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Enzyme-Responsive Amphiphilic PEG-Dendron Hybrids and Their Assembly into Smart Micellar Nanocarriers

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Supporting Information

ABSTRACT: Enzyme-responsive micelles have great potential as drug delivery platforms due to the high selectivity of the activating enzymes. Here we report a highly modular design for the efficient and simple synthesis of amphiphilic block copolymers based on a linear hydrophilic polyethyleneglycol (PEG) and an enzyme-responsive hydrophobic dendron. These amphiphilic hybrids self-assemble in water into micellar nanocontainers that can disassemble and release encapsulated molecular cargo upon enzymatic activation. The utilization of monodisperse dendrons as the stimuli-responsive block enabled a detailed kinetic study of the molecular mechanism of the enzymatically triggered disassembly. The modularity of these PEG-dendron hybrids allows control over the disassembly rate of the formed micelles by simply tuning the PEG length. Such smart amphiphilic hybrids could potentially be applied for the fabrication of nanocarriers with adjustable release rates for delivery applications.

Stimuli-responsive micelles that can disassemble and release their encapsulated cargo upon external stimuli have gained increasing attention due to their potential utilization as nanocarriers for drug delivery.[1] These responsive materials are inspired by the ability of many supramolecular assemblies in Nature to alter their structures and activity in response to changes in their environment. Most reports in the literature usually describe stimuli-responsive polymers that respond to changes in pH,[2] temperature,[3] irradiated light,[4] or their combination.[5] While these approaches offer great control over the triggering of the disassembly processes, substantial advantages could be achieved by utilizing enzymes as stimuli. As many diseases are characterized by imbalances in the expression and activity of specific enzymes in the diseased tissue, this overexpression could potentially be translated into the selective activation of advanced drug delivery platforms.[6] However, to date, little has been reported on enzyme-responsive synthetic micellar nanostructures[7] and systems are often based on breaking the amphiphilic block copolymer into a soluble hydrophilic polymer and an insoluble hydrophobic block.[8]

Here we report a modular design for the efficient synthesis of enzyme-responsive amphiphilic hybrids composed of linear polyethyleneglycol (PEG) and a stimulus-responsive dendron with enzyme-cleavable hydrophobic end groups. These amphiphilic PEG-dendron hybrids are expected to self-assemble in water into micelles with a hydrophilic PEG shell and a hydrophobic core and potentially can be utilized to encapsulate hydrophobic cargo molecules.[9] In the presence of the activating enzyme, the hydrophobic end groups will be cleaved from the dendron, making it more hydrophilic. This change in amphiphilicity should result in destabilization of the micellar aggregates, leading to their disassembly and release of soluble PEG-dendron hybrids and the encapsulated cargo (Figure 1).

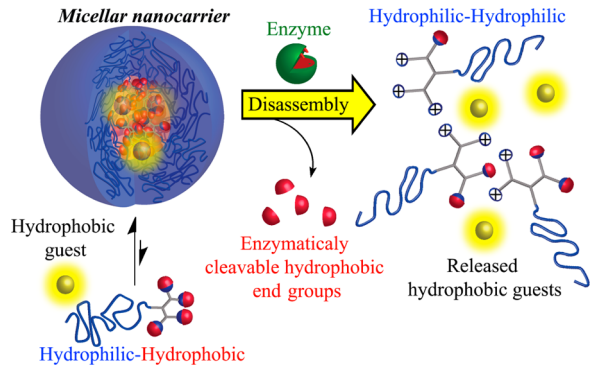

Figure 1. Schematic representation of the encapsulation of hydrophobic guests in the hydrophobic core of a smart micellar nanocarrier. Upon enzymatic cleavage of the hydrophobic end groups, the nanocarrier disassembles and the guest molecules are released.

The synthetic methodology combines the utilization of the PEG chain as a soluble support with thiol-yne chemistry, yielding an accelerated dendron synthesis. Based on this synthetic approach, we have synthesized a series of three PEG-dendron hybrids, **1a–c**, with PEG chains of three molecular weights (2, 5, and 10 kDa) and a second-generation dendron with four end groups. We chose to use phenyl acetamide as end

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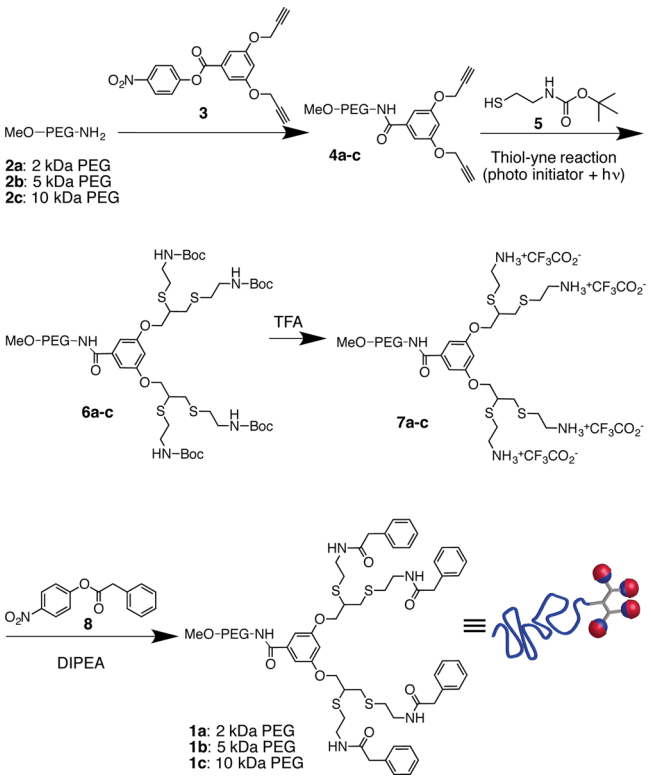
groups that can be cleaved by the enzyme penicilin G amidase (PGA).[10] The hybrid block copolymers were synthesized utilizing monomethyl ether PEG-amine, **2a–c**, as starting materials. Conjugation with an active ester of 3,5-bis(prop-2-yn-1-yloxy)benzoic acid, **3**,[11] yielded PEG-diynes, **4a–c**. The latter were further modified by thiol-yne reaction[12] with N-Boc cysteamine, **5**, to give tetrafunctionalized PEG-dendrons, **6a–c**. Deprotection of the Boc yielded PEG-tetra-amines, **7a–c**. In the last step of the synthesis, 4-nitrophenyl ester of phenyl acetic acid, **8**, was used to introduce the enzyme cleavable hydrophobic end groups. PEG-dendron hybrids, **1a–c**, were obtained as off-white solids with overall yields of 76%, 86%, and 93%, respectively, demonstrating the very efficient and simple synthetic methodology (Scheme 1). The synthesized polymers

Scheme 1. Synthesis of Amphiphilic PEG-Dendron Hybrids



and hybrids were characterized by ^{1}H and ^{13}C NMR, GPC, IR, and MALDI in order to confirm their structures, and the experimental values are in good agreement with the theoretical ones (Supporting Information).

To evaluate the ability of the amphiphilic hybrids, **1a–c**, to self-assemble into micelles, the PEG-dendron hybrids were dissolved directly in aqueous buffer (PBS, pH 7.4). Self-assembly was first examined by utilizing solubilization experiments with the solvachromic hydrophobic dye Nile red.[13] These measurements indicated the formation of micelles with critical micelle concentrations of 7.2, 12.4, and 21.7 μM for hybrids **1a–c** respectively (Table 1, Figures S18–S20).

Next, we studied the self-assembled structures using dynamic light scattering (DLS) and transmission electron microscopy (TEM). These analyses indicated the formation of spherical nanostructures with diameters of 11, 14, and 18 nm for hybrids **1a–c**, respectively (Figures 2 and S26, Table 1). Further support for the formation of micelles with a PEG-shell and dendron-based core was obtained from ^{1}H NMR spectra of the

Table 1. CMCs and Micelles Diameters for PEG-Dendron Hybrids 1a–c

hybrid	1a	1b	1c
CMC[a]	7.2 μM	12.4 μM	21.7 μM
micelle diameter[b]	11 nm	14 nm	18 nm

[a]Calculated by using Nile red. [b]Diameters of the micelles as measured by DLS.

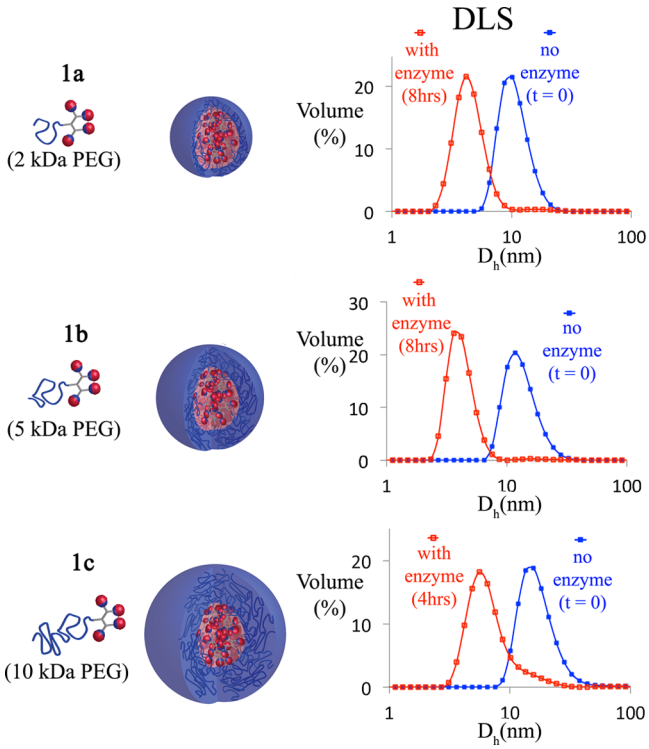


Figure 2. Schematic representation of PEG-dendron hybrids, **1a–c**, and their micellar assemblies (left). Size of the micelles by DLS at a hybrid's concentration of 160 μM before (in blue) and after (in red) the addition of the activating enzyme (right).

PEG-dendron hybrids in D_2O, which showed only the peaks of the PEG's protons (Figures S12–S14).

After the self-assembly of the three PEG-dendron hybrids **1a–c** into micelles was confirmed, we studied their response to enzymatic activity using DLS, fluorescence spectroscopy, and HPLC. The enzymatic cleavage of the hydrophobic phenyl acetamide end groups should decrease the hydrophobicity of the dendron and destabilize the micelles, leading to their disassembly into the corresponding monomeric hybrids. DLS measurements of solutions of the amphiphilic hybrids in the presence of PGA clearly indicated the decrease in the height of the peaks that correlate with the larger micellar aggregates and the formation of new peaks that correlate with the smaller sizes of the nonassembled monomeric chains and the enzyme (Figure 2).

The enzyme-responsive disassembly was further supported by the change in the fluorescence of encapsulated Nile red dyes. As the dye molecules are released into the aqueous environment upon the disassembly of the micelles, their fluorescence intensity is expected to decrease.[14] As anticipated, time-dependent decreases in fluorescence were observed for all three PEG-dendron hybrids, **1a–c**, in the presence of PGA, indicating that the Nile red molecules were released from the

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hydrophobic cores of the micelles as they disassembled upon the addition of the activating enzyme (Figures 3, S27, and S28).

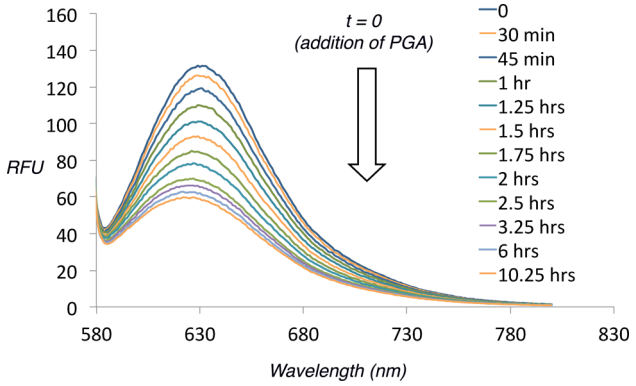

Figure 3. Fluorescence spectra of Nile red (1.25 μM) in the presence of PEG-dendron hybrid **1b** (160 μM) as a function of time after the addition of the activating enzyme, PGA (0.14 μM). Fluorescence intensity decreased as Nile red was released into solution.

We next used HPLC to follow the enzymatic degradation of the PEG-dendron hybrids, **1a–c**. HPLC analyses revealed the relatively fast disappearance of the amphiphilic hybrids, **1a–c**, upon incubation with the activating enzyme. Furthermore, we were encouraged to see the formation of only three major intermediates of increasing polarity (Figures S32–S35). Based on their relative polarities, rate of formation, the monodispersity, and symmetry of the dendrons, these intermediates are partially cleaved hybrids with three, two, and one phenyl acetamide end group (Figures 4, S38, and S39).

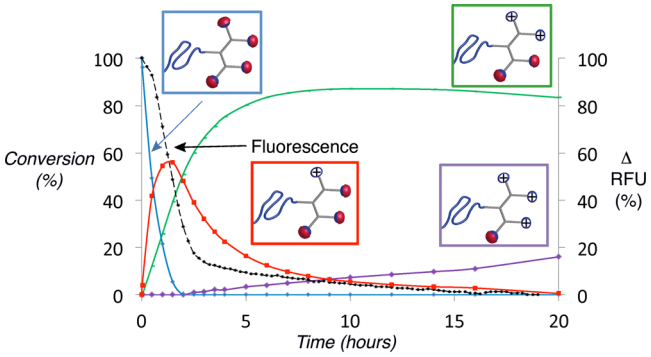

Figure 4. Change in fluorescence intensity and HPLC analysis of the enzymatic degradation of the PEG-dendron hybrid **1b**. Partially degraded intermediates are shown schematically.

Comparisons of the HPLC and fluorescence data showed good correlations between the decrease in fluorescence and the disappearance of the tetra-functionalized hybrid (i.e., hybrids **1a–c**) and the first intermediate with three hydrophobic end groups (Figure 4). These correlations indicate that only these two molecular species contribute to the formation of the micelles. Once their concentrations decrease, the micelles disassemble and their fluorescence cargo is released. It is important to note that at this relatively low enzyme concentration (0.14 μM), we did not observe the formation of the fully degraded tetra-amine hybrids, **7a–c**, even after 24 h. However, at the high enzyme concentration of 1.4 μM, the formation of fully degraded hybrids was observed (Figure S33).

As control experiments, micelles based on hybrid **1b** were incubated either in the absence of PGA or with an esterase that cannot break amide bonds (PLE), in order to examine the selectivity of the enzymatic activation. In both cases the micellar assemblies were found to be stable and no disassembly or degradation was observed by fluorescence spectroscopy and HPLC (Figures S30, S31, and S37). In addition, micelles based on amphiphilic Boc protected hybrid **6b** were incubated with PGA and were also found to be stable (Figures S24, S29, and S36). These control experiments further demonstrate the selectivity of the reported hybrids. Furthermore, the presence of a PEG shell gives the micelles stealth properties and helps to avoid nonspecific activation due to binding to proteins.

Comparison of the disassembly and hydrolysis rates for the PEG-dendron hybrids **1a–c** provided insight into the molecular mechanism of the disassembly. One might expect that micelles with thinner PEG shells would be activated faster then those with thicker ones, as their hydrophobic cores are more accessible to the enzyme. Interestingly, the opposite trend was observed and micelles assembled from hybrids with longer PEG chains disassembled faster (Figure 5).

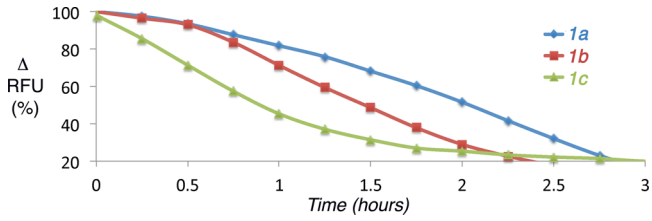

Figure 5. Comparison of the disassembly rates (fluorescence assay) of micelles formed by PEG-dendron hybrids **1a–c**.

These rate differences indicate that the cleavage of the hydrophobic end groups by the enzyme does not occur by penetration of the enzyme through the PEG shell into the hydrophobic core (Figure 6: pathway a). Instead, the enzymatic

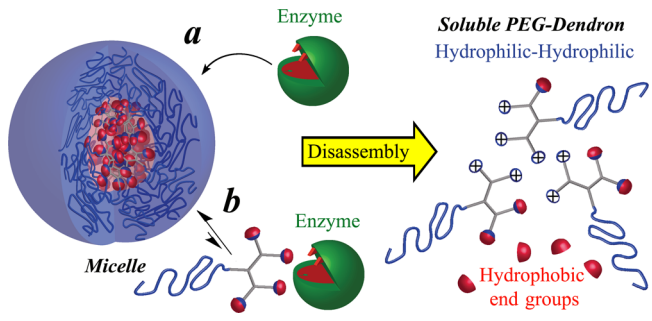

Figure 6. Schematic representation of two hypothetical enzymatic activation pathways: (a) the enzyme penetrates through the PEG shell or (b) equilibrium-based degradation in which the enzyme cleaves the hydrophobic end groups of the monomeric form of the amphiphilic hybrids.

degradation takes place at the "monomeric" form of the amphiphilic hybrids, which are in equilibrium with the micellar aggregates (Figure 6: pathway b). This hypothesis of equilibrium-based activation is in good agreement with other micellar systems[15] and is further supported by the differences in the CMC values, which indicate that hybrids with the longer PEG chains are more available than those with shorter chains (Table 1).

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To summarize, a new family of amphiphilic PEG-dendron hybrids that can self-assemble into enzyme-responsive micellar nanocarriers were synthesized through a very efficient and simple synthetic methodology. The utilization of an enzyme responsive dendron allowed for unprecedented control over the structure and monodispersity of the stimulus-responsive block and enabled a detailed mechanistic study. The disassembly mechanism proceeded through equilibrium dependent enzymatic degradation, which is in good agreement with other enzyme-responsive systems.[15] As the disassembly and release rates depended on the CMC of the amphiphilic hybrids, the release rates can be simply tuned by adjusting the length of the PEG block. Such enzyme-responsive hybrids and their self-assembled micelles have great potential in the field of drug delivery. Further studies of dendrons of various generations and end groups are currently in progress.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed experimental information, characterization data, and control experiments. 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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