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Substrate hydrolysis triggered formation of fluorescent gold nanoclusters – a new platform for the sensing of enzyme activity†

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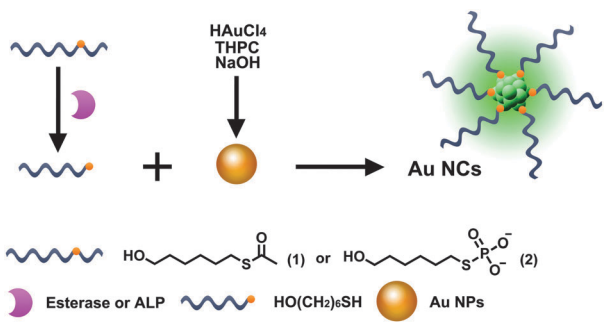
Substrate hydrolysis was utilized to trigger a "top-down" etching process for the generation of fluorescent gold nanoclusters, and the changes in emission intensity of the assay solution provide a facile way for the sensing of enzyme activity.

Functional nanoscale materials have attracted great interest due to their potential applications in optomagnetic devices, labeling, imaging, and sensing.[1] Similar to semiconductor quantum dots (QDs), metal nanoclusters (NCs) exhibit molecular-like properties different from those of the bulk materials. The size of the NCs is comparable to the Fermi wavelength of the electrons. As a result, the continuous densities of states are broken up into discrete energy levels, which leads to the observation of strong fluorescence.[2] These NCs exhibit many attractive properties such as tunable emission, high photoluminescence quantum yield, good photostability, large Stokes shift, good biocompatibility, and low toxicity, and they can be easily prepared. Owing to these interesting features, fluorescent NCs have been utilized for the construction of a number of novel sensors.[3] Similar to the larger nanoparticles, the NCs can be prepared through two routes: the "bottom-up" approach and the "top-down" approach. The "bottom-up" approach utilizes a wet chemical reduction process. The metal precursors are reduced to zero valent atoms with an appropriately selected reducing reagent and the NCs emerge as a result of the accumulation of the zero valent metal atoms.[4] In a "top-down" method, a suitable capping agent is usually selected to etch the larger nanoparticles down to small NCs through a ligand-induced etching process.[5]

Enzymes are one of the most important classes of biomacromolecules. They play vital roles in maintaining proper body functions. Sensing of enzyme activity has been routinely used for the diagnosis, prognosis, and treatment of diseases.[6] Therefore, the development of sensitive, selective, inexpensive, and convenient methods for the detection of enzyme activity is of great importance. There are many traditional enzyme sensing techniques reported in the literature.[7] Nanomaterials have been employed in recent years as promising alternatives for the construction of novel enzyme sensors.[8] Recently, enzyme detection using fluorescent metal NCs has drawn increasing attention.[9] However, these methods employed existing NCs and a signal-off detection mode, which could considerably increase the likelihood of false positive signals associated with the signal-off detection.

Herein, we report that for the first time, substrate hydrolysis is utilized to trigger a "top-down" etching process for the generation of fluorescent NCs, and the changes in emission intensity of the assay solution provide a facile way for the sensing of enzyme activity. Esterase and alkaline phosphatase (ALP) were used as the model enzymes. Proper substrates were designed and synthesized. An alkanethiol ligand was released because of the substrate hydrolysis. The alkanethiol ligand could etch the AuNPs and fluorescent AuNCs formed *in situ*. Our method is simple, inexpensive, sensitive, and selective.

The assay strategy is schematically depicted in Scheme 1. Esterase and ALP catalyze the hydrolysis of substrate 1 and substrate 2, respectively, to generate an alkanethiol ligand 6-mercapto-1-hexanol (MCH) (Scheme S1, ESI†). Literature reports have shown that alkanethiol ligands can be used to etch small size AuNPs to



Scheme 1 Substrate hydrolysis induced generation of fluorescent NCs for the sensing of enzyme activity.

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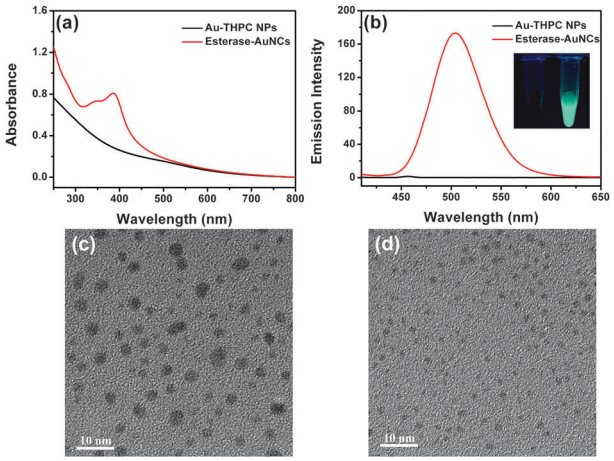

Fig. 1 UV-Vis absorption (a) and emission (b) spectra of the AuNPs and the esterase-AuNCs. Inset in (b): the corresponding photograph of the AuNP and the AuNC samples under 365 nm UV light illumination. (c) and (d): TEM images of the AuNPs and the AuNCs, respectively.

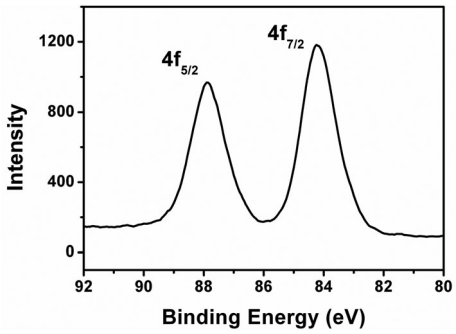

Fig. 2 XPS spectrum of the esterase-AuNCs.

fluorescent AuNCs through a ligand-induced etching process.[5b] The increase in AuNC emission intensity could therefore be directly related to the amount of enzyme in the assay solution.

Esterase is a group of hydrolases that catalyzes the hydrolysis of various esters. It is mainly localized in the endoplasmic reticulum and cytosol of various tissues and is important in detoxification and metabolism of many drugs.[10] Esterase has broad substrate tolerance, and has been widely used as biocatalyst.[11] Fig. 1a shows the UV-Vis absorption spectra of the AuNPs and the esterase generated AuNCs (esterase-AuNCs). The as-prepared AuNPs show no obvious absorption band in the 250–800 nm region. In contrast, esterase-AuNCs exhibit a clear absorption band with band maximum at 385 nm. The absorption band of the AuNCs is likely originated from the metal–metal interactions and/or ligand–metal charge-transfer transitions.[5,12] The AuNPs show no emission, whereas the esterase-AuNCs exhibit intense green fluorescence with a peak maximum at 503 nm under 395 nm excitation (Fig. 1b, ~1500-fold emission intensity enhancement). The fluorescence quantum yield was calculated to be ~5.6% using 9,10-diphenylanthracene as a reference. The fluorescence lifetime of the esterase-AuNCs was determined (Fig. S1, ESI†). The fluorescence decay of the gold clusters shows biexponential behaviour with an average decay time of about 552 ns. The relatively long radiative lifetime of the AuNCs is characteristic of the Au(I)–thiol complexes.[3b,4b] The TEM study shows that the average diameter of the AuNPs is ~2.9 nm (Fig. 1c) and the size of the NPs decreases to ~1.3 nm after the esterase triggered etching process (Fig. 1d). The results clearly demonstrate that the AuNPs have been successfully etched to the AuNCs as a result of the esterase catalyzed substrate hydrolysis. An X-ray photoelectron spectroscopy (XPS) study was carried out to analyze the valence states of the AuNC surface (Fig. 2). The binding energy of Au($4f_{7/2}$) is located at 84.25 eV. It falls within the region of 84.0 eV [Au(0) film] to 85.0 eV [Au(I) thiolate complex]. The results indicate the coexistence of Au(0) and Au(I) (Fig. S2, ESI†).[4b,5a,13] Literature reports have shown that the presence of Au(I) on the surface could help stabilize the NCs.[14] A control experiment shows that without the addition of substrate 1, esterase itself could not generate the AuNCs (Fig. S3, ESI†). The results indicate that esterase alone could not initiate the etching process; the NCs were generated from the substrate hydrolysis induced etching of the gold nanoparticles.

The properties of the AuNPs on the formation of the AuNCs were investigated (Fig. S4, ESI†). The results indicate that the size of the NPs is important for the MCH induced AuNP etching. AuNPs prepared using a 1 : 1.5 ratio of THPC to $HAuCl_4$ were used for the current investigation.

The amount of the alkanethiol ligand used could affect the etching results. The emission intensity of the AuNCs reached a maximum value when 4–5 mM MCH was used (Fig. S5, ESI†). The esterase triggered etching process showed similar results when 4–5 mM of substrate 1 was used. The esterase triggered core etching process was completed in 4 h. No obvious changes in emission spectra were observed with prolonged reaction time (Fig. S6 and S7, ESI†).

The results show that with the increase of the esterase concentration, increased AuNC emission was observed (Fig. S8, ESI†). The maximum emission of the esterase-AuNCs is in direct proportion to the esterase concentration in the range of 0.1–10 mU mL^{-1}. The limit of detection (LOD) is estimated to be 0.04 mU mL^{-1}, which is quite sensitive compared with the reported literature methods.[15]

The enzyme triggered *in situ* formation of the AuNCs could also be used to probe alkaline phosphatase activity. ALP is widely distributed in human and animal tissues and plays important roles in certain pathological conditions. An abnormal ALP level could be correlated to a number of diseases such as bone disease, liver dysfunction, and diabetes.[16] UV-Vis absorption and emission spectra of the ALP-AuNCs are shown in Fig. S9 (ESI†). The shape of the absorption band is similar to that of the esterase-AuNCs, and intense green emission was also observed. A control experiment shows that no emission of the AuNCs was observed in the absence of substrate 2 (Fig. S10, ESI†). The results again suggest that the enzyme (ALP) alone cannot initiate the etching process. The NCs were generated as a result of the ALP catalyzed substrate hydrolysis. Fig. 3 shows that with the increase of the assay solution ALP concentration, increased AuNC emission was observed. And the emission intensity of the ALP-AuNCs is in direct proportion to the ALP concentration in the range of 0.01–10 mU mL^{-1}. Our assay is highly sensitive, and the LOD is estimated to be 0.005 mU mL^{-1}, which is one of the most sensitive nanomaterial-based ALP assays reported to date.[8c,9c,17]

The selectivity of the assay was studied. A number of proteins such as esterase, collagenase, acetylcholinesterase

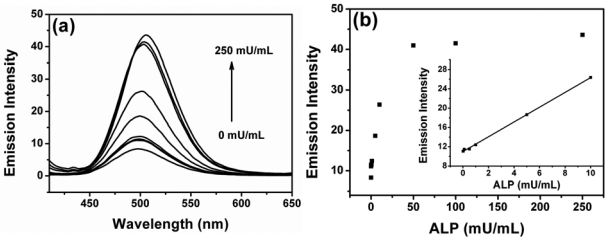

Fig. 3 (a) Changes in the emission spectrum of the AuNCs upon the addition of ALP in different concentrations (0, 0.01, 0.1, 0.5, 1, 5, 10, 50, 100, and 250 mU mL^{-1}). (b) Maximum emission intensity changes of (a) as a function of the ALP concentration. Inset: expanded linear region of the calibration curve. Linear regression equation $I = 55.06 + 7.66C$ ($R^2 = 0.999$).

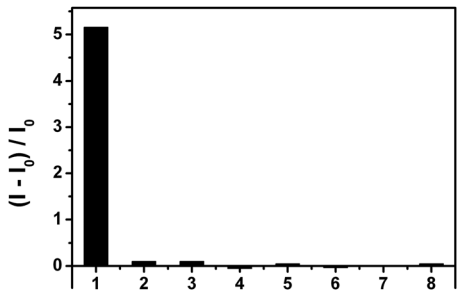

Fig. 4 Selectivity of the assay. Samples 1–8: ALP, esterase, collagenase, AChE, lysozyme, trypsin, lipase, and BSA, respectively. Protein concentration: samples 1–7, 100 mU mL^{-1} each; sample 8: 0.2 mg mL^{-1} BSA. I_0 and I were the maximum emission intensities of the sample solutions without and with the addition of the proteins.

(AChE), lysozyme, trypsin, lipase, and bovine serum albumin (BSA) were tested. Our results show that none of these proteins had the ability to induce the core etching process (Fig. 4). In addition, a number of thiol-containing compounds were tested, and none gave noticeable interference (Fig. S11, ESI†). Thus the assay is quite selective.

Our assay could be used to analyze enzyme activity in complex sample mixtures. Diluted calf serum (2%) and A549 cell lysate (2%) were used. Fig. S12 (ESI†) shows that with the addition of increasing concentrations of ALP, significantly increased AuNCs emission intensity was observed. The results indicate that with more ALP added, more substrate 2 was hydrolyzed, and higher concentrations of the AuNCs were generated.

Our assay could also be used to evaluate the inhibition effect of the ALP inhibitors. Na_3VO_4, a commonly used ALP inhibitor was tested.[17b] When the activity of ALP was inhibited by Na_3VO_4, less amount of substrate 2 was hydrolyzed, and less amount of MCH was generated. As a result, less amount of the AuNCs was produced. Fig. S13 and S14 (ESI†) show that the emission intensity of the ALP-AuNCs decreased with the increase of the inhibitor concentration. 10 mM Na_3VO_4 could almost completely inhibit the activity of 50 mU mL^{-1} ALP.

In conclusion, a novel fluorometric turn-on assay for enzyme activity based on the *in situ* "top-down" generation of the AuNCs has been developed. A hydrolytic enzyme (esterase and ALP as examples) catalyzed the hydrolysis of the substrate. An alkanethiol compound was released. The AuNCs were created through a ligand-induced etching process. Intense green emission was observed. And the changes in emission intensity could be directly related to the amount of enzyme in the assay sample solution. Our method is highly sensitive, simple, inexpensive, and selective. It provides a new nanoscale platform for the sensing of other hydrolases with the appropriately designed substrates, and for the construction of novel hydrolytic enzyme based biosensors for various biochemical and biomedical applications.

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