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COMMUNICATION

Peptide-templated gold nanoclusters as a novel label-free biosensor for the detection of protease activity

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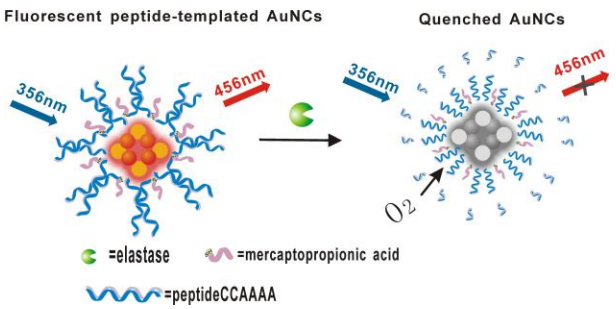
A novel label-free fluorescent biosensor platform has been developed for protease activity assay using peptide-templated gold nanoclusters (AuNCs). The biosensor was demonstrated with elastase as the model to have high sensitivity, excellent specificity, simplicity and rapidness.

Proteases are a class of enzymes that catalyze the hydrolysis of peptide bonds by recognizing the side chains of specific amino acid to break down proteins into smaller fragments. Approximately 2% of human genes encode proteases, which are involved in various biological processes, such as development, immunity, blood clotting, and wound healing.[1] Alterated proteases activity levels are associated with a number of pathological conditions, including cancer, arthritis, neurodegenerative diseases, pulmonary diseases as well as cardiovascular diseases.[2] Sensitive, specific and convenient detection of protease activity is highly desirable for early diagnosis of protease-relevant diseases and high-throughput screening of potential drugs. Various techniques including chromatography, mass spectrometry, electrochemical methods as well as immunoassays,[3] and mostly commonly fluorescence resonance energy transfer (FRET)-based assays,[4] have been reported to the detection of protease activity. However, these assays require the synthesis of label substrates such as attaching fluorescence donor and acceptor to different sites, and some methods are either time-consuming or not sensitive enough.

Gold nanoclusters (AuNCs) offer a highly attractive option for chemical and biomedical applications owing to their ultrasmall size , good biocompatibility, and bright fluorescence.[5] Recently, biomolecules such as peptides, DNA and proteins have been demonstrated for templated synthesis of AuNCs.[6] These biomolecular templates not only provide structure-defined scaffolds for nucleation and growth of AuNCs, but also have the potential to impart the AuNCs with designed biological function such as selective targeting of cells.[6a-c] Along this direction, there are increasing interest in the development of AuNCs based analytical techniques for metal ions and enzymes.[7]

Recently, protein-protected AuNCs have been used as for fluorescence assay of proteases.[8] However, the selectivity of this assay is limited due to non-specific degradation of the protein templates. Our group has reported a simple peptide-templated method to direct rapid synthesis of highly fluorescent AuNC beacons that are responsive to kinases and deacetylases.[9] This technology may have the potential as a single-step, label-free biosensor platform for detecting proteases and their inhibitors. Compared with other FRET or protein-templated AuNCs based protease assays, this technology may be more sensitive, rapid, cost efficient and selective. To testify the hypothesis, herein we chose a protease substrate peptide to template the synthesis of AuNCs and investigate their responsiveness to the target protease. Elastase, a potential biomarker closely associated with tumor progression, was used as a model protease. A peptide substrate (CCAAAA) with two functional domains was used to construct the template for AuNCs. The domain CC offers a strong ligand for AuNCs through the assembly of thiol groups,[10] and the domain AAAA is the substrate of elastase.[11] As schematically illustrated in Scheme. 1, the AuNCs synthesized with the peptide templates is presumed to form a core-shell structure through the interactions between the amino acids and the AuNC core. The peptides act as a compact coating layer, protecting the AuNCs from O_2-mediated fluorescence quenching.[8b] Cleavage of the substrate peptides by elastase destroys the protective peptide coating, which allows O_2 to diffuse into contact with the AuNC cores and quench their fluorescence.



Scheme1. A schematic illustration of the sensing mechanism for prorteases using peptide-protected AuNCs

A key step to the development of label-free biosensors for proteases is a modified method for peptide-templated synthesis of highly fluorescent AuNCs. In this study, the peptide-templated AuNCs were prepared from $HAuCl_4$, the peptide template CCAAAA and an auxiliary ligand 3-mercaptopropionic acid (MPA) using $NaBH_4$ as the reducing agent.[9] The synthetic reaction was accomplished in a single step within 40 min, which was much faster and simpler than the reported methods for peptide or protein protected-AuNCs.[6a] No fluorescent AuNC was obtained in the absence of MPA or the peptide template, indicating the essential roles of both the peptide template and the auxiliary ligand in the formation of fluorescent AuNCs. MALDI-TOF mass spectrometry analysis revealed that AuNCs

synthesized using the peptide-templated method were highly fluorescent Au_8 clusters with surface ligands consisting of two peptides and two MPA molecules.[9] So, the amount of AuNCs used in the experiment was quantified as 100 nM by determined the peptide concentration by mass spectrometry.[9]

As revealed by transmission electron microscopy (TEM), the as-prepared AuNCs had an average size of ~1.4 nm, and the lattice fringes (~2.4 A) were consistent with metallic gold having the same d spacing for the (111) crystal plane of fcc Au (Figure 1a).[12] An intensive blue fluorescence AuNCs was observed for the AuNCs with an excitation peak at 356 nm and an emission peak at 456 nm (Figure 1b). The quantum efficiency was determined to be ~15.8% using quinine sulphate as the reference (Figure S2), which was much higher than those reported for peptide-templated AuNCs.[6] These results suggested the potential of our method in rapid synthesis of highly fluorescent AuNCs.

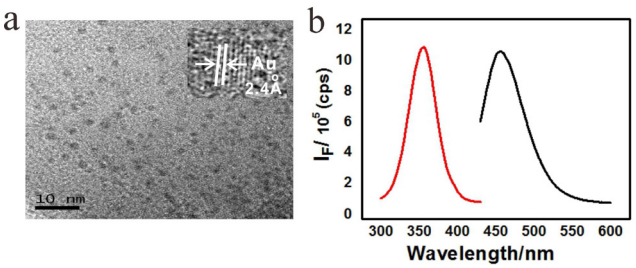


Fig. 1 (a) TEM images of peptide CCAAAA templated AuNCs. Inset: high resolution TEM image. (b) Fluorescence excitation (red, emission collected at 456 nm) and emission spectra (black, excited at 356 nm) of peptide CCAAAA templated AuNCs dispersed in ultrapure water.

Figure 2a depicts typical spectral signals of the peptide-templated AuNCs in response to elastase. After the AuNCs were incubated with 100 nM elastase at 25 °C for 40 min, the fluorescence peak was substantially quenched (by ~75% from an initial value 1100000 to 250000). In control experiments where the AuNCs were incubated with other proteases such as trypsin, pepsin, thrombin and matrix metalprotease 14 or bovine serum albumin (BSA) for 40 min, no appreciable changes appeared for the fluorescence peak. In another control where the AuNCs were incubated with 100 nM elastase in the presence of 1 mM elafin, a potent inhibitor of elastase,[13] there was only a slight decrease of the fluorescence signal, which was ascribed to incomplete inhibition of elastase (Figure 2b). These data suggested that the fluorescence quenching response of AuNCs was selective to activity elastase. To further validate the specificity of the fluorescence quenching response to peptide cleavage, we designed another control peptides, CCGGGG and CCGSGS which were not the substrate of elastase. These control peptide were used in the synthesis of AuNCs according to the above-developed method. The resulting AuNCs also showed strong fluorescence peak with the same excitation and emission maxima. In contrast, after the CCGGGG and CCGSGS-protected AuNCs incubated with 100 nM elastase for 40 min, no significant decrease in the fluorescent peak was observed (Figure 2a). Taken together, these results implied that the fluorescence quenching response of the peptide-protected AuNCs was selective to the cleavage of the peptide by its target protease, indicating the potential of the developed peptide-templated AuNCs as a label-free biosensor for protease activity assay or inhibitor screening.

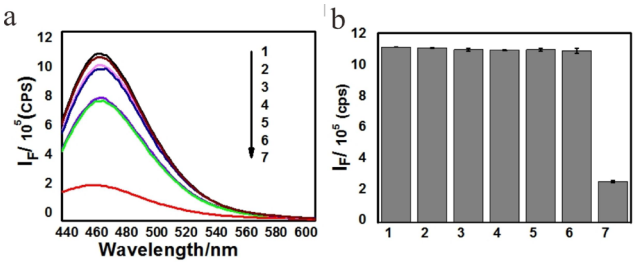


Fig. 2 a: Fluorescent spectral responses of (1) CCAAAA-AuNCs, (2) CCAAAA-AuNCs incubated with 100 nM elastase in the presence of 1 mM elafin, (3) CCGGGG-AuNCs, (4) CCGGGG-AuNCs incubated with 100 nM elastase, (5) CCGSGS-AuNCs, (6) CCGSGS-AuNCs incubated with 100 nM elastase, (7) CCAAAA-AuNCs incubated with 100 nM elastase; b: Fluorescence responses of CCAAAA-AuNCs (excitation at 356 nm, emission at 456 nm) of (1) blank, (2) MMP-14, (3) trypsin, (4) thrombin, (5) BSA, (6) pepsin, (7) elastase. Error bars are standard deviation across three repetitive experiments.

To elucidate the mechanism of the biosensor, we performed electrospray ionization mass spectrometric analysis of the peptide template by decomposing the AuNC cores using KCN-$K_3(FeCN)_6$.[14] Before the CCAAAA-protected AuNCs were treated with elastase, a mother ion peak 531.663 Da corresponding to the peptide was obtained (Figure 3a). After the reaction between the peptide-protected AuNCs with elastase, the mother ion peak for the CCAAAA peptide almost disappeared and another strong peak appeared at 438.7482 Da, which was originated the cleavage of the substrate peptide CCAAAA into CCAAA (Figure 3b).

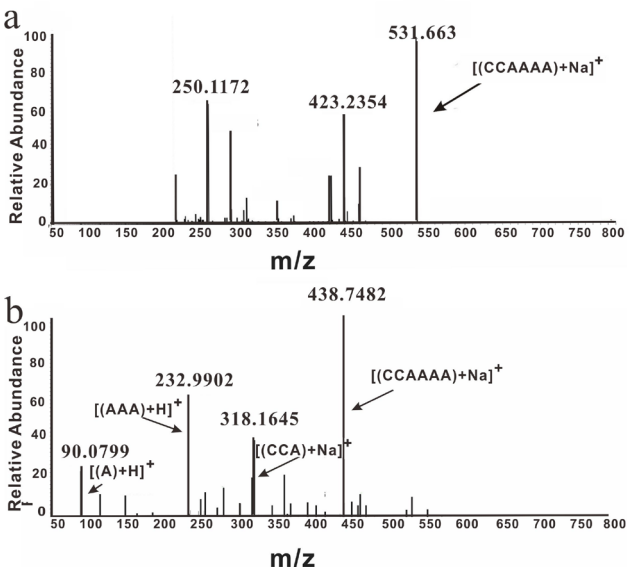


Fig. 3 ESI-MS spectra of CCAAAA and its hydrolysis products induced by elastase. (a) CCAAAA-AuNCs were decomposed with KCN-$K_3(FeCN)_6$; (b) CCAAAA-AuNCs were incubated with elastase followed by digestion with KCN- $K_3(FeCN)_6$.

These data confirmed that the peptides tethering on the AuNC cores were still active substrate of elastase. Fluorescence

anisotropy measurements revealed that the fluorescence anisotropy profile of the AuNCs after incubated with elastase, exhibited a slight decrease (Figure S3). This finding indicated that the AuNCs did not form large aggregates, precluding the possibility of fluorescence quenching via AuNCs aggregation.[13] A further inspection of the mechanism was performed by bubbling nitrogen into the AuNCs solution in a deoxygenation chamber followed by the incubation with elastase. It was observed that in the deoxygenized solution the fluorescence signal of the AuNCs decreased only slightly after 40-min reaction (Figure S4), while the drop of fluorescence peak under air saturated conditions was much more pronounced. This observation disclosed the essential role of O_2 in the fluorescence quenching response of AuNCs to elastase, and verified the presumed mechanism of the label-free biosensor.

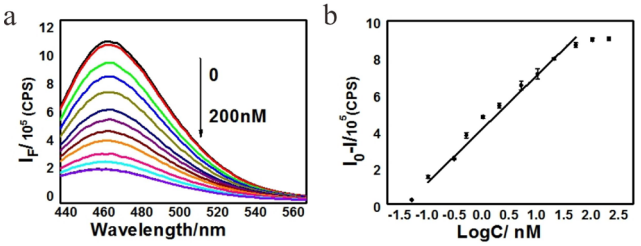


Fig. 4 (a) Plots of I_0-I as a function of the log of elastase concentrations.Here I_0 and I represent fluorescence intensities from peptide-AuNCs in the absence and presence of elastase, respectively. Error bars are standard deviation across three repetitive experiments**.** (b) Fluorescence responses of CCAAAA-AuNCs to different concentrations of elastase.(range from 0 to 200 nM)

Figure 4 depicts the fluorescence response of the peptide-templated AuNCs as a label-free biosensor to elastase of different concentrations. It was observed that the fluorescence intensity of the AuNCs decreased with increasing elastase concentration. A linear correlation was obtained for the fluorescence intensity changes (excitation at 356 nm, emission at 456 nm) versus logarithmic concentrations of elastase in a concentration range from 50 pM to 100 nM. The detection limit was calculated as 30 pM, which was among the best assays for elastase.[4] The relative standard deviation (RSD) ranged from 0.7% to 3%, indicating a desirable reproducibility of the biosensor. Note that, instead of the fluorescence intensity changes, the ratio of fluorescence intensity changes obtained before and after the enzyme reaction could also be used for quantification, which might show improved stability against the perturbation of instrument parameters.

In summary, we developed a novel peptide-templated synthetic method for AuNCs, which allowed the synthesis of highly fluorescent AuNCs in a single step within 40 min. The peptide-protected AuNCs was found to show selective fluorescence quenching response to protease such as elastase. The mechanism of fluorescence quenching was found to be ascribed to the elastase-catalyzed destruction of the protective peptide coating on the AuNC cores that induced O_2-mediated quenching of the fluorescent clusters. This protease-responsive AuNCs were demonstrated to have high sensitivity and selectivity for label-free detection of elastase with wide linear detection ranges. By using substrate peptides for other proteases, the protease-responsive AuNCs could be adapted for the developed label-free biosensors for different proteases. In virtue of the important biological roles of proteases, this technology indeed created a useful label-free biosensor platform for the detection of proteases and their inhibitors, implying its great potential for protease-targeted clinical diagnostics and drug development.

Notes and references

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†Electronic Supplementary Information (ESI) available: Experimental details and additional Figures. See DOI: 10.1039/b000000x/

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