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# A General Colorimetric Method for Detecting Protease Activity Based on Peptide-induced Gold Nanoparticle Aggregation

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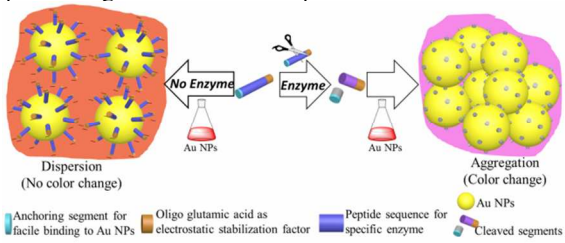
**Herein we describe a modularly designed colorimetric approach for facile and sensitive detection of proteases. The method employs non-crosslinking AuNP aggregation induced by peptide treated with specific protease. A visible colour change of the AuNPs solution from wine-red to violet-blue is readily observed when the protease-digested peptide is added. This method allows facile and visualized assay of the enzyme activity without any sophisticated instrument, affording both convenience and simplicity. The platform can be readily extended to detect virtually any protease.**

Proteases are a class of enzymes which selectively catalyse the hydrolysis of peptide bonds in proteins and polypeptides.[1] Ubiquitous in all living organisms, proteases play a significant role in numerous metabolic and physiological processes, including digestion, cell signalling, cell differentiation, immunological defence, apoptosis, and blood coagulation.[2] Self-regulation of enzymatic activities by organisms is essential for biological metabolism. Abnormal expression of proteases, on the other hand, is often associated with various diseases, including cancer, cardiovascular disease, Alzheimer's disease, inflammatory diseases,[3] etc. Proteases are also found to play a vital role in viral and bacterial infections,[4] and they can act as highly efficient biomarkers for identifying microorganisms in clinical samples.[5] Therefore, protease detection can be critical for disease diagnosis and pathogenic microorganism detection.

Different research groups have developed various methods for protease detection, such as enzyme-linked immunosorbent assays (ELISA)[6], surface-enhanced Raman Scattering (SERS)[7], and the commonly reported fluorescence-based methods, which make use of fluorescence resonance energy transfer (FRET) reporters[8] and fluorogenic probes that can be turned on by target protease[9]. These methods offer high sensitivity, but they also demand delicate fabrication procedure and sophisticated instruments. Their applications are therefore confined to well-equipped laboratories.

Colorimetric assays based on gold nanoparticles (AuNPs) aggregation have recently attracted considerable interests in diagnostic applications due to their simplicity, versatility, and comparable sensitivity with the aforementioned methods. The colorimetric method utilizes the colour change resulting from the aggregation of gold nanoparticles when they are mixed with the analyte.[10] The method has been applied to detecting a wide variety of biologically relevant molecules, such as nucleic acids, proteins, small molecules, metal ions and cells.[11] Stevens and co-workers[12], for example, reported a protease detection method based on the re-dispersion of AuNPs assemblies triggered by enzymes. This method has good sensitivity, though accessibility to cleavage sites may be hindered to some extent by the aggregates.[12] Scrmin and co-workers developed another approach by cross linking a two-cysteine-containing peptide ligand to gold nanoparticles. When enzymes were added, the peptide underwent cleavage and lost its ability to induce nanoparticle aggregation.[13] Recently, Lin and co-workers[14] have devised a new approach for protease detection based on gelatin-functionalized AuNPs. Through protein modification, the stability of AuNPs was increased. However, the protein substrate may not be specific enough for a given protease, since the protein might contain multiple cleavage sites for different proteases.




**Scheme 1.** Illustration of colorimetric detection of proteases using peptide-induced AuNP aggregation approach.

The stability of gold nanoparticle solution can be fine-tuned by functionalizing the nanoparticle surface with different thiol-containing peptides.[15] We hypothesize that functionalization with thiol-containing peptide, which carry multiple negative charges, can substantially enhance the stability of AuNPs via electrostatic repulsion. Based on this hypothesis, we herein present a novel non-crosslinking AuNPs aggregation approach for detecting proteases. In this method, we designed a special peptide ligand which can induce AuNPs aggregation when the ligand is cleaved by the target protease. The peptide ligand consists of three parts: (i) a cysteine moiety to be anchored onto AuNPs; (ii) a peptide linker as the substrate for

specific protease cleavage; (iii) a stabilizing part, $(Glu)_4$, with multiple negative charges to stabilize AuNPs via electrostatic repulsion.[16] As shown in **Scheme 1**, in the absence of enzymes, the ligand with the stabilizing part can maintain the repulsive force between AuNPs, resulting in a clear wine-red colour. When enzymes are added to the peptide solution, the stabilizing part of the peptide ligand will be cleaved off. As a result, when AuNPs are subsequently added, aggregation will occur due to the absence of the stabilizing part.[17]

In our proof-of-concept experiment, we used trypsin as a model enzyme. Peptide I with sequence EEEEGLLGALGKC was synthesized for detecting trypsin, which is known to cleave the peptide on the *C*-terminus of lysine (K) residue. When the peptide was added to citrate-stabilized gold nanoparticles by ligand exchange method, the resulting AuNPs were well-dispersed in aqueous buffer to derive a wine-red coloured solution. UV-Vis spectra revealed a slight red-shift of SPR peak from 520 nm to 525 nm, indicating ligand exchange was successful and no aggregation occurred (Fig. S1a, ESI†).[14,16] TEM image also clearly showed that the obtained gold nanoparticles were well-dispersed (Fig. S1b, ESI†). The zeta potential of the AuNPs decreased after the peptide was functionalized on the surface. The original AuNPs had a zeta potential value of -40 mv, while the peptide-functionalized AuNPs measured around -60 mv. The decrease of zeta potential can probably be attributed to the increased net negative charge of peptides, which provided further electrostatic repulsion to stabilize the AuNPs.[18] On the other hand, when a control peptide without oligo-glutamic acid segment was added to AuNPs by ligand exchange method, the AuNPs immediately aggregated and precipitated (Fig. S2, ESI†). The observation proved our hypothesis that the stability of AuNPs can be substantially enhanced in the presence of peptide ligands with multiple negative charges.

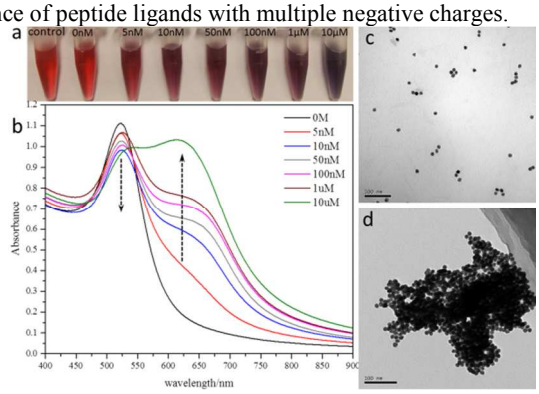



**Fig.1** Colorimetric assay of trypsin. (a) Colour changes induced by addition of trypsin-digested peptides (various concentrations of trypsin were used in the assay: 5 nM, 10 nM, 50 nM, 100 nM, 1 μM and 10 μM). (b) UV-Vis spectrum of AuNP solutions after addition of trypsin-digested peptides. (c) TEM image of AuNP solution mixed with peptide alone. (d) TEM image of AuNP aggregation induced by trypsin-digested peptide (trypsin concentration: 100 nM).

Encouraged by these results, we next carried out enzyme assays with AuNPs. The peptide was first incubated with different amounts of trypsin and then added to AuNPs solution. Aggregation of AuNPs occurred immediately, and a visible colour change from wine-red to purple or violet-blue was observed (Fig 1a). The protease activity can be qualitatively detected by the naked eye without using any instrument, as shown in Fig. 1a. AuNPs solution remained wine-red when only trypsin or peptide is added, indicating the solution was stable under the assay conditions. Addition of the peptide pre-treated with trypsin of 5 nM or higher induced a colour change from red to violet-blue. The colour becomes darker when higher concentrations of trypsin were used. The increased aggregation is caused by the increased amount of peptide cleaved by trypsin and the loss of negatively charged peptide segment. Further quantitative measurement of enzymatic activities was performed with UV-Vis spectrophotometer. As shown in Fig.1b**,** a decrease of absorbance at 525 nm (characteristic wavelength of dispersed AuNPs), and an increase of absorbance at 625nm (characteristic wavelength of aggregated AuNPs) were observed at the same time.[19] The shifted absorbance to higher wavelength with increasing trypsin concentration correlated well with the trend of the colour change (Fig 1a**)**, demonstrating the reliability of using colour change for qualitative analysis of trypsin. TEM images revealed that the AuNPs aggregate was formed when the AuNPs solution was mixed with trypsin-digested peptide (Fig. 1d), but the peptide itself did not induce any aggregation (Fig.1c). The TEM images unambiguously proved that the colour change was a result of AuNPs aggregation caused by trypsin. Cleavage of peptide by trypsin was also monitored and proved by MS analysis (Fig.S3, ESI†).

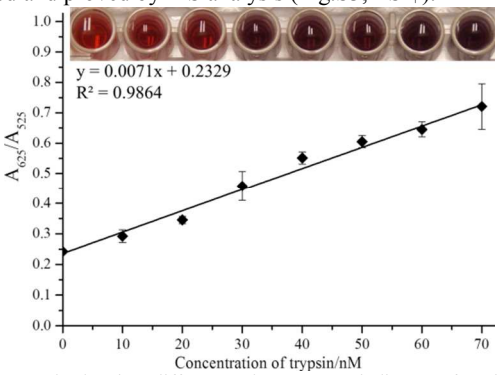



**Fig.2** Photograph showing different colours as an indicator of varied trypsin concentrations and plot of $A_{625}/A_{525}$ as a function of trypsin concentration indicating a near-liner correlation in the range of 0 – 70 nM.

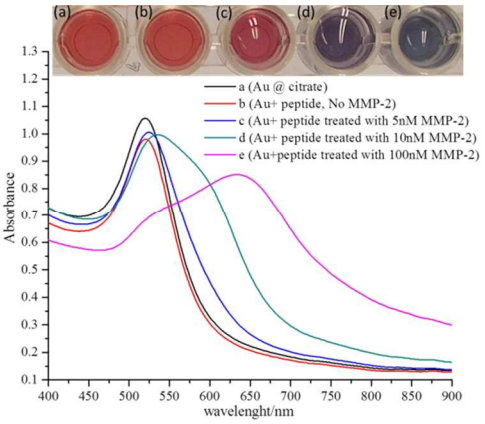



**Fig.3** Photograph showing different colours as an indicator of varied MMP-2 concentrations, and UV-Vis spectra of corresponding samples: (a) AuNPs@citrate, (b) AuNP solution mixed with peptide in TCNB buffer, (c) AuNP solution mixed with peptide digested with 5 nM of MMP-2, (d) AuNP solution mixed with peptide digested with 10 nM of MMP-2, (e) AuNP solution mixed with peptide digested with 100 nM of MMP-2.

Quantitative assay of trypsin activity was further studied by analysing the absorbance at 525nm and 625nm of each sample. The absorbance ratio of the two wavelengths ($A_{625}/A_{525}$) was used to assess the degree of AuNPs aggregation in our study. This method has been proven more accurate in previous reports.[19] The ratio of absorbance, $A_{625}/A_{525}$, was plotted as a function of the concentration of trypsin. As shown in Fig 2, the AuNPs solution became darker when the concentration of trypsin increased from 10 nM to 70 nM. Further data analysis revealed an excellent linear relationship between the ratio of the absorbance wavelength and the



concentration of the enzyme in the range of 0 to 70 nM ($y = 0.0071x + 0.2329$, $R^2 = 0.986$).

In order to prove that our design can be applied to detecting proteases other than trypsin, we designed peptide II with the sequence EEEEGPLGLAGGC for detecting Matrix Metalloproteinase-2 (MMP-2). MMP-2 is a class of proteases which are over-expressed in cancer cells and play a key role in promoting cancer progression. The protease recognizes the sequence of PLGLAG, and cleaves at the C-terminus of the underlined glycine residue.[20] Enzyme assays were performed according to established method. An immediate colour change was observed when the MMP-2-digested peptide was added to the AuNPs solution. Fig.3 shows the resulting colours and corresponding UV-Vis spectrum of AuNPs solution in the presence of peptide pre-treated with various amounts of MMP-2. As shown in the photographs, the presence of MMP-2 at a concentration of as low as 5 nM (Sample c) can be visualized, and increasing concentrations of the enzymes is reflected by both visible colour change and absorbance spectroscopy.

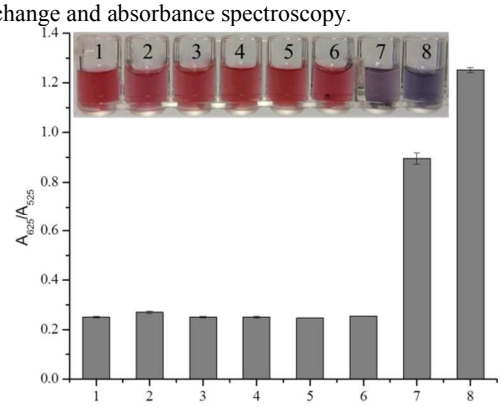



**Fig. 4**. Specificity of the colorimetric platform for detecting proteases activity. 1) trypsin (1 μM) + peptide II; 2) MMP-2(10 nM) + peptide I; 3) BSA (100 nM) + peptide I; 4) BSA (100 nM) + peptide II; 5)denatured trypsin (100 nM) + peptide I; 6) denatured MMP-2 (10 nM) + peptide II; 7) trypsin (100 nM) + peptide I; 8) MMP-2 (10 nM) + peptide II.

To further assess the specificity of our method, we performed control experiment with BSA and denatured protease. The peptides were first incubated with BSA and denatured proteases (heated and kept at 90 ºC for 10 minutes), and then added to AuNPs suspension to examine whether it could trigger AuNPs aggregation. As shown in Fig. 4, the non-protease BSA and the denatured protease did not induce any aggregation, indicating that our assay is specific and activity-dependent. In addition, only peptides incubated with the corresponding proteases were found to induce colour change and increase the $A_{625}/A_{525}$ values. For example, when peptide I (a trypsin substrate) was incubated with MMP-2 and added to AuNPs suspension, the solution remained dispersed because the peptide could not be cleaved by the enzymes. Similar results were also observed for trypsin. These experiments clearly showed that our approach for detecting protease is highly specific. It is noted that some proteins could induce AuNPs aggregation, resulting in broadening of absorption spectrum.[21] However, in our control experiments, the AuNPs were treated directly with the enzymes (50nM), we did not observe any change in color or broadening of absorption spectrum (Fig. S5, ESI). It clearly indicates that the proteases used in our study will not cause AuNPs aggregation and interfere with the assay.

In summary, we have designed a novel and facile colorimetric approach for assaying protease activity based on AuNPs aggregation induced by enzyme cleavage. The method is simple and convenient, and it allows enzyme detection with the naked eye. Importantly, the method is highly sensitive. It can detect trypsin/MMP-2 at a concentration of as low as 5 nM. The platform can be readily extended to detect virtually any protease. We believe this novel method can be a valuable tool for enzyme/microorganism detection and inhibitor screening.

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## Notes and references

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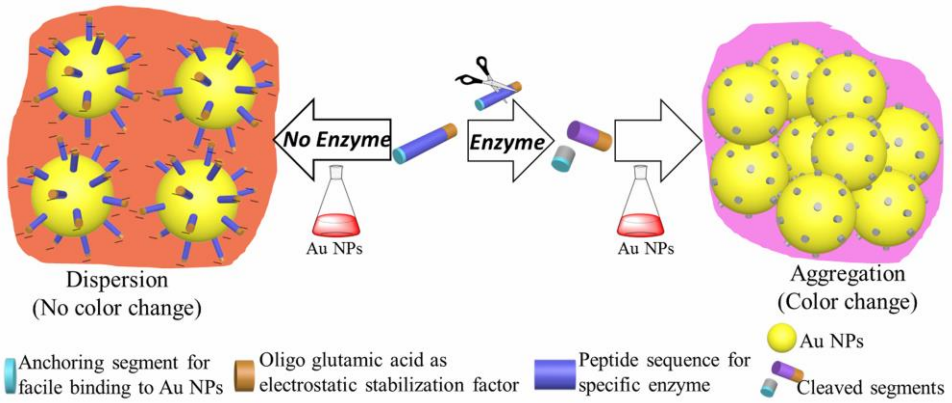


A novel colorimetric approach is developed for detecting protease. The method uses gold nanoparticle aggregation induced by protease-digested peptide.