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Real-time colorimetric screening of endopeptidase inhibitors using adenosine triphosphate (ATP)-stabilized gold nanoparticles

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ABSTRACT

The gold nanoparticles (AuNPs) that were stabilized with adenosine triphosphate (ATP) were stable over a wide range of pHs for the buffer, even in the presence of high concentrations of salt and protein. However, these stabilized AuNPs immediately aggregated when they were exposed to thiol-containing compounds, such as thiophenol. Endoprotease hydrolyzed the thioester bond in the CBZ–Phe–S–Ph substrate, and the hydrolyzed product (thiophenol) reacted with the AuNPs that were stabilized with ATP, causing them to aggregate, which in turn resulted in a visible color change in the AuNPs solution. This method enabled the real-time monitoring of the inhibition potencies of various endopeptidase inhibitors and the activity of endoprotease. This assay discriminated between the inhibition activities of various protease inhibitors for endoprotease on the basis of the color change of the assay solution.

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The plasmon absorption bands of gold nanoparticles (AuNPs) depend on their shape and size.[1] Typically, discrete AuNPs exhibit an absorption band with a high extinction coefficient, which is 3–5 orders higher than the absorption band of organic dye molecules, at around 520 nm. However, the typical absorption band corresponding to the aggregated AuNPs appears at longer wavelengths because of the intense color (blue-purple) of the NP solution. AuNPs have widely been used for the colorimetric detection of DNA sequences, proteins, and metal ions because of their high extinction coefficients and distance-dependent optical properties.[2]

Colorimetric enzyme assays using AuNPs have gained considerable attention in bioassay studies because of their simplicity, high sensitivity, and low cost. However, few colorimetric systems have been developed for the evaluation of the enzymatic activities.[3] A previous study showed that the colorimetric bioassays can be divided into two types corresponding to modified and unmodified AuNPs. In the first method, the aggregates of the AuNPs that are modified using the enzyme substrates are used to monitor the enzymatic activity. The AuNP substrate bond can be cleaved using a suitable enzyme in order to disperse the AuNPs in the solution.[3a–c] This process brings about a change in the color of the AuNPs solution, and therefore, the real-time monitoring of the enzymatic activities is possible. In the second method, the enzyme substrate and the unmodified AuNPs are used to monitor the enzymatic activities.[3d–f] The substrate is pretreated with the target enzyme and then exposed to the unmodified AuNPs. The activity of the target enzyme is monitored on the basis of the color change of the AuNPs that results from the two-stage process. Although the colorimetric assays that use unmodified AuNPs are simpler than the assays that use modified AuNPs, they cannot be used for the real-time monitoring of the activity of the target enzyme because the AuNPs are highly sensitive to various factors such as the electrolyte concentration, the pH of the buffer, and the protein concentration.[1,2a] Hence, a change in any of the above-mentioned parameters results in the irreversible aggregation of the unmodified AuNPs and a significant red shift in the absorbance spectrum. Therefore, the unmodified AuNPs are not easily used for the real-time monitoring of the activity of the target enzyme.

Recently, Zhao et al. reported that a real-time enzyme assay method using the ATP-stabilized AuNPs could be prepared by simply mixing the AuNPs with adenosine triphosphate. The stabilized AuNPs are stable in the buffer solution in the presence of high concentrations of salt and protein.[4] However, the stabilized AuNPs aggregate immediately when they are exposed to the enzyme that hydrolyzes the phosphate bond in ATP because of the change in the solubility of the stabilized AuNPs in buffer solution at higher salt concentrations. With this information on the properties of the ATP-stabilized AuNPs, an operationally simple colorimetric assay was examined for the simultaneous real-time monitoring of the endopeptidase activity and the estimation of the potency of the endopeptidase inhibitors. The ATP-stabilized AuNPs (henceforth referred to as the 'stabilized AuNPs (sAuNPs)') and an endoprotease substrate (**1**) were used in this assay. Endoprotease hydrolyzed the

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thioester bond in the CBZ–Phe–S–Ph substrate (**1**).[5] The hydrolyzed product (thiophenol) reacted with the sAuNPs, causing them to aggregate because of the change in the solubility of the sAuNPs, which in turn resulted in a visible color change in the AuNP solution (Scheme 1). The color change was used to screen the endopeptidase inhibitors and the endoprotease activity.

The stability of sAuNPs to **1** was dependent on the concentration of ATP and without α-chymotrypsin, the thioester reacted with the sAuNPs, causing them to aggregate because the gold surface catalyzed the thioester hydrolysis and then the hydrolysis product can attach to AuNPs.[3f] The stability of the sAuNPs to **1** was evaluated at various ATP concentrations in order to avoid the color change that was induced by **1** in the absence of α-chymotrypsin.[3f] When AuNPs were stabilized at ATP concentrations that were lower than 10 μM in the presence with **1**, the surface plasmon resonance (SPR) band of the sAuNPs changed in 30 min. However, for the AuNPs that were stabilized with ATP concentrations of more than 20 μM, the SPR band did not change after 30 min (Fig. 1A). Therefore, the AuNP substrates were prepared for the endoprotease assay by combining **1** (hydrolyzable substrate) with the sAuNPs that were stabilized by mixing ATP (30 μM) with the AuNPs (diameter: 13 nm).[4] The dispersion of the sAuNPs was confirmed using transmission electron microscopy (TEM; Fig. 1B) and the appearance of the SPR band at 520 nm in the UV–vis spectrum. However, the sAuNPs aggregated upon the addition of α-chymotrypsin to the solution (Fig. 1C), and the SPR band red shifted from 520 to 680 nm (see Supplementary data). Therefore, the thioester was hydrolyzed by the endopeptidase to produce the corresponding thiol, which then reacted with the sAuNPs.

This reaction caused the aggregation of the sAuNPs, causing the color change to purple and the red shift in the SPR band. This red shift is a well-known phenomenon and is used to confirm the formation of the nanoparticle aggregates.[1,2] The color change in the AuNPs was detected by the naked eye, and the extinction coefficients were measured from the UV–vis spectrum at 680 nm.

The AuNP substrates (sAuNPs + **1**) were used to evaluate the enzymatic activity of endopeptidase. In a typical experiment, known concentrations of α-chymotrypsin were added to a solution containing the AuNP substrates. The color of the AuNP solution gradually changed from red to purple upon the hydrolysis of the thioester in **1** with α-chymotrypsin. The quantitative analysis of the α-chymotrypsin-catalyzed hydrolysis of **1** was carried out by measuring the absorbance of the solution at 680 nm (Fig. 2). The reaction rate (K_{obs}) increased with the enzyme concentration, and this increase was monitored in real time. From these results, the detection limit for the method with endoprotease was estimated to be 0.38 nM (see Supplementary data), which was 13-fold better than the recently reported the AuNP-based colorimetric detection of endoprotease.[3f]

In addition to the screening of the enzyme activities, the proposed assay was used to evaluate the inhibitory potency of the α-chymotrypsin inhibitors. This method has been demonstrated using chymostatin, a selective and strong α-chymotrypsin inhibitor.[6] The stock solution of α-chymotrypsin was added to the assay mixture containing the sAuNPs (3 nM), the substrate (**1**, 50 μM), and various concentrations of chymostatin in a pH 7.0 buffer solution (10 mM phosphate buffered saline (PBS) + 0.1 M NaCl), with a final concentration of 0.48 nM. Figure 3 shows the enzymatic activity of α-chymotrypsin in the presence of various concentrations of chymostatin. In this figure, the enzymatic activity loss that was caused by chymostatin was monitored using the change in the UV–vis absorbance of the solution. From Figure 3, IC_{50} was about 26 nM for the inhibition of α-chymotrypsin by chymostatin (see Supplementary data). This value was similar to the previously reported value (IC_{50} = 10 nM).[6b]

The proposed assay was used to evaluate the relative activities of various protease inhibitors. In a typical assay, α-chymotrypsin was added to the control solution and the AuNP solutions containing one of the following protease inhibitors: chymostatin (1 μM, chymotrypsin-selective inhibitor), *N*-acetyl-Trp-Glu-His-Asp-al

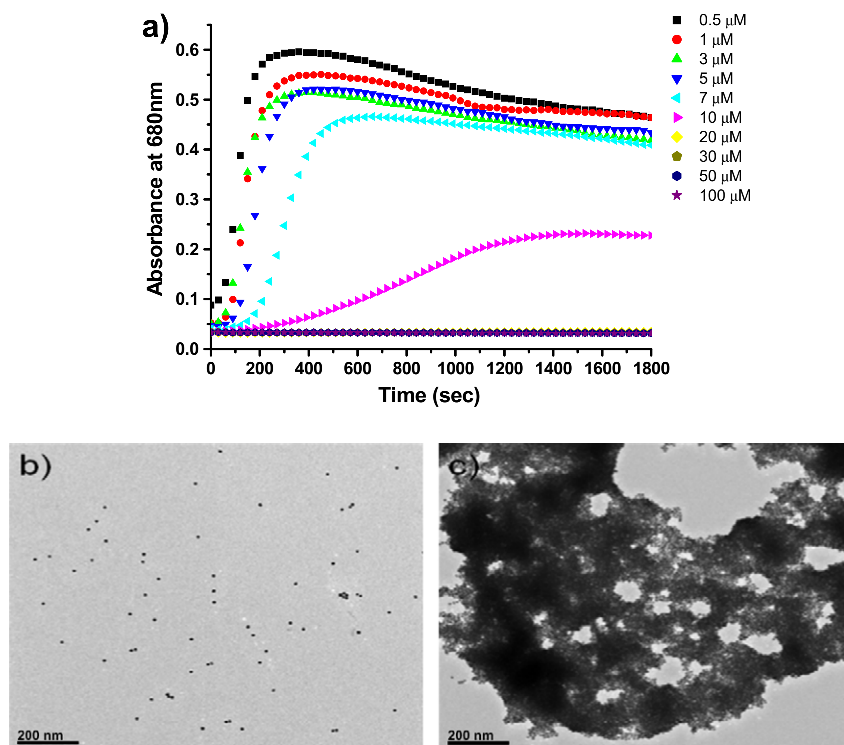


Figure 1. (a) Stabilities of the sAuNPs to **1** in the presence of various concentrations of ATP; (b) TEM image of the mixture of the 13 nm ATP-stabilized AuNPs and **1** at pH 7.0; (c) TEM image of the mixture after the addition of α-chymotrypsin. The scale bar represents 200 nm.

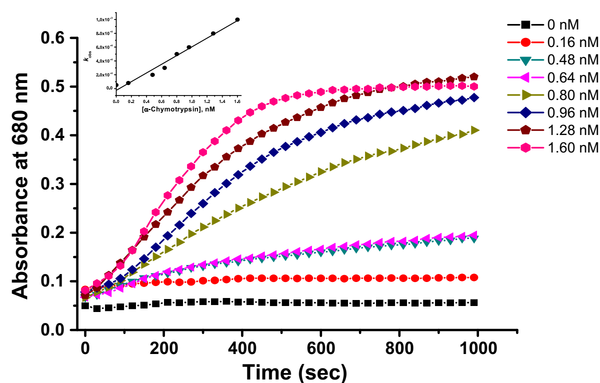


Figure 2. α-Chymotrypsin-catalyzed hydrolysis of 1 (50 μM) to thiophenol at pH 7.0 (10 mM PBS + 0.1 M NaCl; ATP: 30 μM) using the AuNPs (3 nM). The hydrolysis was monitored at an extinction wavelength of 680 nm. Inset: replot of the initial rate against the α-chymotrypsin concentrations (0, 0.16, 0.48, 0.64, 0.8, 0.96, 1.28, and 1.6 nM).

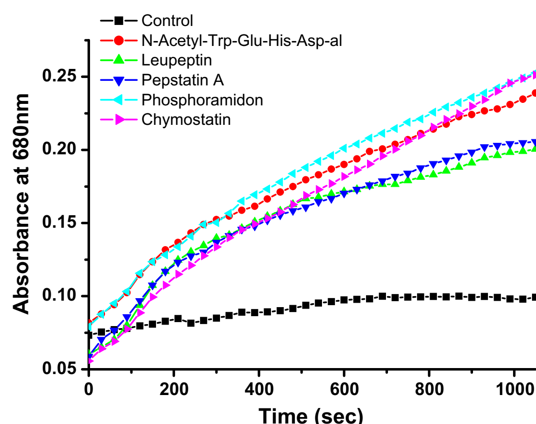


Figure 4. Inhibition of α-chymotrypsin by the endoprotease inhibitors. Plot of the UV absorbance at 680 nm against time. The increase in the absorbance intensity was caused by the addition of the endoprotease inhibitor (1 μM) to the pH 7.0 buffer solution (10 mM PBS + 0.1 M NaCl; ATP 30 μM) containing **1** (50 μM), α-chymotrypsin (0.48 nM), and AuNPs (3 nM).

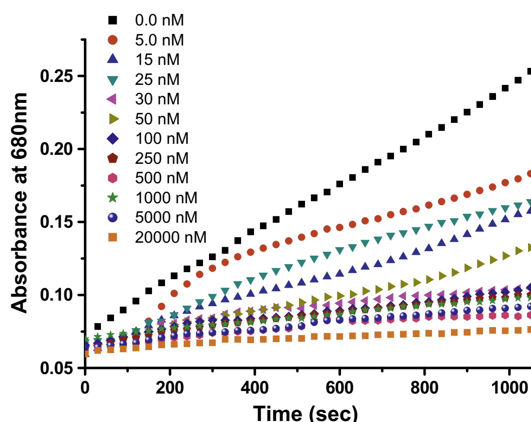


Figure 3. Inhibition of α-chymotrypsin by chymostatin, a selective α-chymotrypsin inhibitor. Plot of the UV absorbance at 680 nm against time. The absorbance intensity increased after the addition of chymostatin (final concentration of the assay mixture: 0–20 μM) to the pH 7.0 buffer solution (10 mM PBS + 0.1 M NaCl; ATP: 30 μM) containing **1** (50 μM), α-chymotrypsin (0.48 nM), and AuNPs (3 nM).

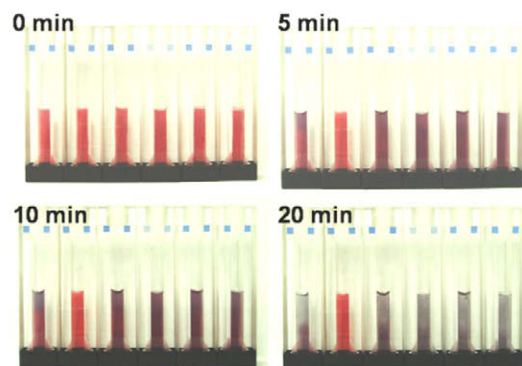
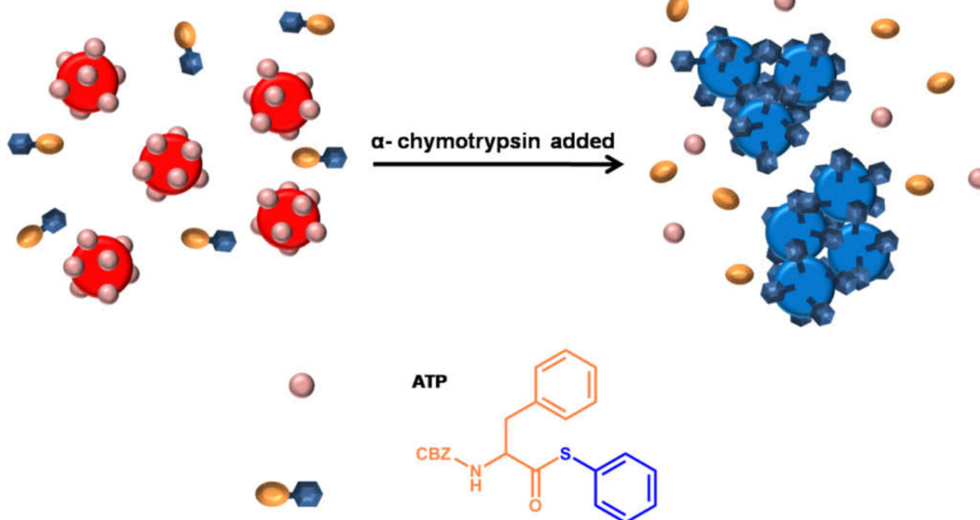


Figure 5. Color change of the AuNP substrates (**1** + sAuNPs) in the absence and presence of the endoprotease inhibitors (1 μM) at specific time intervals after the addition of α-chymotrypsin. (From left to right): control, chymostatin, *N*-acetyl-Trp-Glu-His-Asp-al, leupeptin trifluoroacetate salt, pepstatin A, and phosphoramidon disodium salt.



Scheme 1. Schematic illustration of the aggregation of the AuNP substrates in the colorimetric assay of the endopeptidase activity.

(1 μM, cysteine protease inhibitor),[7] leupeptin trifluoroacetate salt (1 μM, serine protease inhibitor),[8] pepstatin A (1 μM, aspartic protease inhibitor),[9] and phosphoramidon disodium salt (1 μM, metalloprotease inhibitor).[10] The inhibitor activity was monitored at 680 nm as a function of time (sample scan rate: 30 s^{-1}). The slope of the plot (rate) was used to measure the inhibition. Figure 4 shows that only chymostatin effectively inhibited α-chymotrypsin.

High throughput screening (HTS) is widely used to identify the hit compounds from combinatorial libraries.[11] The proposed assay was examined for the HTS of the α-chymotrypsin inhibitors. The catalytic hydrolysis of the substrate (**1**) in the assay solution with α-chymotrypsin brought about a color change from red to purple for the solution. Therefore, if α-chymotrypsin was successfully inhibited, the color of the solution remained unchanged. In Figure 5, the inhibition activities of various protease inhibitors for α-chymotrypsin were differentiated on the basis of the color change of the assay solution.

In conclusion, a new colorimetric assay was developed for screening endoprotease activities and determining the relative inhibitory potencies of the endoprotease inhibitors by monitoring the kinetics of the sAuNP aggregation. This screening method was simpler than the other assays based on the AuNPs and was used for the easy real-time monitoring of the inhibition potencies of various endopeptidase inhibitors. Furthermore, this assay was used for the qualitative and quantitative estimation of the inhibition. This assay can also be adapted to the HTS of potential drug candidates from large combinatorial libraries and important biological endoproteases by simply changing the thiol-bearing substrate.

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Supplementary data

Supplementary data (the experimental details for the colorimetric screening assay, the synthesis of CBZ–Phe–S–Ph (1), the UV–vis spectrum of the SAuNPs versus time in the presence of a-chymotrypsin, the detection limit of the assay method for α-chymotrypsin, and the IC_{50} value of chymostatin for the α-chymotrypsin EDX data of the SAuNPs) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2010.02.061.

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