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Peptide-Templated Gold Nanocluster Beacon as a Sensitive, Label-Free Sensor for Protein Post-translational Modification Enzymes

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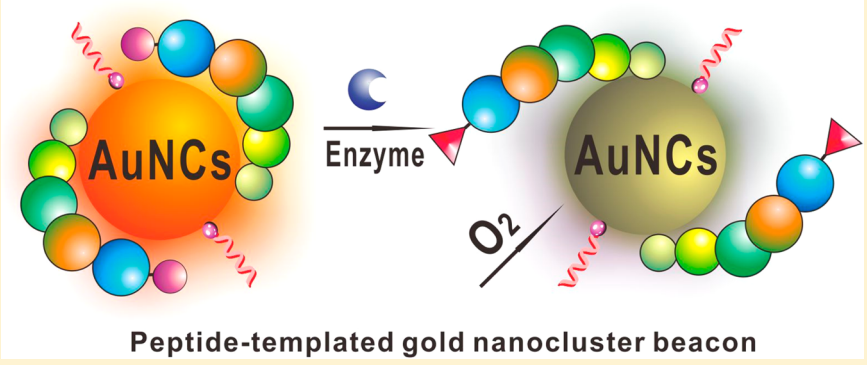
Supporting Information



ABSTRACT: Protein post-translational modifications (PTMs), which are chemical modifications and most often regulated by enzymes, play key roles in functional proteomics. Detection of PTM enzymes, thus, is critical in the study of cell functioning and development of diagnostic and therapeutic tools. Herein, we develop a simple peptide-templated method to direct rapid synthesis of highly fluorescent gold nanoclusters (AuNCs) and interrogate the effect of enzymatic modifications on their luminescence. A new finding is that enzymes are able to exert chemical modifications on the peptide-templated AuNCs and quench their fluorescence, which furnishes the development of a real-time and label-free sensing strategy for PTM enzymes. Two PTM enzymes, histone deacetylase 1 and protein kinase A, have been employed to demonstrate the feasibility of this enzyme-responsive fluorescent nanocluster beacon. The results reveal that the AuNCs' fluorescence can be dynamically decreased with increasing concentration of the enzymes, and subpicomolar detection limits are readily achieved for both enzymes. The developed strategy can thus offer a useful, label-free biosensor platform for the detection of protein-modifying enzymes and their inhibitors in biomedical applications.

Protein post-translational modifications (PTMs) play key roles in functional proteomics, via regulating the activity, localization, interactions with other biomolecules, and degradation of proteins.[1] PTMs are most often mediated by enzymes, including kinases, phosphatases, transferases, ligases, and proteases.[2] Identification and quantification of PTM enzyme activity are critical in the study of regulation mechanisms in cell functioning and the development of diagnostic and therapeutic tools for deseases.[2] Existing techniques for PTM enzyme assays typically rely on specific reagents including antibodies or binding domains to modification sites, labeled cosubstrates, and activity based probes.[3−6] There is a grand need but a daunting challenge in the development of simple and label-free methods that allow rapid and real-time detection of PTM enzymes.

Gold nanoclusters (AuNCs) are a topic of great interest in chemistry and biology because of their unique size-dependent photophysical properties.[7−9] Compared with existing fluorescence probes, AuNCs show better resistance to photobleaching and blinking and can exhibit stronger and more durable fluorescence signals.[10] Due to their chemical inertness and ultrafine size, AuNCs have little effect on the activity of the bioentities, offering superior performance including low toxicity and high biocompatibility.[11] These advantages make AuNCs highly attractive as a new generation of probes for biolabeling and biosensing.

AuNCs are routinely synthesized with the help of stabilizing or templating agents including thiolates and dendrimers.[7,12−14] Recently, biomolecules such as peptides, DNA, and proteins have been proven as structure-defined scaffolds to induce the nucleation and growth of AuNCs, offering useful templates for synthesis of AuNCs.[15−18] A unique advantage with biomolecular templates is their intrinsic biological activity, which may furnish highly versatile functions to the AuNCs. A few but notable examples of bioactive AuNCs include peptide or protein-templated AuNCs, enabling selective targeting of cell membranes or nuclei,[15,16] and insulin-biomineralized AuNCs

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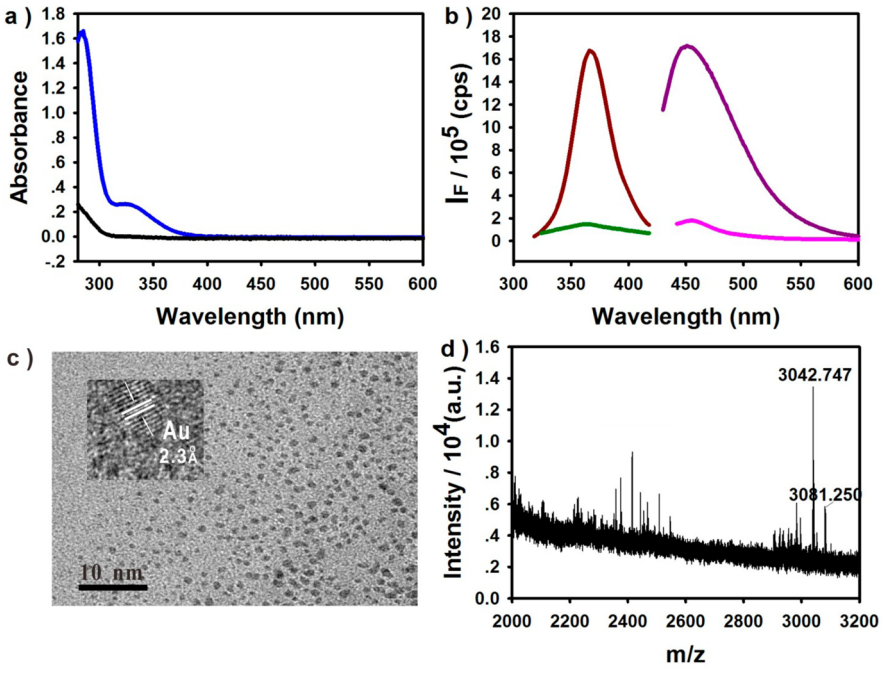

Figure 1. (a) UV−visible absorption spectra of AuNCs templated by peptide **1** (blue) and without peptide **1** (black). b) Fluorescence excitation and emission spectra of AuNCs templated with peptide **1** (brown and violet) and without peptide **1** (green and pink). (c) TEM images of peptide **1** templated AuNCs. Inset: high resolution TEM image. (d) MALDI-TOF MS spectra of peptide **1** templated AuNCs with CHCA matrix.

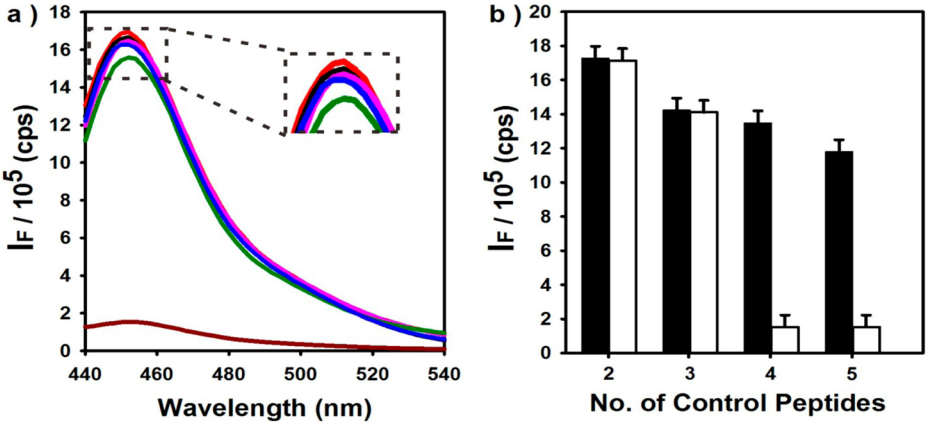

Figure 2. (a) Fluorescence spectral responses of peptide **1** templated AuNCs to different proteins: blank (red), 10 M HAT 1 (black), 10 M BSA (pink), 10 M HDM (blue), 30 nM HDAC 1 plus TSA (green), and 30 nM HDAC 1 (brown). (b) Fluorescence peak intensities of AuNCs templated by different peptides obtained without (black) and with (white) HDAC 1. Error bars indicated standard deviations (SDs) across four repetitive assays.

allowing direct visualization of the preserved protein activity.[17] However, the specific activity of biomolecular templates in biochemical reactions has rarely been reported. Hence, there is a lack of knowledge on how the luminescence of AuNCs is influenced by biochemical reactions with the biomolecular templates.

Herein, we report the development of a simple peptide-templated method to direct rapid synthesis of highly fluorescent AuNCs and the interrogation of effects of enzymatic PTMs on their luminescence properties. It is found for the first time that the resulting peptide−AuNC nanocomposites retain the substrate activity, and enzymatic modifications of the peptides are able to quench the fluorescence of AuNCs. This enzyme-responsive fluorescent nanocluster beacon opens up the possibility to inspect enzymatic PTMs using a direct, label-free approach, creating a simple but useful biosensor platform for sensitive and real-time detection of PTM enzymes. Considering the important biological roles of PTM enzymes, this development may be able to catalyze new biomedical applications of AuNCs in fundamental understandings of biological processes as well as disease diagnostics and therapeutics. In this work, we chose two PTM enzymes, histone deacetylase 1 (HDAC 1) and protein kinase A (PKA), as the model system. Both HDAC 1 and PKA are known to be tightly implicated in cell cycle regulation, cell proliferation, and cancer development and are among the most potential therapeutic targets and diagnostic biomarkers.[19]

We first investigated the synthesis of fluorescent AuNCs with the substrate peptide of HDAC 1.[20] The substrate peptide **1** CCIHK(Ac) was designed to have two cysteine amino acids at

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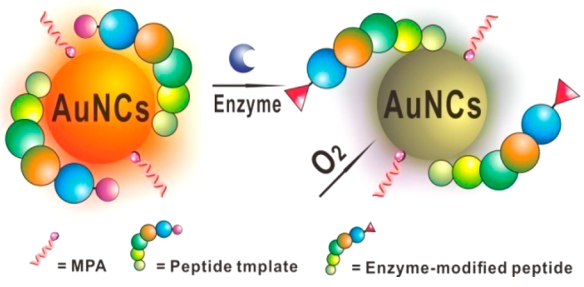
the N-terminal such that it could have high affinity to gold and thus provide a structure-defined scaffold for the growth of AuNCs. As compared with existing synthesis methods for peptide templated AuNCs,[15] a major modification in our approach was peptide-directed rapid (~20 min) synthesis of AuNCs in the presence of an auxiliary ligand 3-mercaptopropionic acid (MPA) and a strong reducing agent $NaBH_4$. The use of this auxiliary ligand could increase the stability of AuNCs and their fluorescence quantum yield due to the formation of a compact, negatively charged coating on the cluster surface. The strong reducing agent was chosen because of its ability to cause fast reduction of $HAuCl_4$ which facilitated efficient nucleation and formation of small AuNCs.[21]

As anticipated, the synthesized AuNCs displayed a broad absorption band with an onset near 400 nm and an intensive blue fluorescence with an excitation peak at 360 nm and an emission maximum at 455 nm (Figure 1a,b), an indicator of Au_8 clusters based on the spherical Jellium model.[22] The quantum efficiency was determined to be ~21.7% using quinine sulfate as the reference (Figure S1 in the Supporting Information), which was much higher than those reported for peptide-templated AuNCs.[15−18] The enhanced luminescence suggested that the auxiliary ligand MPA actually provided a protective coating for AuNCs. In a control experiment where AuNCs were synthesized in the absence of peptide **1**, their fluorescence emission was very weak, implying the essential role of the peptide template for highly luminescent AuNCs. As revealed by transmission electron microscopy (TEM), the AuNCs showed an average size of ~1.1 nm, and their lattice fringes (~2.3 A) were consistent with the interplanar spacing of the (111) crystal plane of face-centered cubic Au (Figure 1c).[23,24] A further inspection of the composition of the AuNCs was performed using a MALDI-TOF mass spectrometry in the positive ion mode with α-cyano-4-hydroxycinnamic acid (CHCA) as the matrix. It was observed that the mass spectra only gave several intense fragment peaks in the m/z range below 3200 Da. The peak with the maximum m/z (obs. 3081.250 Da) and the second maximum m/z (obs. 3042.147 Da) could be assigned to the parent ions, both indicating the presence of Au_8 clusters, $[Au_8\ peptide_2\ MPA_2\ K]^+$ (calc. 3080.714 Da) and $[Au_8\ peptide_2\ MPA_2\ H]^+$ (calc. 3042.624 Da) (Figure 1d). Taken together, all these observations implied that the AuNCs synthesized using our peptide-templated method were highly fluorescent Au_8 clusters with surface ligands consisting of two peptides and two MPA molecules.

Scheme 1. Illustration of the Mechanism for the Peptide-Templated AuNCs as a Label-Free Sensor for PTM Enzyme



An intriguing finding of our work was that the peptides tethering on the as-prepared AuNCs could still act as an effective substrate for HDAC 1, and deacetylation of these peptides would quench the fluorescence of AuNCs. When reacted with 30 nM HDAC 1, the peptide **1** templated AuNCs exhibited gradually decreased fluorescence and ~93% quenching of fluorescence was obtained after 45 min (Figures 2a and S2 in the Supporting Information). On the other hand, we incubated the peptide **1** templated AuNCs with other proteins or enzymes such as bovine serum albumin (BSA), histone acetyltransferase 1 (HAT 1), and histone demethylase 1 (HDM 1). It was found that the AuNCs did not show a significant decrease in the fluorescence signal after a 45 min incubation (Figure 2a). These results revealed that the fluorescence quenching of the AuNCs was specifically originated from deacetylation of the substrate peptides by HDAC 1. Notably, we examined the effect of a potent and specific inhibitor of HDACs, trichostatin A (TSA),[22] in the reaction between the peptide **1** templated AuNCs and HDAC 1. It was found that, in the presence of 100 nM TSA, there was merely a slight decrease in the fluorescence of AuNCs after a 45 min reaction with HDAC 1 (Figure 2a). Such a slight fluorescence decrease was attributed to the weak residual activity of HDAC 1 after being inhibited by TSA. This finding not only confirmed the selectivity of fluorescence quenching of AuNCs to active HDAC 1 but also implied the potential of the fluorescent AuNCs in rapid screening for inhibitors of HDAC 1. To further validate whether the fluorescence quenching was selective to deacetylation reaction, a control experiment was performed using control peptides **2** and **3** that were not the substrate of HDAC 1. The AuNCs synthesized with peptide **2** or **3** also displayed strong fluorescence peaks near 455 nm on excitation at 360 nm. After being treated with 30 nM HDAC 1 for 45 min, however, no remarkable fluorescence quenching was observed for these AuNCs (Figure 2b). In contrast, in control experiments where AuNCs were synthesized with an alternative HDAC 1 substrate peptide, **4** or **5**, appreciable fluorescence quenching was obtained for the AuNCs after a 45 min reaction with HDAC 1. These observations implied that only AuNCs synthesized with active substrate peptides could be quenched by the reaction with HDAC 1.

To unveil the fluorescence quenching mechanism for the AuNCs in response to active HDAC 1, we first investigated the product of peptide **1** tethering on the AuNCs after HDAC 1 mediated reactions. The MALDI-TOF mass spectra for the peptide-templated AuNCs (positive ion mode, CHCA matrix) showed intense peaks at 626.153 and 583.819 Da, respectively, before and after the HDAC 1 mediated reaction (Figure S3 in the Supporting Information). These two peaks were ascribed to the surface peptide ligands, peptide **1** (calc. 626.753 Da) and deacetylated peptide **1** (calc. 583.709 Da), before and after the enzymatic treatment. By using KCN to decompose the AuNCs before and after the enzymatic reaction,[25] a further electrospray ionization (ESI) mass spectrometric analysis of the released peptides gave a clear evidence for deacetylation of peptide **1** after HDAC 1 treatment (Figure S4 in the Supporting Information). Zeta potential measurements of the AuNCs before and after the enzymatic reaction also suggested a substantial increase of surface positive charges in the reaction buffer, indicators of the formation of deacetylated peptide **1** (Figure S5 in the Supporting Information). These findings, taken together, suggested that the peptides used for templated synthesis of AuNCs still retained their substrate activity, despite their close proximity to AuNC cores. Nonlinear fitting of the time-dependent fluorescence responses to HDAC 1 of two concentrations gave estimates of the average Michaelis−

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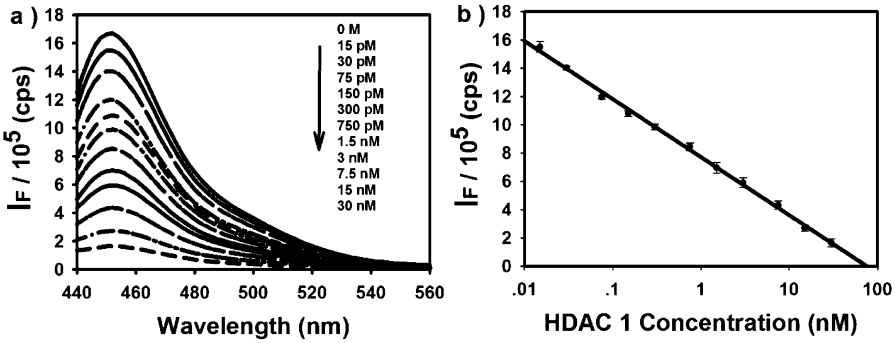

Figure 3. (a) Typical fluorescence spectral responses of peptide **1** templated AuNCs to HDAC 1 of varying concentrations. (b) Fluorescence peak intensities versus HDAC 1 concentrations in logarithmic scale. Error bars indicated SDs across four repetitive assays.

Menten constant K_m and the average catalytic constant k_{cat} as 0.25 μM and 15.7×10^9 min^{-1}, respectively (Figure S2 in the Supporting Information).[26] These estimates, which were approximate to the corresponding values reported for K_m (0.69 μM) and k_{cat} (8.2×10^9 min^{-1}),[20] verify that the AuNCs templating peptide served as a highly active substrate for HDAC.

Close inspection of the fluorescence quenching mechanism was performed by fluorescence lifetime analysis of the AuNCs before and after the enzymatic reaction. By fitting to a biexponential fluorescence decay, we found that the lifetime dropped from 24.5 ns (80.1%) and 3.9 ns (19.9%) for peptide **1** coated AuNCs to 1.0 ns (48.6%) and 3.8 ns (51.4%) for the AuNCs tethered with deacetylated peptide (Figure S6 in the Supporting Information). The decrease in fluorescence lifetime suggested a less protective microenvironment for the AuNCs modified with deacetylated peptides. Fluorescence anisotropy measurements of the AuNCs during different times in the HDAC 1 treatment displayed gradually decreased values of fluorescence anisotropy (Figure S7 in the Supporting Information). This result, presumably attributed to the changed viscosity microenvironment for the AuNCs during the enzymatic reaction, eliminated the possibility of fluorescence quenching due to aggregation of AuNCs.[12] The in-depth chemical states of the AuNCs before and after the enzymatic reaction were further characterized by X-ray photoelectron spectroscopy (XPS). Deconvolution of the XPS spectra for the peptide **1** coated AuNCs gave two distinct components corresponding to a major species Au(0) (~95%) and a minor one Au(I) (~5%) involved in maintaining the stability of AuNCs.[27] Surprisingly, a much larger percentage of Au(I) (~93%) was obtained when the AuNCs were subjected to the reaction with HDAC 1 (Figure S8 in the Supporting Information). These data revealed that the deacetylation of peptide **1** tethering on AuNCs was concomitant with the oxidation of AuNCs, implying a crucial role of oxygen in the fluorescence quenching mechanism. Motivated by this finding, we then performed a deoxygenation test by bubbling nitrogen into the AuNCs solution in a deoxygenation chamber followed by the incubation with HDAC 1. Interestingly, in the deoxygenized solution, the fluorescence signal of the AuNCs exhibited only a slight decrease after a 45 min reaction with 30 nM HDAC 1 (Figure S9 in the Supporting Information). This observation gave immediate evidence that oxygen, though not involved in the deacetylation reaction, was essential for the fluorescence quenching response of the AuNCs. Combining these results, we could presume a mechanism for the AuNCs' fluorescence quenching response to HDAC 1, as shown in Scheme 1. The AuNCs synthesized with the peptide templates might form a "complementary" core−shell structure through the interactions between all amino acids and the AuNC core. The peptides acted as a compact coating, while retaining their substrate activity, protecting the AuNCs from contact with O_2 dissolved in the solution and alleviating O_2-mediated fluorescence quenching. Enzymatic deacetylation of the substrate peptides altered the interactions between the peptides and the core, destroying the compactness of the protective coating. Thus, O_2 was able to diffuse into contact with the AuNC cores, quenching their fluorescence via energy transfer[28] and even oxidizing them into nonfluorescent clusters.

The enzyme-responsive fluorescent AuNCs beacon indeed provided a label-free biosensor platform for quantification of the enzymatic activity. Figure 3 depicts fluorescence responses of the peptide **1** templated AuNCs to HDAC 1 of varying concentrations. One observed dynamically decreased fluorescent peaks with increasing HDAC 1 concentration ranging from 15 pM to 30 nM (0.0005 to 1 U/mL). A linear correlation was obtained for the peak responses versus the logarithmic HDAC 1 concentrations over the three-decade range with a detection limit of 5 pM, implying a wide linear range and high sensitivity of this label-free biosensor.

To demonstrate the generality of the peptide-templated synthesis method for fluorescent AuNCs and the label-free biosensor platform for PTM enzymes, another substrate peptide **3**, CCLRRASLG, was designed for PKA. As anticipated, AuNCs were successfully synthesized by using this peptide as template according to the developed procedure (Figure S10 in the Supporting Information). The resulting AuNCs also showed a selective fluorescence quenching response to active PKA (Figure S11 in the Supporting Information). For the peptide-templated AuNCs used as a label-free biosensor in quantitative analysis of PKA, the fluorescence peak responses exhibited a linear correlation to the logarithmic PKA concentrations in the range of 15 pM to 60 nM (0.001 to 4 U/mL) with a detection limit of 6 pM (Figure S12 in the Supporting Information). Such a low detection limit and a wide dynamic range were much better (at least 10-fold improvement) than existing strategies for PKA assays,[29−32] revealing that the AuNCs beacon created a viable biosensor platform for sensitive enzymatic activity assays.

In conclusion, we developed a simple, rapid method for highly fluorescent AuNCs synthesis with different peptide templates and discovered that PTM enzymes were able to exert chemical modifications on the peptide-templated AuNCs and quench the AuNCs' fluorescence. The peptide-templated synthesis and the enzyme-responsive property of AuNCs

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were successfully demonstrated using two PTM enzymes, HDAC 1 and PKA. It was found that the synthetic method produced highly fluorescent Au_8 clusters with compact peptide coating. Enzymatic modification of the peptides could largely quench the fluorescence of the AuNCs, presumably because chemical modifications destroyed the protective peptide coating on AuNCs and induced O_2-mediated quenching and oxidation of the fluorescent clusters. This enzyme-responsive fluorescent nanocluster beacon was shown to be highly sensitive and selective for label-free and real-time quantification of HDAC 1 and PKA with wide linear detection ranges. In virtue of the important biological roles of PTM enzymes and the advantages of the enzyme-responsive nanocluster beacon, this technology indeed created a useful label-free biosensor platform for the detection of PTM enzymes and their inhibitors, implying its great potential in proteomics, biomedical imaging, and clinical theranostics.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details and other figures. 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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