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Disassembly-Driven Turn-On Fluorescent Nanoprobes for Selective Protein Detection

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Selective protein detection or imaging is of fundamental importance in basic biological research as well as medical diagnosis. Small-molecule “switchable” fluorescent probes, which induce changes in the fluorescence properties (intensity and/or wavelength) only at the intended target protein, are particularly useful for this purpose because they allow sensitive, simple, and specific detection with high target-to-background ratios.[1] Several strategies for generating such smart probes have been described. The most prevalent probes are fluorogenic substrates that allow the monitoring of specific hydrolases (e.g., β-lactamase, glycosidases, and proteases) by either turn-on fluorescence[2] or fluorescence resonance energy transfer (FRET).[3] The fluorescence switching of this type of probe is based on the catalytic cleavage of the molecule by the target enzyme. Substrate-based probes for other enzymes, such as kinases, have also been reported.[4] On the other hand, the design of switchable probes for nonenzymatic protein targets remains more challenging. A typical approach involves the incorporation of a solvatochromic fluorophore to a ligand specific to the target protein.[5] Unfortunately, in most cases, the resultant probes are always fluorescent, causing high background signals, and induce only small-to-moderate fluorescence changes in protein sensing. Recently, peptide-based molecular beacons were prepared as switchable probes for proteins that bind specific short peptide sequences.[6] A limitation of the beacon system is its applicability only to polypeptide-binding protein receptors. Clearly, the establishment of new switching strategies for protein-specific fluorescent probes is highly desired.

Here we introduce a novel mechanism for the design of “turn-on” fluorescent probes that are selective for target proteins. The strategy was inspired by our recent work on self-assembling ^{19}F NMR/MRI probes developed for protein imaging.[7] We demonstrated that an amphiphilic compound consisting of an ^{19}F reporter and a protein ligand forms self-assembled aggregates in aqueous solution (signal off) and that the aggregates disassemble in response to the target protein through the specific protein−ligand interaction (signal on). This switching mechanism based on the self-assembly and recognition-driven disassembly of amphiphilic probes is considered to be unique and applicable to the generation of a new type of switchable fluorescent probe. It is well-known that many fluorophores undergo quenching when they form dimers or aggregates.[8] Therefore, we reasoned that a self-assembling, ligand-tethered fluorophore would function as a protein-specific probe as shown in Scheme 1. Namely, it was expected that the self-assembling probe would display no or only weak fluorescence when aggregated, whereas it would emit bright fluorescence in response to the target protein through the recognition-induced disassembly of the probe.

Scheme 1. Disassembly-Based Strategy for Specific Protein Detection with Self-Assembling Turn-On Fluorescent Probes

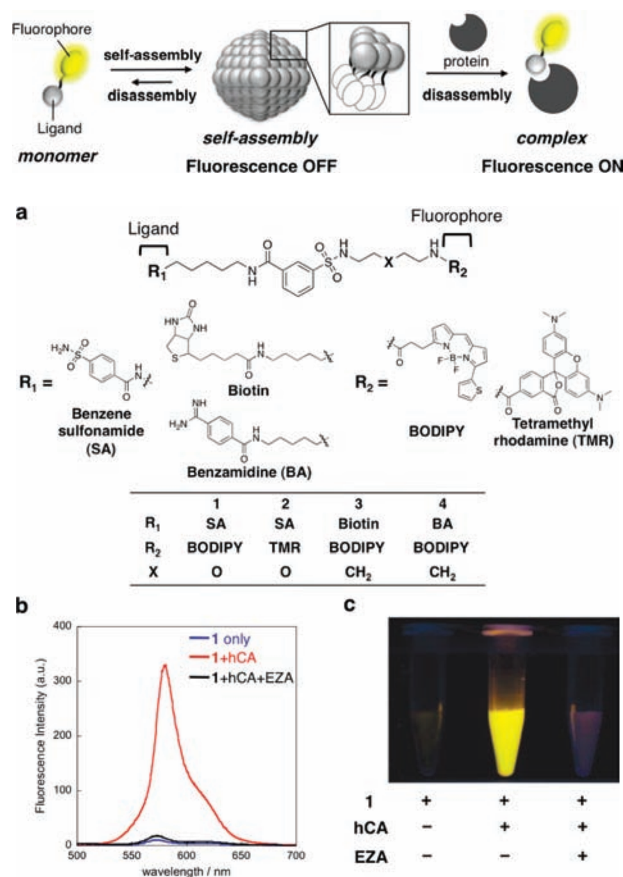

Figure 1. Self-assembling turn-on fluorescent probes for protein detection. (a) Chemical structures of fluorescent probes **1** and **2** for hCA, **3** for avidin, and **4** for trypsin. (b) Fluorescence spectra (λ_{ex} = 468 nm) of probe **1** (25 μM) in 50 mM HEPES buffer (pH 7.2) in the absence (blue) and presence (red) of hCA (25 μM) and after addition of EZA (125 μM) to the solution of **1** and hCA (black). (c) Photograph of probe **1** (25 μM) in the absence (left) and presence (middle) of hCA (25 μM) and after addition of EZA (125 μM) to the solution of **1** and hCA (right). The image was obtained with UV excitation (λ_{ex} = 365 nm).

To test this idea, we chose human carbonic anhydrase I (hCA) as a model protein and designed compounds **1** and **2** (Figure 1a and Schemes S1 and S2 in the Supporting Information). On the basis of the previous study,[7] a fluorophore, BODIPY in **1** or

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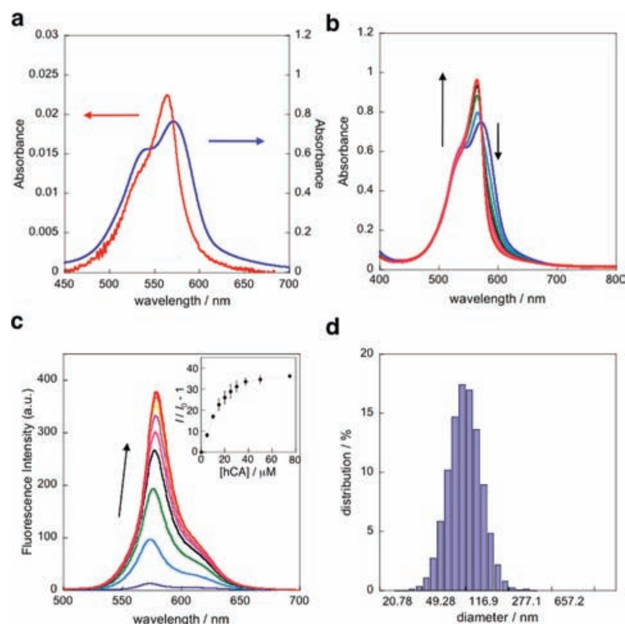

Figure 2. Spectroscopic analyses of hCA-specific probe **1**. (a) UV−vis absorption spectra of probe **1** alone: 0.5 μM (red); 25 μM (blue). (b) UV−vis absorption spectral changes of probe **1** (25 μM) upon addition of hCA (0−75 μM). (c) Fluorescence spectral changes of probe **1** (25 μM) upon the addition of hCA (0−75 μM) (λ_{ex} = 468 nm). Fluorescence measurements were performed 1 min after adding hCA to the probe solution. The inset shows the fluorescence titration curve (λ_{em} = 580 nm); error bars represent standard deviations of three experiments. (d) DLS analysis of the particle size distribution of self-assembled probe **1** (25 μM). All of the experiments were performed in 50 mM HEPES buffer (pH 7.2).

tetramethylrhodamine (TMR) in **2**, was attached as a hydrophobic moiety to benzenesulfonamide (SA), an hCA-targeting ligand (hydrophilic head),[9] through a relatively hydrophobic linker. We first examined the response of each probe to hCA by monitoring the UV and fluorescence spectral changes in aqueous solution. As shown in Figure 1b, the emission was extremely weak [the fluorescence quantum yield (Φ) was 0.001] when **1** was dissolved in aqueous buffer but dramatically increased (by 38-fold) upon the addition of hCA (Φ = 0.04). The fluorescence recovery was so efficient that the brighter emission in the presence of hCA was easily detectable by the naked eye (Figure 1c). On the other hand, probe **2** showed a relatively large fluorescence even in the absence of hCA, and thus, no significant fluorescence enhancement occurred upon addition of the protein (only by 1.5-fold) (Figure S1 in the Supporting Information). These results indicated that the rather hydrophobic BODIPY, not the positively charged TMR, was suitable as a fluorophore for clear off/on response. Interestingly, the enhanced fluorescence of **1** was almost completely quenched again by the addition of EZA, a strong competitive inhibitor for hCA (Figure 1b).[9] This demonstrates that the turn-on fluorescence switching is reversible and controlled by the recognition of the ligand moiety of the probe by hCA. The target specificity of probe **1** was next assessed under protein mixture conditions. Only weak fluorescence was observed when **1** was mixed with a solution containing bovine serum albumin, hemoglobin, concanavalin A, and chymotrypsin. In sharp contrast, a strong emission appeared upon the addition of **1** to a mixture containing the four proteins and hCA, which reveals that **1** is hCA-specific (Figure S2).

Next we performed titration experiments in detail. First, the concentration-dependent changes in the absorption and emission properties of **1** alone in buffer were investigated. As shown in Figure 2a, we observed a red shift of the absorption maximum from 565 nm (0.5 μM) to 572 nm (25 μM) with increasing the probe concentration. Probe **1** dissolved in DMSO (i.e., in a monomeric state) showed an absorption maximum at 565 nm (data not shown). Therefore, the observed red shift of the absorption maximum suggested that the BODIPY fluorophore changed from the monomeric state to the aggregated state, in particular, in a J-type orientation,[8] as the concentration increased. In agreement with this behavior, a substantial decrease in the fluorescence was observed with increasing probe concentration (above 2.5 μM) after a linear increase in the fluorescence intensity up to a probe concentration of 1 μM (Figure S3). These data strongly support the conclusion that **1** undergoes self-assembly in aqueous solution, which causes efficient fluorescence quenching. Subsequently, an hCA titration experiment was carried out. Absorption spectra of a solution containing **1** (25 μM) showed that when the hCA concentration was increased, the broad red-shifted absorption at 572 nm gradually decreased and a sharp peak at 565 nm linearly increased (Figure 2b). These data suggest the disruption of the aggregates of **1** by hCA. Consistent with this, the emission of **1** at 580 nm, which was originally quenched in the absence of hCA, was significantly increased by the addition of hCA (Figure 2c). The fluorescence response was rapid, becoming complete within 1 min following the hCA addition. The change in the emission intensity was saturated at a 1:1 **1**/hCA ratio. The fluorescence enhancement showed a linear relationship with hCA concentration up to 25 μM (1 equiv). From this plot, we determined the detection limit for hCA to be 70 nM (Figure S4), which is much superior (by ~70-fold) to that achieved with the hCA-specific ^{19}F NMR probe (5 μM).[7]

The self-assembly/disassembly properties of **1** were further confirmed with several other techniques. Atomic force microscopy (AFM) revealed the formation of spherical or oval aggregates of **1** with diameters ranging from 100 to 200 nm (Figure S5). In dynamic light scattering (DLS) measurements, a buffer solution containing only **1** (25 μM) showed aggregates with a mean diameter of 100 nm (Figure 2d), whereas negligible DLS intensity was observed after addition of hCA to the solution. On the basis of all the data, we can illustrate the fluorescence off/on switching mechanism as follows: (1) probe **1** self-assembles to form submicrometer-sized aggregates in aqueous solution;[10] (2) in the aggregated state, **1** emits almost no fluorescence because of efficient quenching; (3) the aggregates disassemble in the presence of hCA through binding of the protein to the ligand moiety of **1**; and (4) the disassembly process leads to the recovery of fluorescence.

Because of the modular feature of the probe design, we successfully synthesized the similar probes targeting different proteins simply by replacing the ligand motif. In order to target avidin, for example, compound **3** containing the biotin ligand was prepared (Figure 1a and Scheme S3).[11] Similarly, a benzamidine derivative was used as a ligand for the detection of trypsin (i.e., compound **4**) (Scheme S4).[12] As expected, in both cases, the fluorescence intensity was very weak when the probe was alone in solution. On the other hand, the fluorescence was dramatically enhanced by 22- and 23-fold upon the addition of the corresponding target proteins avidin and trypsin (Figure 3a and Figure S6). In the latter case, the enhanced fluorescence was quenched again by the addition of an excess amount of free benzamidine (data not shown). More significantly, none of the probes **1**, **3**, or **4** responded to nontarget proteins, demonstrating the high orthogonality and target specificity of these probes (Figure 3b and Figure S7). A series of orthogonal, turn-on fluorescence detection of proteins was clearly observable by the naked eye.

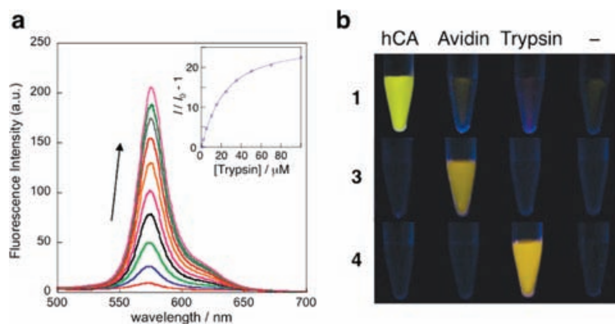

Figure 3. Detection of several different proteins using turn-on fluorescent probes. (a) Fluorescence spectral changes of probe **4** (10 μM) upon the addition of trypsin (0−100 μM) in 50 mM HEPES buffer, 0.5 M NaCl (pH 7.2) (λ_{ex} = 480 nm). The inset shows the fluorescence titration curve (λ_{em} = 575 nm). (b) Photographs showing the orthogonal, specific protein detection of hCA, avidin, and trypsin using probes **1**, **3**, and **4**, respectively. The images were obtained with UV excitation (λ_{ex} = 365 nm). For detailed conditions, see the Supporting Information.

In summary, we have developed new fluorescent molecules that detect specific proteins with turn-on fluorescence signals. The switching mechanism is based on the self-assembly (fluorescence off) and recognition-driven disassembly (fluorescence on) of amphiphilic, ligand-tethered fluorophores. The probe design is modular and thus applicable for specific detection of various proteins, including enzymes and nonenzymatic proteins. It is expected that not only the ligand motif but also the fluorophore unit can be replaced with other fluorescent dyes with different emission wavelengths. We anticipate that the present supramolecular approach[13] may facilitate the development of new protein-specific switchable fluorescent probes that are useful for a wide range of applications, such as diagnosis and molecular imaging. In addition, along with widely used inorganic particles and polymer-based assemblies, self-assembled aggregates formed by small organic compounds may hold great promise as a new type of nanometer-sized materials in the field of nanobiotechnology.

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Supporting Information Available: Experimental details, Figures S1−S7, and Schemes S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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