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Fluorescence amplified detection of proteases by the catalytic activation of a semisynthetic sensor†

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A general approach to mimic the sensing scheme of allosteric enzymes is developed. Through the covalent labeling of a sulfonamide inhibitor to the enzyme HCAII *via* SNAP-tag protein, the enzyme is rendered inactive. Catalytic activation is triggered only when a protease is present to cleave the recognized peptide sequence.

To increase assay sensitivity in detecting ultralow concentration of analytes or biomarkers, enzyme-catalyzed signal amplification using enzyme conjugates is a common tool in many bioanalytical methods.[1] Enzyme catalysis provides several advantages such as high substrate specificity and rapid catalysis to generate optical[2] or electrical signals.[3] A classical example of an enzyme-catalyzed signal amplification is the enzyme-linked immunosorbent assay (ELISA) which uses a covalently labeled enzyme–antibody conjugate to carry out specific analyte detection and signal amplification.[4] Although the ELISA-type assays have been widely employed in many research and clinical laboratories, shortcomings such as laborious operating procedures and extensive washing steps to remove the unbound enzyme-conjugate before initializing signal amplification have limited their application to only in laboratories. Thus, the development of a new enzyme-catalyzed signal amplification sensor which does not require extensive washing steps and has a straightforward operation protocol would be an important step toward the fabrication of a point-of-care diagnostic device to detect ultralow concentration of biomarkers.

In contrast to the enzyme–antibody conjugate detection scheme, a more elegant sensing mechanism performed by many natural enzymes to control complex cellular processes is *via* allosteric regulation,[5] in which a polypeptide pseudosubstrate of the enzyme binds to the active site to inhibit the enzyme activity. Enzyme activation takes place only when an activator interacts with the allosteric site, typically the site between the enzyme and the pseudosubstrate, to induce a conformational change or cleavage event to remove the pseudosubstrate from the active site. To date, several allosteric enzyme mimics have been developed by using the protein engineering technique for the sensing application.[6] However, one major drawback of using the protein engineering approach alone to create a biomimetic allosteric sensor is the limited application of only genetically encoded polypeptides at the allosteric sites and as pseudosubstrates which narrow the scope and versatility to construct better allosteric enzyme mimic sensors.

Herein, we describe a rational design of a semisynthetic sensor to mimic the allosteric sensing scheme of natural enzymes by covalently labeling a synthetic sulfonamide inhibitor to the enzyme human carbonic anhydrase II (HCAII) (Fig. 1a). Previous semisynthetic approaches have demonstrated the amplified detection of DNA,[7] where the semisynthetic DNA sensors were prepared by attaching the activated synthetic inhibitor-nucleic acid molecules to the proteases through nucleophilic amino acids (cysteine or lysine). However, many proteins are not amenable to this strategy due to multiple inherent cysteine or lysine residues.[8] A site-specific, easy purification and quantitative protein labeling method is crucial for obtaining a useful semisynthetic sensor. In our allosteric enzyme mimic sensor, covalent labeling of the synthetic sulfonamide inhibitor to the HCAII enzyme was achieved by

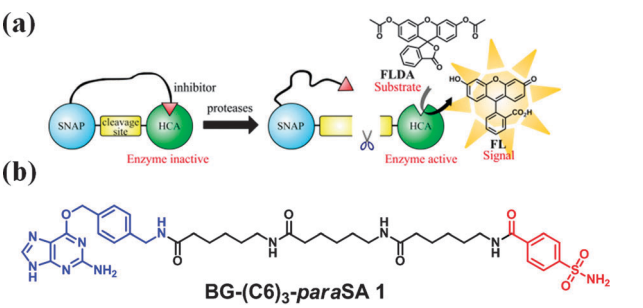

Fig. 1 (a) Schematic representation of the semisynthetic sensor mimicking natural allosteric enzymes for caspase-3 detection. (b) Molecular structure of BG-$(C6)_3$-*para*SA **1**. The synthetic molecule is used to modulate HCAII activity in the presence or absence of caspase-3.

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introducing a self-labeling protein SNAP-tag into HCAII to facilitate quantitative and site-specific labeling *via* a benzylguanine (BG) moiety.[9] It has been shown that SNAP-tag labeling with the BG moiety is efficient with high reproducibility and requires only simple purification to obtain the labeled semisynthetic sensor.[10]

As a proof-of-principle, we demonstrate this semisynthetic design by applying the sensor to the detection of protease activity of caspase-3[11] which is a key mediator and a well-established cellular marker of apoptosis.[12] The recombinant sensor protein SNAP_DEVD_HCAII (Fig. 1a) is functionalized with the covalent linkage of a synthetic sulfonamide inhibitor BG-$(C6)_3$-*para*SA **1** (Fig. 1b) to the SNAP-tag protein *via* the corresponding benzylguanine moiety (BG) whereupon the sulfonamide inhibitor can bind intramolecularly to the active site of HCAII. The amino acid residue sequence DEVD, a caspase-3 cleavage sequence, is incorporated between SNAP-tag and HCAII for caspase-3 recognition and proteolysis. In the absence of caspase-3, the proteolytic sensor exists in a closed conformation with the HCAII activity strongly inhibited by the sulfonamide inhibitor due to the high effective concentration in the intramolecular closed conformation.[13] In the presence of caspase-3, the protease recognizes the DEVD sequence and cleaves the recombinant sensor protein into two fragments. In this intermolecular case, the sulfonamide inhibitor exhibits weaker inhibition to HCAII, which in turn can hydrolyze non-fluorescent fluorescein diacetate (FLDA) to generate strongly fluorescent fluorescein (FL). HCAII was chosen as the signaling enzyme because of its esterase function to hydrolyze FLDA.[14] In addition, numerous other sulfonamide inhibitors have been reported to inhibit HCAII esterase activity,[15] which provide us with the means to control the sensitivity of our sensor.

To test our allosteric enzyme mimic sensor, we incubated our semisynthetic sensor with caspase-3 for 1 hour at 37 °C followed by addition of FLDA to initiate fluorescence signal amplification. In the presence of caspase-3, we observed a dramatic fluorescence enhancement (by 17-fold) as compared to the sensor without caspase-3 (Fig. 2a). The proteolysis of the DEVD peptide at the sensor by caspase-3 releases the sulfonamide inhibitor from HCAII and activates the enzyme toward the hydrolysis of FLDA and the generation of fluorescent FL. In the absence of caspase-3, the activity of HCAII was completely inhibited by the intramolecular sulfonamide inhibitor, and only very weak fluorescence was observed. Negative control

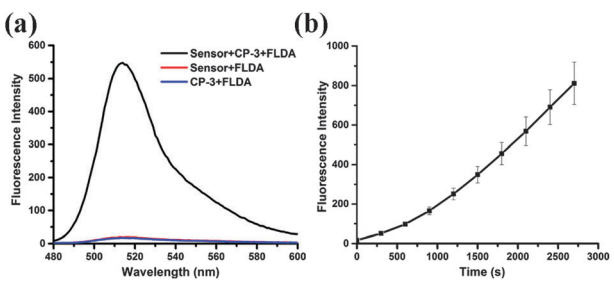

Fig. 2 (a) Fluorescence spectra of the semisynthetic sensor in the presence or absence of caspase-3 (CP-3). (b) Time course of the fluorescence intensity in the presence of 5 ng μL^{-1} caspase-3. The error bar was calculated from three independent experiments.

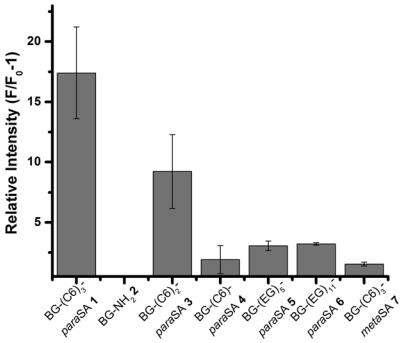

Fig. 3 Relative fluorescence intensities obtained using different synthetic sulfonamide inhibitors. BG-$(C6)_3$-*para*SA **1** shows an average of 17-fold fluorescence amplification after 45 minutes.

with caspase-3 and FLDA only shows that caspase-3 cannot hydrolyze FLDA directly. We also tried different acetate protected fluorophores, and found that FLDA is the most suitable substrate for HCAII (Fig. S2, ESI†). Fig. 2b shows the time course of the fluorescence intensity. The fluorescence amplification results showed a gradual increase in fluorescence between 0 and 45 minutes. These results indicate that the enzyme activity can be modulated by covalently linking a synthetic inhibitor to the enzyme and fluorescence amplification occurs upon treatment of a specific activator.

In order to optimize the fluorescence signals, we synthesized a series of inhibitors with different linker lengths and sulfonamides. When BG-NH_2 **2**, a compound without a sulfonamide inhibitor, was labeled to the sensor protein, the semisynthetic sensor was not able to differentiate between the presence or absence of caspase-3 (Fig. 3). We also studied the effect of linker length on the fluorescence enhancement and found that BG-$(C6)_3$-*para*SA **1** showed the highest signal amplification with an average of 17-fold intensity enhancement. The dissociated SNAP-sulfonamide fragment can still block the HCAII activity, though with weaker affinity as compared to the intramolecular sulfonamide. We generally observed residual HCAII activity of about 30% under our caspase-3 sensing conditions as compared to the non-inhibitor modified SNAP_DEVD_HCAII (Fig. S3, ESI†). Shortening the linker to BG-$(C6)_2$-*para*SA **3** and BG-(C6)-*para*SA **4** or introducing hydrophilic ethylene glycol chain BG-$(EG)_5$-*para*SA **5** or BG-$(EG)_{11}$-*para*SA **6** does not provide significant fluorescent amplification. When the weaker *meta*-sulfonamide inhibitor BG-$(C6)_3$-*meta*SA **7** was employed to label the sensor protein, the fluorescence was amplified by merely 2-fold. The lower fluorescent amplification of compounds **3–7** is attributed to the incapability of the inhibitors to completely inactivate the enzyme. In these cases, we generally observed higher background fluorescence in the absence of caspase-3. Therefore, the sensitivity of the semisynthetic sensor depended mainly on the activity of liberated HCAII upon caspase-3 cleavage for fluorescence amplification and the level of background fluorescence associated with the strong inhibition by sulfonamide inhibitors in the closed conformation.

A series of different concentrations of caspase-3 was used to calibrate the detection range of the sensor. As the concentration of caspase-3 increases, the resulting fluorescence is enhanced due to the increase of catalytically active free HCAII (Fig. 4a).

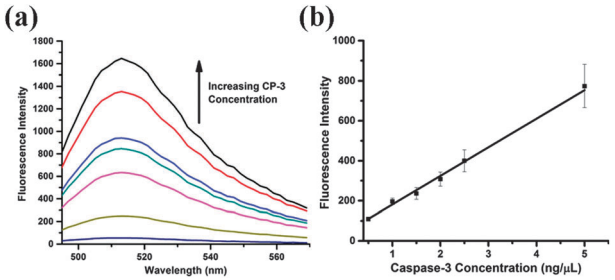

Fig. 4 (a) Fluorescence spectra of the semisynthetic sensor with increasing concentrations of caspase-3. (b) Linear relationship between the fluorescence intensity and the concentration of caspase-3 within the range of 0.5 ng μL^{-1} to 5 ng μL^{-1}. The error bar was calculated from three independent experiments.

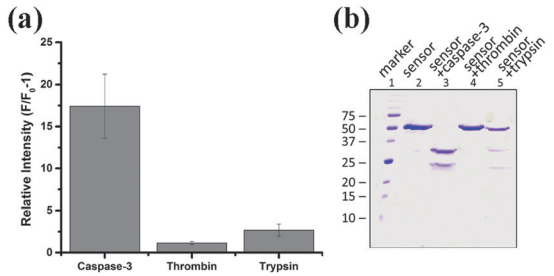

Fig. 5 (a) Relative fluorescence intensity of the semisynthetic sensor in the presence of 5 ng μL^{-1} concentration of caspase-3, thrombin and trypsin. (b) SDS-PAGE gel analysis of the sensor in the presence of caspase-3, thrombin and trypsin. The molecular weight of the sensor protein is about 53 kD.

A linear relationship was found between the relative fluorescence intensity and the concentration of caspase-3 within the range from 0.5 ng μL^{-1} to 5 ng μL^{-1}, with a correlation coefficient of 0.99 (Fig. 4b). The detection limit was estimated to be 0.2 ng μL^{-1} from three times the standard deviation corresponding to the blank sample with 5 μM FLDA. We also compared the caspase-3 detection sensitivity by using 50 μM synthetic caspase-3 fluorescence substrate, Ac-DMQD_AMC.[16] The result shows that our semisynthetic sensor (0.5 μM) is at least 10-times more sensitive than the synthetic caspase-3 substrate (Fig. S4, ESI†).

We also investigated the selectivity of the sensor toward caspase-3 by testing the influence of two other common proteases, thrombin and trypsin. A high fluorescence signal was obtained only when caspase-3 was added, whereas the fluorescence signals were negligible in the presence of thrombin and only about 3-fold for trypsin (Fig. 5a). SDS-PAGE gel analysis revealed that caspase-3 cuts the DEVD sequence of semisynthetic sensor protein SNAP_DEVD_HCAII into two fragments with similar molecular weight as SNAP-tag protein and HCAII (Fig. 5b). Thrombin is unable to cleave the DEVD sequence, whereas trypsin shows a trace amount of unspecific proteolysis of the semisynthetic sensor. The measured data demonstrated that the fluorescence signal was specifically triggered by the cleavage of the DEVD sequence to activate HCAII, which indicated that the semisynthetic sensing system could offer high specificity.

In summary, we have shown that the allosteric sensing scheme of natural enzymes can be mimicked using our semisynthetic sensor by tethering a sulfonamide inhibitor to HCAII enzyme. The semisynthetic sensor is modular and can only be catalytically activated when the target protein caspase-3 is present. This offers a novel bioanalytical approach to avoid time-consuming and laborious washing steps encountered in most of the enzyme-catalyzed signal amplification assays. By genetically introducing a self-labeling SNAP-tag protein into the enzyme, we have established a general scheme to assemble an enzyme, a sensing site and an inhibitor into one single functional unit to mimic natural allosteric enzymes. Thus, our semisynthetic sensor design not only provides a general approach to mimic allosteric enzymes but also highlights the potential of semisynthetic sensors in practical applications where sensitive and simple detection methods are required.

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