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Detection of Kinase Activity Using a Synthetic System of Gold Nanoparticles in HEPES Buffer

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The reduction of tetrachloroaurate(III) ions ($AuCl_4^-$) with 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) was used to detect protein kinase A activity. The phosphorylation of substrate peptides was identified by changes in the solution colors resulting from the aggregation of synthesized gold nanoparticles in HEPES buffer.

The highly sensitive detection of enzymatic activities is crucial for the development of drug discovery and diagnosis. In particular, colorimetric detection based on the synthesis and aggregation of gold nanoparticles (AuNPs) is known to be a simple but sensitive method because of its exquisite photochromic properties, stability, and low toxicity.[1] The visual detection system using AuNPs was based on changes in solubilities, sizes, and/or shapes before and after enzyme treatment.[2]

Recently, it has been reported that HEPES, a Good's buffer, was frequently used as a reducing agent of tetrachloroaurate(III) ions ($AuCl_4^-$) to Au(0) under extremely mild conditions (ambient temperature and neutral pH) via the oxidation of its piperazine ring to a N-centered cationic free radical, producing branch-shaped AuNPs capped with anionic HEPES.[3] We successfully developed a method for the visual and defined detection for the activities of an enzyme, alkaline phosphatase (ALP), based on the synthesis of well-dispersed AuNPs or Au precipitates in HEPES buffer containing its substrate or product using de novo designed dipeptide and adenosine triphosphate (ATP) substrates.[4] HEPES reduction triggered by adding $AuCl_4^-$ into the solution before and after dephosphorylation under extremely mild conditions without the preparation of well-designed AuNPs makes this novel assay simple and versatile. However, ALP has lower specificities against the substrates and needs no additive biological regents. To demonstrate whether a system based on synthesis of AuNPs can generally detect enzymatic activity, other enzymes with substrate specificities and complex biological fluids are required. For this purpose, protein kinase A (PKA) is the best candidate, because it requires ATP and magnesium chloride ($MgCl_2$) for enzymatic activity.

Here, we report the detection of enzyme activity based on the substrate- or product-dependent synthesis of AuNPs in HEPES buffer. Highly cationic substrate peptides of PKA (S6, sequence: RRRLSSLRA, *N*- and *C*-termini were free and amidated, respectively) were used in this system, such that phosphorylation produced a slightly cationic phosphorylated S6 (pS6). It was anticipated that the HEPES-capped AuNPs would grow slower with S6 due to the strong electrostatic interactions between the peptide and the AuNPs. In other words, growth of the AuNPs might be inhibited by the peptide. In contrast, the AuNPs synthesized with the product, pS6, would grow faster because of the weak interactions between the peptides and the AuNPs. Importantly, despite the fact that the phosphorylation of PKA requires complex biological additives, the detection of the enzymatic activities was achieved (Figure 1).

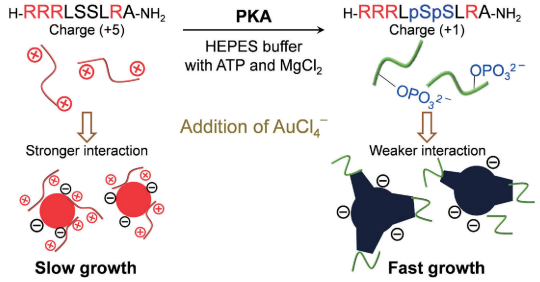

Figure 1. A schematic illustration of the detection of PKA activities based on the synthesis of AuNPs in HEPES buffer.

$AuCl_4^-$ (0.5 mM) was reduced in HEPES buffer (100 mM, pH 7.2) containing substrate S6 (final concentration: 5 μM) at 25 °C for 30 min before (without PKA) and after PKA treatment (400 U mL^{-1}, 37 °C, 1 h, containing 2 mM ATP and 10 mM $MgCl_2$). Solutions with the pale violet color of AuNPs with a λ_{max} at 600 nm were obtained before the PKA treatment (Figure 2a). It has been reported that solutions of branch-shaped AuNPs capped with HEPES showed a λ_{max} at 680 nm and weak absorption at 520 nm, which were attributed to the major and minor axes of the branches, respectively.[5] Therefore, positively charged S6 might strongly inhibit the growth of the branched structures, suggesting that the S6 coadsorbed onto the AuNP surfaces with HEPES molecules.

In contrast, dark violet solutions of AuNPs with a λ_{max} at 560 nm were observed after the PKA treatment (Figure 2b). The absorbance of the solutions after the PKA treatment was much higher than before the treatment, demonstrating well-dispersed AuNPs. It was suggested that the interactions of the anionically charged HEPES-capped AuNPs with the product pS6 became weaker than with the substrate S6. Additionally, the λ_{max} (560 nm) was different from that of the HEPES-capped AuNPs (branched shape, 680 and 520 nm) and before the PKA treatment (600 nm), implying that each synthesized AuNP had different structures such as sizes and morphologies. The mass spectra of the concentrated solutions after the PKA treatment provided the molecular weight of the completely phosphorylated substrate pS6 (data not shown), supporting the adsorption of pS6

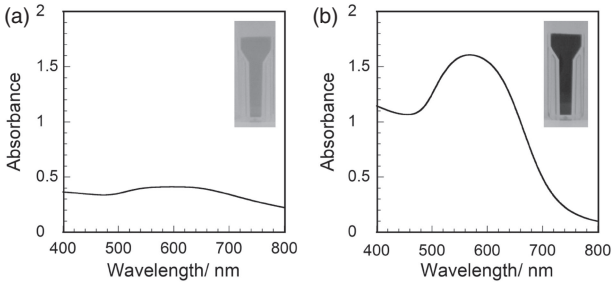

Figure 2. Ultraviolet–visible spectra and photographs of solutions (a) before and (b) after the PKA treatment.

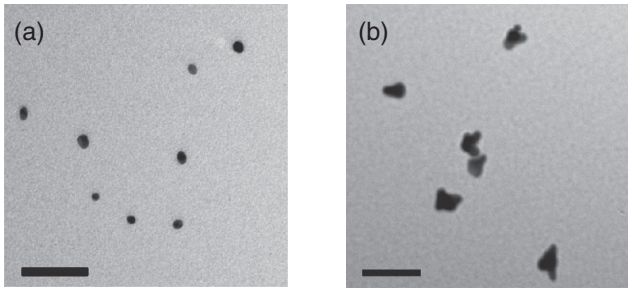

Figure 3. TEM images of AuNPs synthesized (a) before and (b) after PKA treatment with the substrate, S6 (500 μM). Each scale bar represents 50 nm.

phosphorylated by PKA onto the AuNPs. To understand these differences, additional experimental evidence was collected.

The changes in the solutions before and after the PKA treatment may be due to possible contributions of ATP, $MgCl_2$, or PKA. Accordingly, control experiments were performed to determine the effect of the PKA treatment. The HEPES reductions of $AuCl_4^-$ with ATP, $MgCl_2$, or PKA were then carried out. As a result, the interactions of the substrates with the AuNPs were stronger than with ATP, $MgCl_2$, and PKA, demonstrating that the difference in the solutions before and after PKA treatment was dependent on the structural changes of the substrates (Figure S1[6]). The visual difference in the AuNP solutions before and after treatment clearly demonstrated that the HEPES reduction system generally detects enzymatic activities regardless of the substrate specificities and requirement for complex biological fluids.

The supernatants before and after the PKA treatment at various concentrations of S6 were then transferred to microtiter plates, and photographs of the plates were taken with an image scanner (Figure S2[6]). The dependence on the substrate concentration was clearly visualized. The supernatants before the PKA treatment were clear at $>5\,\mu M$, whereas those after the treatment were pale violet due to the faster growth of the AuNPs attributed to the changes in the interactions with the substrate peptides. Accordingly, this HEPES reduction system successfully detected PKA activity depending on the substrate concentrations. Moreover, the absorbance of the AuNP solutions generated with chemically synthesized pS6 (1.54 ± 0.10) was the same as that of solutions generated with the PKA-treated S6 (1.53 ± 0.05), demonstrating the detection of completely phosphorylated peptides. Furthermore, the absorbance values at λ_{max} for various concentrations of PKA were also measured (Figure S3[6]). The larger absorbance values indicate progression in the enzyme reactions due to faster growth of the AuNPs. The values increased at $50\,U\,mL^{-1}$ and were dependent on the concentrations of PKA. Thus, the detection system using the AuNPs can estimate the enzyme concentration.

Figure 3 shows transmission electron microscopic (TEM) images of AuNPs synthesized at the substrate concentration of 5 μM, which is the threshold for detection. Sphere-shaped AuNPs of ca. 10 nm were observed before the PKA treatment (Figure 3a). The structures were more isotropic and smaller than those capped with HEPES, suggesting that the growth of the AuNPs was inhibited by the strong interactions with highly cationic S6 adsorbed onto the AuNPs. On the other hand, branch-shaped AuNPs of ca. 20 nm were observed after the PKA treatment. The branches were slightly shorter than those of conventional HEPES-capped AuNPs, suggesting the coadsorption of HEPES and pS6 onto the AuNPs. As a result, the growth of the AuNPs was modestly inhibited. These observations agree with the changes in the λ_{max} described above. These results suggested that highly positive S6 binds strongly to the anionically charged AuNPs, whereas the less positive pS6 binds weakly to the AuNPs. In fact, the adsorbed amounts of S6 and pS6 were quantified by using a fluorescamine method, and the amount of S6 was much greater than that of pS6 (Table S1[6]).

In conclusion, we successfully constructed the detection system using the synthetic system of AuNPs in HEPES buffer for PKA activity that requires specificity against substrates and complex biological fluids. Ultraviolet–visible spectral analyses and TEM observations revealed that the AuNPs synthesized by the HEPES reduction system before and after the PKA treatment clearly showed different morphologies. The substrate before the PKA treatment (net charge: 5) bound strongly to anionically charged AuNPs through electrostatic interactions, resulting in considerable inhibition of the AuNP growth. In contrast, the product after PKA treatment (net charge: 1) bound weakly to the AuNPs and modestly inhibited the growth. Our synthetic system does not require a well-designed substrate for the detection of enzymatic activities. Furthermore, the system can act with whatever complex biological additives required for enzymatic activities. Therefore, HEPES reduction systems triggered by adding $AuCl_4^-$ into the solutions before and after enzymatic reactions under extremely mild conditions can be used as a general detection system for enzymatic activity.

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6 Supporting Information is available electronically on the CSJ-Journal Web site, http://www.csj.jp/journals/chem-lett/index.html.

