Enzyme Activity

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Sensing Phosphatase Activity by Using Gold Nanoparticles**

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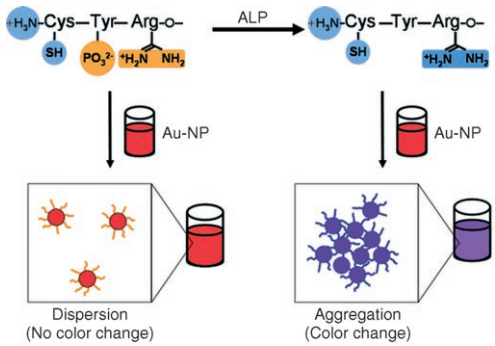
Phosphorylation and dephosphorylation play pivotal roles in cellular regulation and signaling processes.[1,2] Numerous inhibitors have been proposed or applied to regulate the process of phosphorylation/dephosphorylation.[3] A sensitive assay to report change of the phosphorylation state will be extremely valuable for biomedical applications. As the chemical difference in the process is only a phosphate group, developing a simple and sensitive assay is challenging. Herein we present a novel design of a gold-nanoparticle-based colorimetric assay to detect phosphatase activity. Gold nanoparticles (Au-NP) have interesting optical and electronic properties, that is, a strong surface plasmon resonance at around 520 nm in aqueous solutions, which is attributed to collective oscillations of surface electrons induced by incoming visible light.[4,5] The exact plasmon resonance band is dependant on the size, shape, surrounding medium, and distance of the particles.[6–8] Upon aggregation of Au-NP, a significant red shift in absorbance is observed. This colorimetric nano-gold-aggregation (NGA) phenomenon has been applied to several assays, such as solution-based immunoassays,[9] DNA-hybridization assay,[10] and glucose-sensing.[11] To form NGA, binding between two molecules, for example, antigen–antibody, complimentary oligonucleotides, or guest–host complexes, is needed. Recently, a new design to detect enzymatic activity by using the NGA phenomenon but not utilizing binding has been reported.[12] In that design, acetylated cysteine residues were added to both termini of the peptide substrate. As the cysteine could bind to Au-NP effectively, the intact peptide substrates cross-linked Au-NP through the two cysteine arms, resulting in colorimetric changes. Conversely, the proteolyzed peptide fragments, with only one cysteine residue, could not bridge Au-NP, resulting in no colorimetric change. Based on this NGA-induced absorption change, protease activity can be conveniently determined by measuring the reduction of aggregation. Recently, Levy et al. reported that not only the thiol group but also positively charged amine groups that have a strong affinity for gold.[13] Short peptides containing a cysteine and a lysine or an arginine residue at both ends of the peptide were reported to induce immediate aggregation when mixed with Au-NP. Learning from these prior studies, we believe that a novel NGA assay could be developed to detect the fate of the phosphate group. Taking the advantage of difference in charges, we hypothesize that the aggregation of Au-NP could be controlled by changing the interaction between the negatively charged phosphate group and the positively charged amino group.

To demonstrate the concept of charge-induced NGA assay, alkaline phosphatase (ALP) was used as a model enzyme. ALP removes the phosphate group efficiently and has been widely used as a biomarker in enzyme immunoassays and molecular biology.[14–16] As a proof-of-concept experiment, a short phosphorylated tripeptide, H_2N-Cys-Tyr(PO_3^{2-})-Arg-OH, was synthesized. The N-terminal cysteine residue was included for Au-NP binding. The positively charged guanidine group on the arginine side chain was arranged right next to the negatively charged phosphate group on the tyrosine side chain (Scheme 1) to prohibit the binding between the positively charged guanidine group and Au-NP. Au-NP would stay dispersed upon mixing with the initial phosphorylated peptide. When the corresponding enzyme (i.e., alkaline phosphatase) removed the negatively charged phosphate group from the peptide, aggregation was triggered upon addition of the resulting dephosphorylated peptide into the Au-NP suspension. The color of the Au-NP suspension would depend on the amount of dephosphorylated peptide, which enables qualitative and quantitative measurement of enzymatic activity.



Scheme 1. Alkaline phosphatase (ALP) assay based on color changes during peptide-induced Au-NP aggregation. Negatively charged amino acids (i.e., phosphotyrosine) in the short peptide act as a molecular switch for Au-NP aggregation. The phosphate group prohibits binding between the guanidine group on the arginine side chain and Au-NP, thereby only the cysteine residue can interact with gold. When the negatively charged phosphate group is removed from the peptide by enzymatic hydrolysis, the resulting dephosphorylated short peptide bridges Au-NP and induces Au-NP aggregation.

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To monitor colormetric changes, H_2N-Cys-Tyr(PO_3^{2-})-Arg-OH (54 μM) was initially incubated with different amounts of alkaline phosphatase (0, 1.7, 3.4, and 17 μM) in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer solution (10 mM; pH 7.4, 2 mM $MgCl_2$). After 30 min of enzyme treatment, the resulting peptide solution (210 μL) was added to the Au-NP suspension (800 μL, 20-nm size, 1.16 nM) to induce Au-NP aggregation. As shown in Figure 1a, the solution color of the Au-NP alone remained unchanged with time, indicating that the Au-NP is stable under these conditions. When the intact phosphorylated peptide was added, a slight darkening of the solution was observed, but the color stayed constant with time. This result suggested that the phosphorylated peptide was stable under the experimental conditions, which has been further confirmed by HPLC analysis (data not shown). In contrast, in the presence of the peptide and ALP, the color of the Au-NP solution changed from pink-red to violet-blue. Darker color was observed with higher concentration of ALP and longer incubation time. This was presumably caused by the increased amount of dephosphorylated peptide produced by ALP.

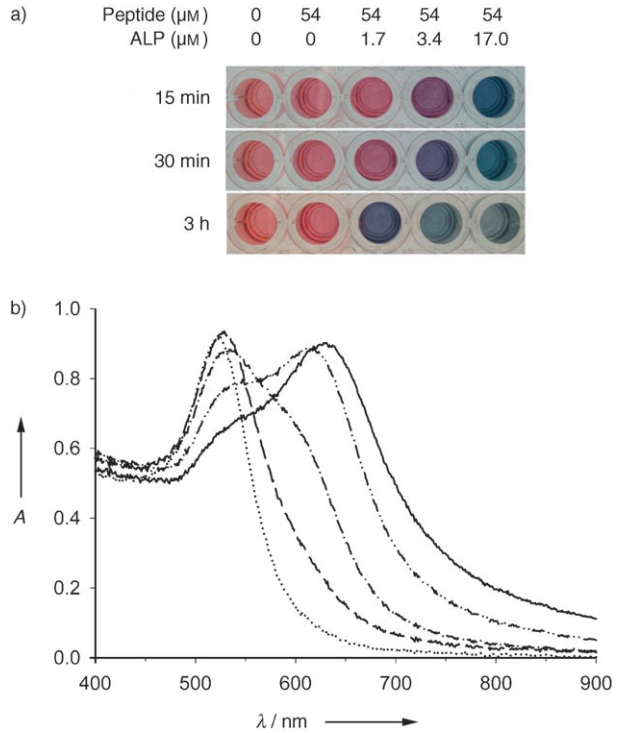

Figure 1. Colorimetric assay of ALP. a) Color of the Au-NP solution with or without peptide addition. The peptide was first incubated with ALP at different concentrations for 30 min. The reaction solution was then mixed with the Au-NP suspension, and the color of Au-NP was observed at 15 min, 30 min, and 3 h. b) UV/Vis spectra of Au-NP solutions at the 30-min time point of the Au-NP aggregation test. Au-NP suspension without peptide (••••); Au-NP suspension mixed with peptide treated with 0 μM ALP (-----), 1.70 μM ALP (–•–•), 3.40 μM ALP (–••–••), and 17 μM ALP (——) for 30 min.

Spectroscopically, significant changes in UV/Vis absorption were identified 30 min after mixing the enzyme-treated peptide with Au-NP (Figure 1b). Both a decreased absorbance of the plasmon band at 520 nm and an increased absorbance at 650 nm were observed for the ALP-treated samples. In particular, the shifted absorbance to longer wavelength with increasing ALP concentration correlated well with the color changes seen in Figure 1a. These findings support our hypothesis that observation of color changes can be used for qualitative analysis of phosphatase activity.

Detection of enzyme activity was further studied under the same condition with different amounts of ALP (0, 0.034, 0.34, 0.85, 1.7, 3.4, 5.1, 6.8, 10.2, 13.6, and 17 μM). As an indicator of Au-NP aggregation, absorbance at 650 nm was measured every 2 min after mixing the enzyme-treated solution with the Au-NP suspension (Figure 2a). As previously observed, the negative control (peptide alone) caused minimum changes in absorbance with time. ALP without the peptide did not change the absorbance at 650 nm either (see the Supporting Information). Only the Au-NP samples mixed with ALP-treated peptide showed an increase in the absorbance immediately after mixing with peptide solutions. The kinetics of Au-NP aggregation was observed to be faster with a higher concentration of ALP, indicating that more dephosphorylated peptide induced more aggregation. In addition, a

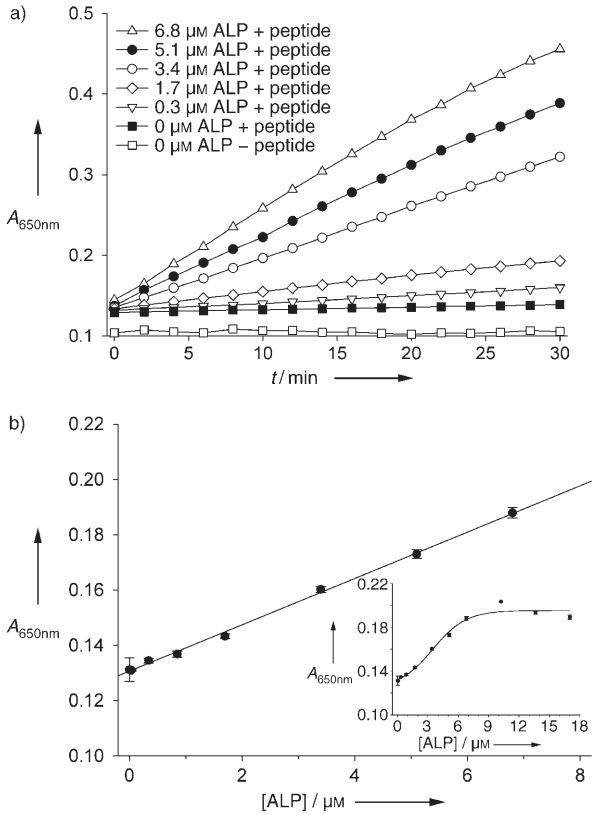

Figure 2. a) Aggregation kinetics of Au-NP after addition of the ALP-treated peptide solution. Representative kinetic profiles of Au-NP aggregation are shown. b) ALP concentration versus absorbance at 650 nm of an Au-NP suspension recorded 4 min after the addition of the ALP-treated peptide solution. The inset shows the plateau observed in the absorbance at ALP concentrations higher than 6.8 μM.

near-linear correlation between the enzyme concentration and the absorbance (Figure 2b, $r^2 = 0.99$) was observed in the range of 0–6.8 μM at the 4-min time point. However, the absorbance reached a plateau when the enzyme concentration was higher than 6.8 μM (Figure 2b, inset).

Based on this observation, we thought that enzyme inhibition could also be determined by using this simple NGA assay. To demonstrate this application, a potent ALP inhibitor, *p*-bromotetramisole oxalate (*p*-BO),[17,18] was selected and the inhibition test was performed by using the currently optimized method. ALP was preincubated with different amounts of *p*-BO (0.0025, 0.25, 5, 25, 50, 250, and 1000 μM) in 20 μL HEPES buffer solution for 22 min at room temperature. The peptide substrate was then added to the *p*-BO-pretreated ALP solution and incubated for an additional 30 min. The final concentrations of ALP and peptide in the solution were 5.1 μM and 54 μM, respectively. After incubation, the reaction solution was mixed with Au-NP and the absorbance at 650 nm was measured with time as mentioned above. A representative kinetic aggregation of the Au-NP solution is shown in Figure 3a. When the absorbances at the 4-min time points versus the *p*-BO concentration were plotted, a sigmoidal profile was obtained (Figure 3b). The IC_{50} was found to be about 40 μM, which was similar to a previously observed value.[17]

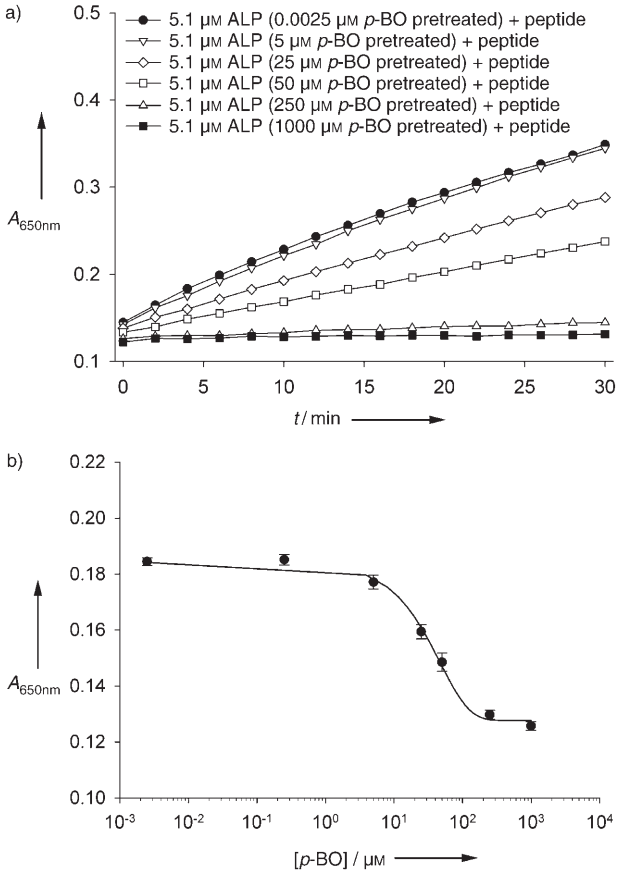

Figure 3. Inhibition assay of ALP activity using Au-NP aggregation. A) Representative kinetic profiles of Au-NP aggregation after addition of the ALP-treated peptide solutions. ALP was pretreated with inhibitor (*p*-BO) at different concentrations before being mixed with the peptide. b) *p*-BO concentration versus absorbance at 650 nm recorded 4 min after mixing the ALP-treated peptide solution with the Au-NP suspension.

In summary, we have demonstrated a unique design of a NGA enzymatic assay. The Au-NP aggregation was tuned by shielding the charge interaction between amino acids and Au-NP. This is a quick and simple assay that does not require individually functionalized Au-NP or specialized instrumentation. Enzyme concentration can be conveniently measured by a single readout. The described ALP assay is only used as an example to prove the concept of the NGA assay. Several other enzymatic assays could be developed by using similar peptide substrates. For example, the same peptide substrate should be useful to sense tyrosine phosphatase; replacing the phosphate group with a sulfonate group could be a useful substrate for estrogen sulfatase. With more sophisticated design on the peptide substrate sequences, detecting other enzymes could also be possible. Potentially, the described NGA assay might become a valuable tool for enzyme sensing and drug screening.

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