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# Protein AND Enzyme Gated Supramolecular Disassembly

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Supporting Information

**ABSTRACT:** An amphiphilic nanoassembly was designed to respond to the concurrent presence of a protein and an enzyme. We present herein a system, where in the presence of these two stimuli supramolecular disassembly and molecular release occur. This molecular release arises in the form a fluorescence response that has been shown to be specific. We also show that this system can be modified to respond only if light stimulus is also present in addition to the protein and the enzyme. Demonstration of such supramolecular disassembly principles could have broad implications in a variety of biological applications.

Supramolecular assemblies that disassemble and release molecules due to a change in their environment have been of interest in a variety of areas including sensing, diagnostics, catalysis, and drug delivery.[1−4] The supramolecular disassembly is often the result of a chemical change that occurs in the key functional groups of the assembly in response to the change in the environment, often referred to as the stimulus. Selectivity in this stimulus responsive disassembly is important for most of the targeted applications. A versatile approach to enhancing selectivity involves the imposition that the disassembly will occur only in the presence of two or more stimuli. This is reminiscent of molecular AND gates, where systems involving fluorescence sensing have been previously demonstrated.[5] These systems were mainly focused on changing a non-fluorescent small molecule to a fluorescent one or vice versa in response to the concurrent presence of two stimuli. These systems are useful in certain sensing and chemical logic gate applications, but are not easily extended to protein-based systems as they are mostly demonstrated in apolar solvents. Supramolecular disassembly due to the presence of two or more stimuli, especially involving polymeric assemblies in aqueous phase, is also known.[6] However, these systems have mainly focused on stimuli such as light, pH, redox conditions, and temperature.[7−10] While these stimuli have been of interest due to their implications in biology, the imbalances in these factors in biology are considered to be secondary indicators. The primary indicators of biological imbalances, and thus diseased tissue locations, are variations in protein concentrations or enzymatic activity. Therefore, protein-responsive disassembly has been of recent interest.[11] Considering the decisive advantages of multistimuli responsive assemblies, we were interested in addressing the challenge of designing a system that not only responds to proteins but also would respond only due to the concurrent presence of two different proteins. We demonstrate a versatile new molecular design that allows for such a supramolecular disassembly, illustrated in Figure 1. We also further demonstrate that the same supramolecular system can be modified to include a third AND input to generate the molecular release response.

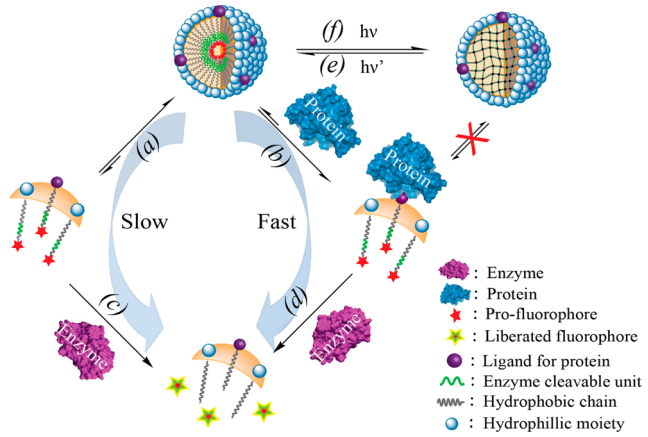


**Figure 1.** Schematic representation of protein AND enzyme gated supramolecular disassembly.

The molecular design strategy that has the potential to serve as a protein-based AND logic gate is shown in Figure 1. We use a facially amphiphilic molecule to test this design concept. In an amphiphilic micelle-like assembly, the hydrophobic units are buried in the core of the assembly and therefore should not be accessible to an enzyme. For this to be true, it is important that the equilibrium between the monomeric state of the amphiphilic molecule and its aggregated state heavily favors the latter (represented by equilibrium *a* in Figure 1). We have previously shown that micelle-like supramolecular aggregates, from facially amphiphilic molecules, can be disassembled due to a ligand−protein binding event.[11c] The disassembly occurs due to the change in the hydrophilic−lipophilic balance caused by the protein binding; i.e., the protein binding causes the equilibrium to shift to the monomeric state of the amphiphile (represented as equilibrium *b* in Figure 1). We hypothesize then that by incorporating an enzyme-sensitive linker in the hydrophobic part of the amphiphile, we will favor the aggregated state in the absence of the protein, but favor the monomeric state in the presence of the protein. This should generate a system that would release a covalently bound molecule only in the presence of both protein stimuli (determined by the difference in steps (*a*,*c*) vs (*b*,*d*) in Figure 1).

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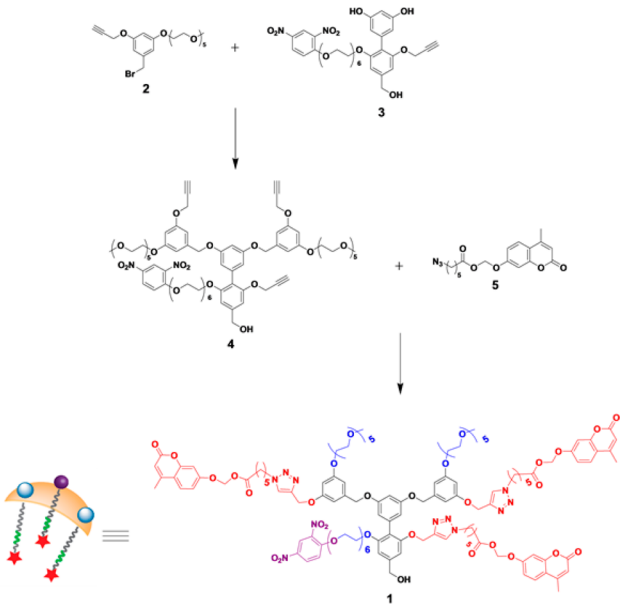
**Scheme 1. Synthesis of Target Amphiphile Molecule 1**



To test this design hypothesis, we targeted the amphiphilic molecule **1** shown in Scheme 1. In this molecule, the dinitrophenyl ligand (complementary and specific to anti-DNP immunoglobulin G (IgG)) molecule is presented at the hydrophilic face of the biaryl unit in **1**. Similarly, our design requires that the enzyme-sensitive unit be placed at the hydrophobic part of **1**. We chose the acetal-functionalized coumarin ester as the hydrophobic moiety, because (*a*) this functional group is capable of being cleaved by an esterase and (*b*) the product of the enzymatic reaction with this substrate is a highly fluorescent coumarin, which allows for directly monitoring the enzyme-induced molecular release. Note that the coumarin moiety is presented at all three repeat units in **1**, as this feature provides the key to introducing a third stimulus into this process (*vide infra*). Moreover, the presence of the pro-fluorophore in all three units also will produce the optimal fluorescence in response to the presence of the protein and the enzyme.

The target molecule **1** was synthesized through a multistep modular synthesis. Briefly, the alkyne-based precursor molecule **4** was achieved by reacting the amphiphilic benzyl bromide molecule **2** to amphiphilic biaryl unit **3** containing a 2,4-dinitrophenol (DNP) ligand using an alkylation reaction, as shown in Scheme 1. The synthetic steps involved in obtaining the amphiphilic repeat units themselves are outlined in the Supporting Information. The alkyne precursor molecule **4** was reacted with the coumarin azide **5** under the Huisgen 1,3-dipolar cycloaddition reaction conditions, the so-called 'click chemistry', to yield the target amphiphile **1**.

Following the synthesis of the molecule **1**, we characterized the amphiphilic assembly formed by this molecule in aqueous phase. Measuring the size of the assembly using dynamic light scattering (DLS) revealed that the size of the aggregates formed from these molecules was about 480 nm, even at concentrations as low as 1 $\mu$M. We have characterized these assemblies as micelle-like aggregates, because these are not simple micelles. However, analogous assemblies do have a hydrophobic core and are solid structures.[11a,c,e,12] The assembly size was monitored for a 24 h time period, and the size of the assembly in solution did not change over this time period (Figure S2 a,b). Probing these by transmission electron microscopy (TEM) showed that these molecules indeed form spherical assemblies (Figure S2c). We attempted to assess the critical aggregation concentration (CAC) of **1** using guest encapsulation measurements without success. We presumed that this could be due to its rather low CAC. Therefore, we resorted to surface tension measurements, and there was no obvious change in the surface tension, compared to pure water within concentration range measured (Figure S2d). These results were considered to suggest that the equilibrium strongly favors the aggregated state in **1** and that its CAC is very low.

The stimuli responsive characteristics of this assembly were then studied in the presence of the protein, enzyme, and the combination using the release of the fluorescent coumarin as the probe. Figure 2a shows the results of this study using a 13

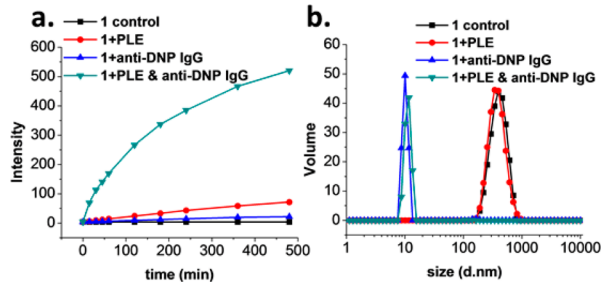



**Figure 2.** Disassembly and coumarin release from **1** (13 $\mu$M) in the presence of anti-DNP IgG (1 $\mu$M) and PLE (50 nM): (a) Fluorescence change with time. (b) Size change after 8 h, monitored by DLS.

$\mu$M solution of **1** in the presence and absence of anti-DNP IgG and porcine liver esterase (PLE). We chose this concentration for **1**, since similar amphiphilic molecules exhibited CACs slightly below this.[11a,c,e] First, no fluorescence was generated in the absence of the stimulus (anti-DNP IgG or PLE) or in the presence of anti-DNP IgG. With PLE, there was a small amount of fluorescence generated, indicating that equilibrium *a* (Figure 1) significantly favors the aggregated state of the amphiphile. However, in the presence of both anti-DNP IgG and PLE, there was rather rapid fluorescence generation in the solution indicating that anti-DNP IgG influences the equilibrium to favor the monomeric state in equilibrium *b*. The initial rate of ester cleavage in the presence of anti-DNP IgG and PLE was found to be about 26 times faster than that in the presence of PLE alone. The stimuli-induced disassembly process was also monitored by DLS. Figure 2b shows that the size of the assembly does not change in the absence of either of the proteins or in the presence of PLE after 8 h. However, the size drastically changed in the presence of anti-DNP IgG, again confirming the dominance of the monomeric state of the amphiphile in solution (equilibrium *b* in Figure 1). Understandably, the size of the assembly was also small in the presence of both anti-DNP IgG and PLE.

In order to examine if the cooperative disassembly and molecular release is specific to the anti-DNP IgG/PLE combination, we studied coumarin release in the presence of PLE and other proteins, viz. pepsin (anionic, pI 2.2−3.0), chymotrypsin (cationic, pI 8.8), and thrombin (neutral, pI 7.1). Figure 3a shows that only the anti-DNP IgG/PLE combination generated the fluorescence response. The responses in the presence of other proteins were indistinguishable from the PLE itself (Figure 2a). Moreover, DLS shows that disassembly does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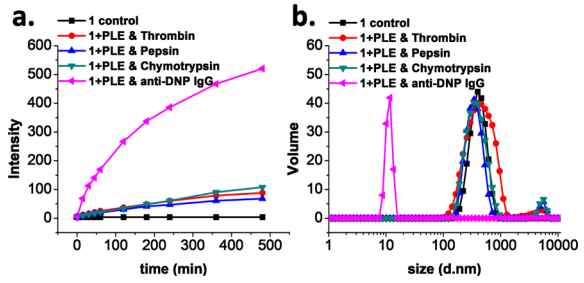


**Figure 3.** Studies of binding specificity enhanced dye release. (a) Fluorescent intensity change upon time. (b) Size after 8 h, monitored by DLS.

not occur in the presence of any of these proteins once again supporting the specificity of the disassembly and molecular release to the anti-DNP IgG/PLE combination (Figure 3b and Figures S5, S6 also). To further test this specificity, we investigated a structurally similar molecule that lacks the ester substrate and the DNP ligand moiety, which showed no change in size with the presence of anti-DNP IgG (Figure S4a, b). We also tested dye release caused by nonspecific binding protein: IgG from rat serum. Nonspecific binding protein IgG incorporation with PLE afforded a small increase in guest release in comparison to PLE alone, which indicates that specific binding is required for the dramatic guest release observed with anti-DNP IgG (Figure S4c, d).

Finally, we were interested in exploring whether we can introduce a third, non-protein input into this scheme. We hypothesized that crosslinking the assemblies will lock the aggregated state of the molecule and thus not allow for binding induced disassembly (step *f*, Figure 1). Then, de-crosslinking this aggregate in step *e* using a third stimulus will be an essential step to access the binding induced monomeric state of equilibrium *b*, as shown in Figure 1. To examine this possibility, the hydrophobic coumarin units in the assembly were photo-crosslinked at 365 nm irradiation.[13] Figure 4a shows that

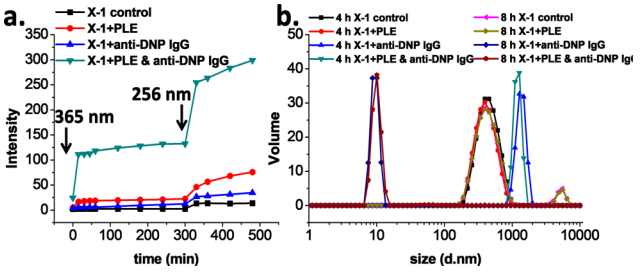


**Figure 4.** Studies of crosslinked **1** (**X-1**) disassembly and coumarin release. (a) Fluorescent intensity change upon time. Samples were irradiated with 365 nm UV light before experiment. Samples were irradiated with 256 nm UV light at 5 h. (b) Size at 4 and 8 h, monitored by DLS.

fluorescence generation quickly reaches a plateau in 15 min, indicating that a small percentage of un-crosslinked molecule **1** was released in response to the protein binding and enzymatic reaction. To test the hypothesis of making the equilibrium available using the light stimulus, the assemblies were un-crosslinked by irradiating the sample with 256 nm UV light at the 5 h time point. Indeed, the coumarin release was observed at this time point in the presence of the anti-DNP IgG/PLE combination. This process was also monitored by DLS. Before de-crosslinking, the anti-DNP IgG or anti-DNP IgG/PLE combination causes the assembly to increase in size, likely due to the aggregation caused by the anti-DNP IgG binding (Figure 4b). After de-crosslinking however, disassembly indeed occurs in the presence of anti-DNP IgG.

In summary, we have shown the following: (*i*) by designing an appropriate amphiphilic molecule containing a protein-specific hydrophilic ligand moiety and enzyme-specific hydrophobic substrate moieties, supramolecular assemblies that exhibit molecular release in the presence of both the complementary protein and the enzyme, but not in the presence of either of these stimuli alone, can be achieved; (*ii*) the dual protein-responsive molecular release is due to the variation in the monomer−aggregate equilibrium in the presence and absence of the complementary protein; (*iii*) a fluorescent signal can be generated by incorporating a pro-fluorophore as the hydrophobic enzyme substrate; (*iv*) the fluorescence response is specific to the protein/enzyme combination; (*iv*) by locking the aggregated state of the equilibrium, the protein response characteristics can be turned off, which can then be reversed by de-crosslinking the aggregate, thus making the system responsive to a third stimulus input. The supramolecular principles, developed here, are likely extendable to a variety of other stimuli and thus have potential applications in a variety of areas including delivery and sensing, which are the foci of current work in our laboratories.

## ■ ASSOCIATED CONTENT

### Supporting Information

Synthetic 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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