less accessible to the activating enzyme. This assumption is supported by the fluorescence data that are discussed later.

To demonstrate the concept of enzymatic triggered self-assembly of block copolymers, polymer **1a** was incubated with Apase in a buffer solution of pH 5. Upon enzymatic cleavage of the phosphate groups, a hydrophobic backbone consisting of 4-hydroxystyrene and the corresponding phosphoric acid repeat units develops (Figure 2); the polymer (**4a**) becomes amphiphilic and undergoes self-assembly into nanostructures. Dynamic light scattering of aqueous solutions of polymer **1a** (0.125 mg/mL) showed no light scattering, indicating that the polymer is soluble at this concentration. However, the limited solubility of APase gave aqueous solutions that showed unstable baselines, and therefore DLS could not be used to reproducibly monitor the activation and self-assembly processes.

To examine the self-assembly process in greater detail, the fluorescence spectra of highly diluted pyrene[24] containing (2 μM) an aqueous solution of **1a** incubated with APase were recorded at different time intervals. Pyrene containing solutions of the polymers without APase, APase itself, and polymer **4c** (Scheme 1) were used as control experiments. Figure 3 presents the ratio of the vibrational band intensities (I_I/I_{III}) of pyrene's fluorescence spectra in aqueous solutions of APase, polymer **1a**, polymer **1a** and APase, and polymer **4c**.

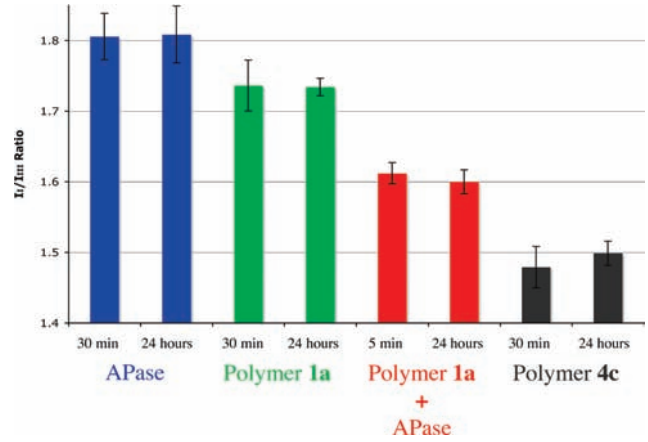

Figure 3. Quotient of pyrene's vibrational band intensities (I_I/I_{III}).

The lower ratio of the vibrational band intensities (I_I/I_{III}) for the polymer **1a** after incubation with APase in comparison to the polymer itself indicates the formation of hydrophobic regions in the activated polymer solution. This result is expected, as the enzyme triggers a change in the chemical and physical properties of the polystyrene based block. These changes, transform the block copolymer from a double-hydophilic to an amphiphilic block copolymer, which self-assembles into colloidal nanostructures having the PEG hydrophilic chains as an outer shell and the poly(4-hydroxystyrene) chains as a hydrophobic core. As pyrene molecules migrate into these hydrophobic regions, a decrease of the ratio (I_I/I_{III}) is observed. It should be noted that the ratio (I_I/I_{III}) for the PEG-*b*-poly(4-hydroxystyrene) **4c** is lower than the ratio (I_I/I_{III}) for activated polymer **1a**. The difference in the ratios (I_I/I_{III}) supports the ^{31}P NMR results, which indicated incomplete activation of all the phosphate groups along the backbone. As mentioned previously, after the enzyme starts cleaving the phosphate groups, the polymer becomes amphiphilic and self-assembles. The presence of phosphate residues leads to a more polar core when compared to *p*-hydroxystyrene, **4c**. This molecular level understanding of the enzymatic activation process shows that the desired self-assembly process does occur under physiological conditions.

An interesting feature observed in the NMR and pyrene fluorescence experiments is the significant disparity in time frames. This is due to the sensitivity difference between the two techniques which necessitates major changes in the relative molar ratios of polymer (substrate) and enzyme. Greater than an order of magnitude difference is observed when going from the NMR experiment (2700 phosphate residues/1 Apase) to the fluorescence study (150 phosphate residues/1 Apase).

To further demonstrate this novel process and to acquire information on the shape and size of the self-assembled

nanostructures, direct visualization with TEM was used. TEM micrographs of the nanostructures were obtained from pH 5 buffer solutions of polymer **1a** in the presence of the enzyme, and a representative example is shown in Figure 4. No structures were observed for the control solutions of the enzyme sample and polymer without the enzyme (data not shown). In direct contrast, spherical colloidal nanostructures with an average diameter of 90 nm were observed for a solution of polymer **1a** after incubation with the activating enzyme (Figure 4). This clearly shows that enzymatic activation is achieved and required for nanostructure formation. Similar structures were observed for the chemically synthesized control polymer **4c** (Supporting Information).

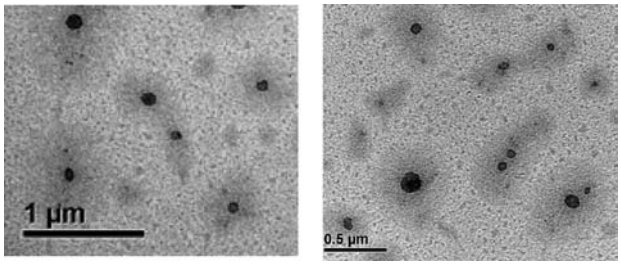

Figure 4. TEM images of block copolymer **1a** after incubation in buffer pH 5 with the enzyme.

In conclusion, we have demonstrated that the polymerization of vinyl monomers with cleavable enzymatic substrates leads to water-soluble double-hydrophilic block copolymers. Significantly, upon enzymatic activation, the diblock copolymer becomes amphiphilic and undergoes self-assembly into colloidal nanostructures. This approach can be extended to various polymeric backbones[25] and enzymatic triggers. The ability to change the chemical and physical characteristics of polymeric materials through enzymatic triggering opens the way for novel and exciting applications such as enzymatic-triggered activation of surfaces, formation of nanostructures *in vivo* in a highly controlled manner, etc. Further studies of parameters that control the size and shape of the formed nanostructures, like block sizes and ratios, as well as the use of other types of polymeric backbones are currently under investigation.

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Supporting Information Available: Synthesis of the monomer, polymerization procedures, characterization details, samples preparation, and ^{1}H NMR/^{13}C NMR/^{31}P NMR/GPC/HRMS/Fluorescense/TEM data. This material is available free of charge via the Internet at http://pubs.acs.org.

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