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Enzyme Colorimetric Assay Using Unmodified Silver Nanoparticles

Hui Wei, Chaogui Chen, Bingyan Han, and Erkang Wang*

State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, Jilin 130022, P. R. China, and Graduate School of the Chinese Academy of Sciences, Beijing 100039, P. R. China

Colorimetric assay based on the unique surface plasmon resonance properties of metallic nanoparticles has received considerable attention in bioassay due to its simplicity, high sensitivity, and low cost. Most of colorimetric methods previously reported employed gold nanoparticles (GNPs) as sensing elements. In this work, we develop a sensitive, selective, simple, and label-free colorimetric assay using unmodified silver nanoparticle (AgNP) probes to detect enzymatic reactions. Enzymatic reactions concerning adenosine triphosphate (ATP) dephosphorylation by calf intestine alkaline phosphatase (CIAP) and peptide phosphorylation by protein kinase A (PKA) were studied. In the absence of the enzymes, unreacted ATP could protect AgNPs from salt-induced aggregation, whereas in the presence of the enzymes, the reaction product of ATP (i.e., adenosine for CIAP and ADP for PKA) could not. Via our method, dephosphorylation and phosphorylation could be readily detected by the color change of AgNPs, with a detection limit of 1 unit/mL for CIAP and a detection limit of 0.022 unit/mL for PKA. More importantly, the enzymatic inhibition by inhibitors and enzymatic activity in complex biological fluids could also be realized. This work is an important step toward a colorimetric assay using AgNPs and might provide a promise for enzyme assay in realistically complex systems and for screening of different enzyme inhibitors in future.

In the past decades, intense research has been focused on nanomaterials due to both their both scientific and potential economic importance.[1,2] Besides continuous efforts in developing various fabrication approaches to nanomaterials, considerable progress has been made in the field of bioassays based on nanomaterials.[3,4] Among all the nanomaterials explored in the bioassay, gold nanoparticles (GNPs) have received much consideration owing to their intrinsic characteristics such as ease of preparation, biocompatibility, catalytic activity, stability, and excellent optical properties.[3–5]

Varieties of colorimetric sensors for different analytes including DNA, small molecules, metal ions, carbohydrates, and proteins have been developed using GNPs' unique surface plasmon resonance (SPR) as sensing elements.[6–27] GNP-based colorimetric sensors rely on the fact that the dispersed GNP solution is red whereas the aggregated GNP solution is purple (or blue).[3,5] The advantages of GNP-based colorimetric sensors include the following: (a) simplicity, (b) high sensitivity due to GNPs' extremely high extinction coefficients, and (c) low cost due to eliminating the use of specific analytical instruments. These colorimetric sensors can be generally divided into two kinds according to the

* To whom correspondence should be addressed. Fax: (+86) 431-85689711. Tel: (+86) 431-85262003, E-mail: ekwang@ciac.jl.cn.

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GNPs used: one uses modified GNPs (type I) and the other uses unmodified GNPs (type II). Mirkin et al. pioneered type I sensors employing DNA-modified GNP conjugates.[6] Other sensors have also been fabricated using GNPs functionalized with appropriately designed ligands.[15,25,28] Rothberg and co-workers reported type II sensors employing unmodified GNPs.[9,10] Recently, type II sensors have been extended to the detection of metal ions, small molecules, and proteins.[14,19,29] These GNP-based colorimetric sensors could provide comparable or even better sensitivity and selectivity to conventional molecular fluorescent ones.[3,9,13,16,28,30,31]

Silver nanoparticles (AgNPs) are also of significantly scientific and technological importance to biological detection.[32–36] By monitoring shifts in their SPR after binding of the analytes, triangular silver nanoparticles generated via nanosphere lithography have been used to detect streptavidin, antibiotin, and amyloid β-derived diffusible ligands.[37–40] GNPs with silver enhancement explored by Mirkin et al. have been extensively studied and used in Raman, electrical, and colorimetric bioassays.[32,41] As for colorimetric assays, AgNPs have some advantages over GNPs to a certain degree since they possess higher extinction coefficients relative to GNPs of the same size.[42] However, compared with GNPs, little attention has been paid to AgNP-based colorimetric assays though sensitive and selective colorimetric detection of DNA,[43,44] metal ions,[45] and proteins[46] have been reported using AgNPs functionalized with appropriately ligands. These limitations can be attributed to the facts that (a) the functionalization of AgNPs usually causes chemical degradation and (b) the AgNPs' surface could be easily oxidized. Recently, extremely stable AgNPs−DNA conjugates based on DNA with triple cyclic disulfide moieties have been successfully prepared.[42] Similar to GNPs−DNA conjugates, these AgNPs−DNA conjugates exhibited distant-dependent optical properties and highly cooperative binding properties and could be used as sensing elements in colorimetric assay.

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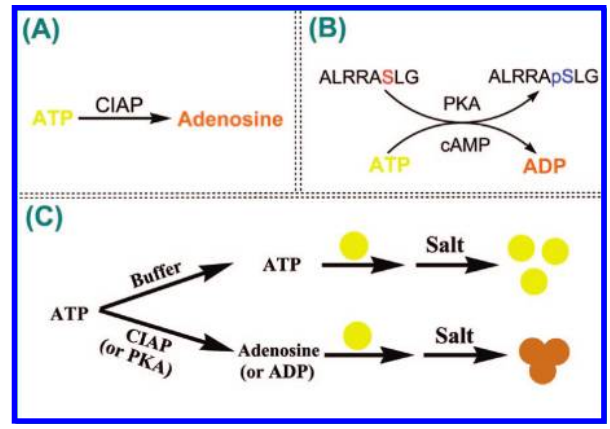

Figure 1. Enzymatic reactions with CIAP (A) and PKA (B), and AgNP-based enzyme colorimetric assay (C).

Until now, all AgNP-based colorimetric assays developed employed AgNPs functionalized with designed ligands. Herein, we present a label-free, sensitive, selective, and simple enzyme colorimetric assay using unmodified AgNPs based on the fact that the dispersed AgNP solution is yellow whereas the aggregated AgN solution is pale red (or brown). Enzymatic reactions concerning dephosphorylation and phosphorylation, specifically adenosine triphosphate (ATP) dephosphorylation by calf intestine alkaline phosphatase (CIAP) and peptide phosphorylation by protein kinase A (PKA) (Figure 1), were chosen as model systems due to their significant importance to cellular regulation, cellular signaling, and biomedical applications.[47,48] In the absence of the enzymes, unreacted ATP could protect AgNPs from salt-induced aggregation, whereas in the presence of the enzymes, the reaction product of ATP (i.e., adenosine for CIAP and ADP for PKA) could not (Figure 1). With our method, dephosphorylation and phosphorylation could be sensitively and selectively detected by the color change of AgNPs, thereby the protein assay is realized. At the same time, common steps such as functionalization and separation could be successfully avoided, which could potentially broaden the applicability of AgNP colorimetric sensors in the future.

EXPERIMENTAL SECTION

Chemicals and Materials. Silver nitrate, sodium citrate, sodium orthovanadate (Na_3VO_4), and sodium chloride were purchased from Beijing Chemical Reagent Co. (Beijing, China). Sodium borohydride, ATP, adenosine diphosphate (ADP), cyclic adenosine monophosphate (cAMP), adenosine, *N*-2-hydroxyethylpiperazine-*N*′-2′-ethanesulfonic acid (HEPES), bovine serum albumin (BSA), α-thrombin, trypsin, and protein kinase A from bovine heart (PKA) were purchased from Sigma-Aldrich (Milwaukee, WI). CIAP was purchased from TaKaRa Biotechnology Co. Ltd. (Dalian, China). Fetal bovine serum was obtained from Beijing Dingguo Biotechnology Co. Ltd. (Beijing, China). PKA substrate H-Leu-Arg-Arg-Ala-Ser-Leu-Gly-OH (peptide **1**) and the corresponding phosphorylated peptide H-Leu-Arg-Arg-Ala-Ser(PO_3H_2)-Leu-Gly-OH (peptide **1′**) were synthesized by GL Biochem. Co. Ltd. (Shanghai, China). Other reagents and chemicals were at least analytical reagent

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grade. The water used throughout all experiments was purified by a Milli-Q system (Millipore, Bedford, MA).

The 1× reaction buffer for CIAP was 50 mM Tris-HCl (pH 9.0) containing 1 mM $MgCl_2$. The reaction buffer for PKA was 50 mM HEPES (pH 7.2).

Note: One unit of CIAP is defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of 4-nitrophenyl phosphate/min at pH 9.8 and 37 °C. One unit of PKA is defined as the amount of enzyme that transfers 1.0 pmol of phosphate from γ-^{32}P-ATP to hydrolyzed and partially dephosphorylated casein per minute at pH 6.5 and 30 °C in the presence of cAMP.

Preparation of Citrate-Stabilized AgNPs. The AgNPs were prepared via a previously reported method.[49] Briefly, 250 μL of 100 mM $AgNO_3$ and 250 μL of 100 mM trisodium citrate were added into 100 mL of water under stirring. Then 6 mL of freshly prepared 5 mM $NaBH_4$ was added into the above aqueous solution under vigorous stirring. The resulting yellow colloidal silver solution was further stirred for 30 min and then was left undisturbed overnight.

Colorimetric Detection of CIAP. A typical colorimetric analysis was realized as following procedure (referred to as test I): First, 5 μL of 5 mM ATP and 5 μL of 1 unit/μL CIAP (in buffer) were added into 40 μL of 1× reaction buffer. The mixed reaction solution was incubated in a 37 °C water bath for 20 min. Second, 200 μL of AgNPs was added into the incubated solution, and then 15 μL of 0.5 M NaCl was added to produce the color change. Finally, 250 μL of the resulting CIAP/ATP(adenosine)/AgNPs/NaCl solution was mixed with 750 μL of water to record absorption spectra.

To examine the influence of incubation time on the colorimetric analysis, enzymatic reactions incubated for different reaction times were investigated, under conditions identical to those used for test I.

To examine the influence of the amounts of CIAP on the colorimetric analysis, various amounts of CIAP were investigated, under conditions identical to those used for test I.

To examine the specificity of CIAP catalytic enzymatic reaction, BSA, α-thrombin, and trypsin were investigated instead of CIAP, under other conditions identical to those used for test I.

Inhibition Assay of CIAP. Enzymatic inhibition assay was conducted as follows: First, 5 μL of 1 unit/μL CIAP and 5 μL of various concentrations of Na_3VO_4 were mixed and incubated in a 37 °C water bath for 10 min. Second, 5 μL of 5 mM ATP was added to the mixture, and the resulting mixed solution was incubated in a 37 °C water bath for different reaction times. Third, 200 μL of AgNPs was added into the incubated solution, and then 15 μL of 0.5 M NaCl was added to produce the color change. Finally, 250 μL of the resulting CIAP/ATP/Na_3VO_4/AgNPs/NaCl solution was mixed with 750 μL of water to record absorption spectra.

CIAP Assay in Biological Fluids. CIAP samples in biological fluids were prepared by mixing 5 μL of CIAP of different concentrations and 5 μL of 1% fetal calf serum. Then 10 μL of the biological sample was added into 20 μL of 10 mM ATP and 35 μL of 1× reaction buffer. The mixed reaction solution was incubated in a 37 °C water bath for 10 min. The 200 μL of AgNPs was added into the incubated solution, and 60 μL of 0.5 M NaCl was added to produce the color change. Finally, 250 μL of the resulting CIAP/ATP(adenosine)/AgNPs/NaCl solution was mixed with 750 μL of water to record absorption spectra.

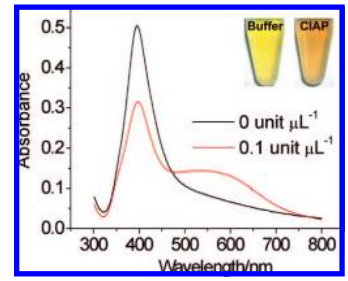

Figure 2. Typical absorption spectra and photographs (inset) of AgNPs mixed with 0.5 mM ATP in the absence and presence of 0.1 unit/μL CIAP.

Colorimetric Detection of PKA. A typical colorimetric analysis was realized as the following procedure (referred to as test II): First, 10 μL of 2 mM peptide **1**, 2.5 μL of 1 mM cAMP, 2.5 μL of 1 mM $MgCl_2$, 2.5 μL of 10 mM ATP, and 5 μL of PKA of different concentrations were mixed and incubated in a 30 °C water bath for 2 h. Second, 100 μL of AgNPs was added into 10 μL of the incubated solution, and the resulting mixed solution was incubated in a 30 °C water bath for 10 min to produce the color change. Finally, 100 μL of the resulting PKA/peptide **1** (or peptide **1′**)/AgNP solution was mixed with 800 μL of water to record absorption spectra.

Instrumentation. Absorption spectra were recorded on a Cary 500 scan UV−vis−NIR spectrophotometer (Varian, Harbor City, CA).

RESULTS AND DISCUSSION

Colorimetric Detection of CIAP. To testify that our unmodified AgNPs could be readily used in an enzymatic activity assay as colorimetric sensing probes, the catalytic hydrolysis of ATP by CIAP was first investigated (Figure 1). Figure 2 shows a typical colorimetric analysis of CIAP. The AgNP colloidal solution mixed with the reaction solution of ATP in the presence of CIAP changed from yellow to pale red when 15 μL of 0.5 M NaCl was added, while the one in the absence of CIAP remained yellow after adding the same amount of salt. Through this color change phenomenon, the presence of CIAP could be directly observed with the naked eye, realizing the detection of ATP dephosphorylation by CIAP in a very convenient way.

To quantitatively detect CIAP using our AgNP colorimetric approach, absorption spectra of AgNPs in the absence and presence of different concentrations of CIAP after addition of 15 μL of 0.5 M NaCl were recorded (Figures 2, 3, and Supporting Information Figure S1). As shown in Figure 3, the A_{550}/A_{397} values of solutions (i.e., the color change of the AgNPs) were a sensitive function of CIAP concentration. A detection limit of 1 unit/mL for CIAP could be obtained, which is as low as the GNP-based colorimetric method previously reported.[21]

To examine the specific detection of the CIAP catalytic enzymatic reaction using our unmodified AgNP probes, control experiments were taken using BSA, α-thrombin, and trypsin. As shown in Figure 4 (also see Supporting Information Figures S2−S5), as high as 25 μM BSA, 10 μM α-thrombin, and 2.1 μM

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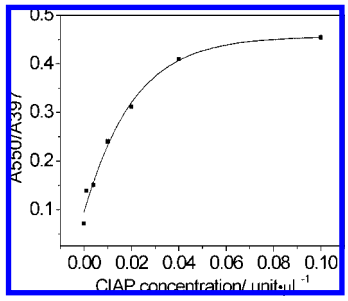

Figure 3. Absorption ratio (A_{550}/A_{397}) vs CIAP concentration.

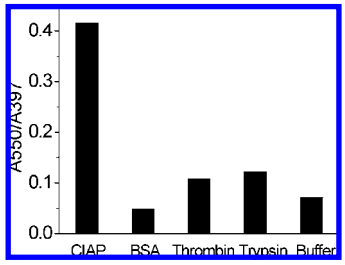

Figure 4. Absorption ratio (A_{550}/A_{397}) of AgNPs mixed with 0.5 mM ATP in the presence of different proteins (200 nM (0.1 unit/μL) CIAP, 25 μM BSA, 10 μM α-thrombin, and 2.1 μM trypsin).

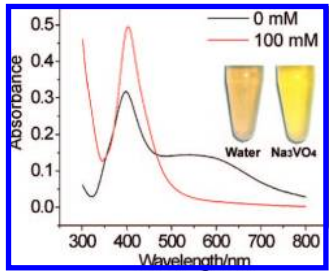

Figure 5. Typical absorption spectra and photographs (inset) of AgNPs in the absence and presence of 100 mM Na_3VO_4.

trypsin did not exhibit a significant response while 200 nM CIAP could give a good detectable signal. Thus, the colorimetric approach developed here using unmodified AgNP probes showed good selectivity toward CIAP detection.

Inhibition Assay of CIAP. The colorimetric approach developed was further used to evaluate the enzymatic inhibition by Na_3VO_4, a well-known inhibitor for CIAP.[21] When the activity of CIAP was inhibited by Na_3VO_4, the ATP could not be dephosphorylated by CIAP; thus, the unreacted ATP could protect AgNPs from salt-induced aggregation and the AgNP colloidal solution appeared yellow (Figure 5). It was found that 5 mM Na_3VO_4 could significantly inhibit the activity of 0.5 unit/μL CIAP while 50 mM Na_3VO_4 could completely inhibit the activity of 0.5 unit/μL CIAP (Figures 5 and 6; also see Supporting Information Figures S6 and S7).

CIAP Assay in Biological Fluids. As proven above, the AgNP colorimetric method developed showed good selectivity toward CIAP detection because the ATP dephosphorylation assay was based on the specific catalytic reaction of the enzyme CIAP. To investigate that the colorimetric method could be further used in more complex samples, the CIAP in spiked 1% fetal calf serum was analyzed. As shown in Figure 7, 0.008 unit/μL CIAP spiked could produce a large increase (51.8% compared with the buffer

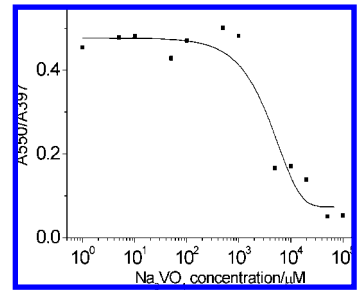

Figure 6. Absorption ratio (A_{550}/A_{397}) vs Na_3VO_4 concentration.

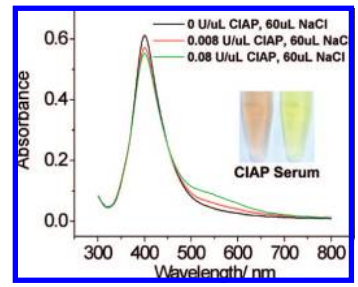

Figure 7. Typical absorption spectra of AgNPs mixed with 2.7 mM ATP in the absence and presence of 0.008 and 0.08 unit/μL CIAP. Inset: photographs of AgNPs mixed with 2.7 mM ATP in the absence and presence of 0.08 unit/μL CIAP.

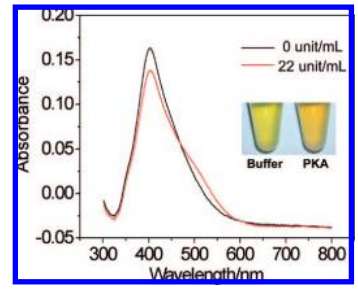

Figure 8. Typical absorption spectra and photographs (inset) of AgNPs mixed with 1.1 mM ATP and 0.89 mM peptide **1** in the absence and presence of 22 units/mL PKA.

sample) in A_{550}/A_{397} absorption ratio (also see Figure S8, Supporting Information). The more CIAP spiked, the larger the increase in A_{550}/A_{397} absorption ratio obtained. This clearly shows that the AgNP-based colorimetric method might become a promising analytical platform for the enzymatic activity assay in realistically complex and clinical systems.

Colorimetric Detection of PKA. To testify that the AgNP-based colorimetric method developed is a general one, the phosphorylation of peptide **1** by PKA was studied (Figure 1). When 1 mol of peptide **1** was phosphorylated by PKA, 1 mol of ATP was changed into 1 mol of ADP. As expected, the color of the AgNP solution appeared yellow in the absence of PKA and changed from yellow to yellow brown in the presence of PKA because ATP could protect AgNPs from salt-induced aggregation effectively (here the salt was the electrolytes included in the reaction solution) while ADP could not (Figure 8). To quantitatively detect PKA using our AgNP colorimetric approach, absorption spectra of AgNPs in the absence and presence of different concentrations of PKA were recorded. As shown in Figure 9, the A_{520}/A_{402} values of solutions (i.e., the color change of the AgNPs)

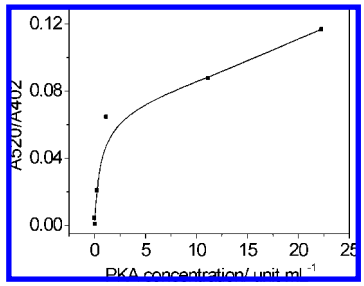

Figure 9. Absorption ratio (A_{520}/A_{402}) vs PKA concentration.

were a sensitive function of PKA concentration, and as low as 0.022 unit/mL CIAP could be detected.

Thus, our unmodified AgNP probes provide a sensitive and selective colorimetric approach for the detection of both ATP dephosphorylation by CIAP and peptide phosphorylation by PKA.

CONCLUSIONS

A sensitive, selective, simple, and label-free colorimetric assay using unmodified AgNP probes to detect enzymatic reactions was developed for the first time. By use of our method, dephosphorylation and phosphorylation could be readily monitored by the color change of AgNPs, with a detection limit of 1 unit/mL for CIAP and 0.022 unit/mL for PKA. More importantly, the assay of enzymatic inhibition by inhibitors and enzymatic activity in complex biological fluids could also be realized. Besides the common advantages of colorimetric assay such as simplicity, high sensitivity, and low cost, our method using unmodified AgNPs could further simplify the experimentation since it is a label-free design and provide an important and attractive alternative to the most popular GNPs as sensing probes. Besides widely developed electrochemical and optical sensors, this study provides another kind of colorimetric sensor for alkaline phosphatase and protein kinases.[50–53] This work might provide a promise for enzyme assay in realistically complex systems and for screening of different enzyme inhibitors in future.

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