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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. 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. 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, etc. Proteases are also found to play a vital role in viral and bacterial infections, and they can act as highly efficient biomarkers for identifying microorganisms in clinical samples. 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), surface-enhanced Raman Scattering (SERS), and the commonly reported fluorescence-based methods, which make use of fluorescence resonance energy transfer (FRET) reporters and fluorogenic probes that can be turned on by target protease. 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. 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. Stevens and co-workers, for example, reported a protease detection method based on the redispersion 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. 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. Recently, Lin and co-workers 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.

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

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specific protease cleavage; (iii) a stabilizing part, (Glu)_k with multiple negative charges to stabilize AuNPs via electrostatic repulsion. 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.

In our proof-of-concept experiment, we used trypsin as a model enzyme. Peptide 1 with sequence EEEGGLGALGK 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†). 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. 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.

![Image](Image 344x240 to 527x396)

**Fig 1** Colorimetric assay of trypsin. (a) Colour changes induced by addition of trypsin-digested peptides (various concentrations of trypsins 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 625 nm (characteristic wavelength of aggregated AuNPs) were observed at the same time.† 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†).

Quantitative assay of trypsin activity was further studied by analysing the absorbance at 525 nm and 625 nm of each sample. The absorbance ratio of the two wavelengths (A_{525}/A_{625}) was used to assess the degree of AuNPs aggregation in our study. This method has been proven more accurate in previous reports. The ratio of absorbance, A_{525}/A_{625}, was plotted as a function of trypsin concentration indicating a near-linear correlation in the range of 0–70 nM.

![Image](Image 77x257 to 274x394)

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

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concentration of the enzyme in the range of 0 to 70 nM (y = 0.0071x + 0.2329, R² = 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 EEEGPGLAGGC 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 PLILAG, and cleaves at the C-terminus of the underlined glycine residue. 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.

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 cause AuNPs aggregation and absorbance spectroscopy.

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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.