Simple and rapid colorimetric enzyme sensing assays using non-crosslinking gold nanoparticle aggregation†

Weian Zhao,[a] William Chiuman,[b] Jeffrey C. F. Lam,[b] Michael A. Brook*[a] and Yingfu Li*[ab]

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Non-crosslinking gold nanoparticle (AuNP) aggregation induced by the loss (or screen) of surface charges is applied for enzymatic activity sensing and potentially inhibitor screening.

AuNP-based colorimetric assay has been recently used for the detection of various substances,[1] based on the unique phenomenon that well-dispersed AuNP solution is red in color whereas aggregated AuNPs appear a blue (or purple) color.[2] The major advantage of AuNP-based assays is that molecular recognition events can be transformed into color changes, which can be observed by the naked eye, and therefore no sophisticated instruments are required.[1] Moreover, because of the extremely high extinction coefficients of AuNPs, AuNP-based assays could provide sensitivity comparable to (or even higher than) conventional analytical methods such as fluorescence assays.[1b] Mirkin and co-workers pioneered the study of AuNP-based colorimetric biosensors where they used oligonucleotide-modified AuNPs to detect a target complementary DNA molecule.[1a] Recently, AuNP-based assays have also been applied for the detection of other targets such as metal ions,[1b] proteins,[1d–i] and small molecules.[1j] The sensing of enzymatic activity and study of enzyme inhibitor using AuNP-based assay have also been developed.[3–5] For instance, Brust and co-workers developed an elegant assay to identify kinase inhibitors taking advantage of the fact that kinase modified AuNP can undergo inter-particle crosslinking to form aggregates and therefore generate a red-to-blue color change.[3a] Guarise and coworkers demonstrated that a short peptide, which contains a cysteine group at both ends, can effectively crosslink AuNP leading to AuNP aggregation.[4] They then developed an assay to detect protease that can cleave that peptide. Most recently, Choi and coworkers extended this concept to a phosphate group-containing peptide substrate.[5] They showed that the substrate charge properties, controlled by a phosphatase, can affect the peptide crosslinking of AuNP, and thus the AuNP aggregation and color change.[5]

In all aforementioned assays, AuNP aggregation is induced by inter-particle crosslinking such as DNA hybridization,[1a,b] antibody-antigen interactions,[1e] and peptide bridging with two binding tags.[4,5] However, this crosslinking AuNP aggregation is limited to the use of crosslinkers that possess at least two binding tags in order to bridge AuNP together. To meet this criterion, the crosslinkers (*e.g.* peptide with two binding tags to AuNP) sometimes have to be carefully designed and synthesized.[5,6] Moreover, AuNP aggregation induced by inter-particle crosslinking is a relatively slow process: it sometimes takes a few hours to observe the aggregation-induced color change.[1a]

It is well known that colloidal stability can also be adjusted by modifying surface charges which affect electrostatic stabilization, and that aggregation can be induced due to the loss (or screening) of surface charges.[7] In this communication we exploited this "non-crosslinking" aggregation phenomenon[8] to develop colorimetric assays for sensing enzymatic activities. We speculate that if the substrate and product of an enzymatic reaction differently affect the AuNP stability by changing their electrophoretic properties, such a reaction can be monitored colorimetrically using AuNPs and the enzymatic activity can therefore be determined.

Enzymatic reactions concerning nucleoside triphosphates as substrates, specifically nucleotide dephosphorylation by alkaline phosphatase and DNA polymerization by DNA polymerase, were chosen as model systems due to their vital importance in molecular biology.[9] It is known that nucleobases can bind to citrate-capped AuNPs with the displacement of weakly bound citrate ions *via* metal–ligand interactions.[10] The adsorption of highly charged nucleotides or uncharged nucleosides should then further stabilize AuNPs or cause their aggregation, respectively, due to the gain or loss of surface charges (Fig. 1(A)). We found that the AuNPs capped by adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), or adenosine 5′-monophosphate (AMP) were much more stable than bare AuNP (see ESI†). The stability of AuNPs followed the order AuNP/ATP > AuNP/ADP > AuNP/AMP > bare AuNP. This is further confirmed by surface charge measurements: the zeta potentials of ATP-adsorbed AuNPs and

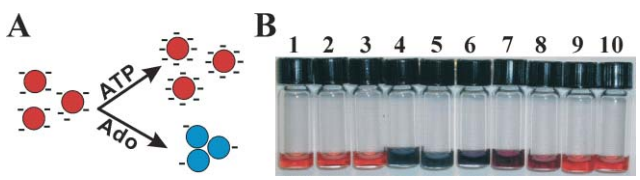

Fig. 1 (A) Schematic illustration of the differential impact of ATP and adenosine (Ado) on AuNP stability. (B) Photographs of AuNP solutions containing 60 μM ATP, ADP, AMP, inosine and adenosine (vials 1–5, respectively). Vial 6–10 are the AuNP solutions containing 30, 20, 10, 5 and 2.5 μM adenosine, respectively. Photographs were taken 1 min after mixing AuNP with a relevant compound.

[a]*Department of Chemistry, McMaster University, 1280 Main St. W. Hamilton, ON, L8N 3Z5, Canada. E-mail: liying@mcmaster.ca; mabrook@mcmaster.ca; Fax: (+1) 905-522-9033*
[b]*Department of Biochemistry and Biomedical Sciences, McMaster University, 1280 Main St. W. Hamilton, ON, L8N 3Z5, Canada*
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bare AuNPs are -32.36 ± 1.43 mV and -24.98 ± 1.54 mV, respectively. This clearly illustrates the direct relationship between the concentration of negatively charged phosphates on AuNP surface and colloidal stability. In contrast, the adsorption of uncharged nucleosides (*e.g.* adenosine and inosine) caused AuNP aggregation, indicated by an instant red-to-blue color change (Fig. 1(B)).

We then sought to take advantage of the observed non-crosslinking AuNP aggregation phenomenon to develop a simple colorimetric assay for monitoring an enzymatic dephosphorylation reaction where ATP was converted into adenosine by calf intestine alkaline phosphatase (CIAP) (Fig. 2(A)). The development of simple, quick and inexpensive assays to monitor dephosphorylation or phosphorylation reactions is technically challenging because the loss or gain of nucleotide phosphate group(s) cannot be easily distinguished by conventional spectroscopic methods. For instance, the substrate (ATP) and the product (adenosine) in CIAP-mediated dephosphorylation reactions have essentially identical absorption spectra (see ESI,† Fig. S2(A)).

In our AuNP-based assay, a typical reaction mixture (20-μL) containing 10 mM ATP, 10 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5), and 2.5 units (~250 nM) of CIAP. To monitor the reaction, 1 μL of the mixture was taken out at a given reaction time, diluted 100 times by H_2O,[11] and then added to 200 μL AuNP solution (14 nM). The UV-Vis adsorption spectra were recorded 1 min after mixing. As expected, with the increase of reaction time, the adsorption of the mixture (originally peaked at ~520 nm) broadened and shifted to longer wavelength (Fig. 2(B)), while the color of the solution changed progressively from red to blue (Fig. 2(C)), indicating the AuNP aggregation during the conversion of ATP to adenosine.

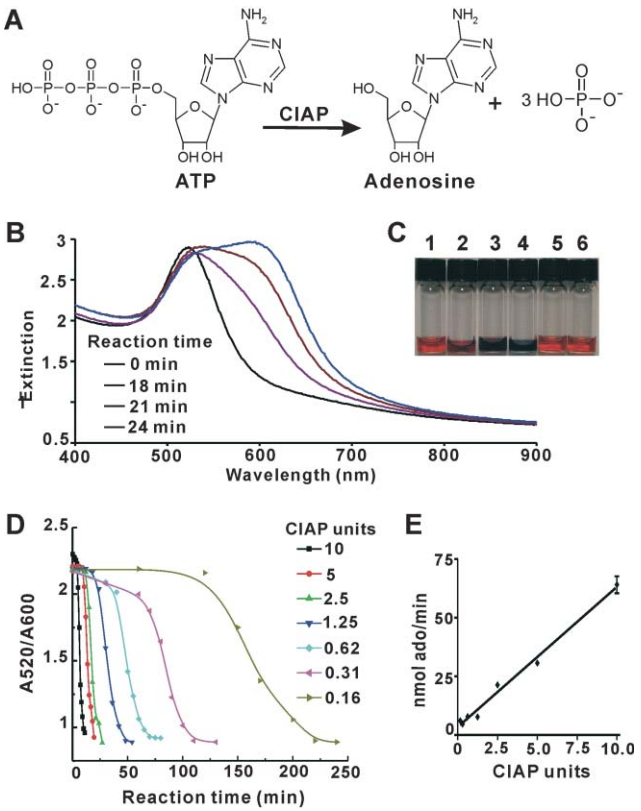

Fig. 2 (A) Conversion of ATP into adenosine by CIAP. (B) UV-Vis spectra of AuNP solutions after addition of an ATP–CIAP mixture incubated at indicated time points. (C) Photographs of AuNP solutions after addition of ATP-CIAP mixture incubated for 0, 18, 21 and 24 min (vials 1–4, respectively). Vials 5 and 6 were the AuNP solutions with addition of the mixture (incubated for 24 min) where ATP or CIAP was omitted, respectively. This control experiment showed that CIAP itself does not cause AuNP aggregation.[13] (D) *A*520/*A*600 *vs.* enzymatic reaction time for the indicated CIAP concentrations. (E) The amount of substrate processed per minute *vs.* CIAP concentration (units/20 μL reaction solution).

To estimate the percentage of ATP converted, a calibration curve relating the ratio of *A*520/*A*600 as a function of ATP/adenosine molar ratio (in the absence of CIAP) was developed (see ESI,† Fig. S3). By fitting the *A*520/*A*600 value calculated from Fig. 2(B) at a certain reaction time, one can easily estimate how far the reaction has progressed.

To quantify the enzyme activities, reactions with various amounts of CIAP were performed and the adsorption spectra are given in Fig. S4 in ESI.† The *A*520/*A*600 ratios as a function of enzymatic reaction time are plotted in Fig. 2(D); the amount of substrate processed per minute (see ESI† for calculations) as a function of CIAP units is shown in Fig. 2(E). A linear relationship was obtained, which strongly suggests that this assay can be applied to quantify the effective enzyme activities. The typical detection limit of CIAP in the current study is ~0.16 units/20 μL (~16 nM).[12]

Notably, this assay can be further used to study enzyme inhibitors. Sodium orthovanadate (Na_3VO_4), a well known inhibitor for CIAP,[14] is used in our proof-of-concept experiment. It was found that 2 mM of Na_3VO_4 (note that, under investigated conditions, Na_3VO_4 itself does not stabilize or aggregate AuNPs) completely inhibited CIAP (1.25 units/20 μL) activity, as there is no significant spectra shift (Fig. 3(A)) or color change (data not shown) in the AuNP test. The titration of inhibitor concentration showed that, at the investigated enzyme concentration (1.25 units/20 μL), the detectable inhibitor concentration is ~300 μM or above (Fig. 3(B)). Note that the detectable inhibitor concentration is highly dependent on the enzyme concentration used for the reaction, that is, less inhibitor is required for effective inhibition when enzyme with lower concentration is used (data not shown).

We reasoned that the concept illustrated above could also be adapted to other enzymatic reactions where substrates and products impact differently on the AuNP stability. To provide a second demonstration, we applied the same method to monitor an

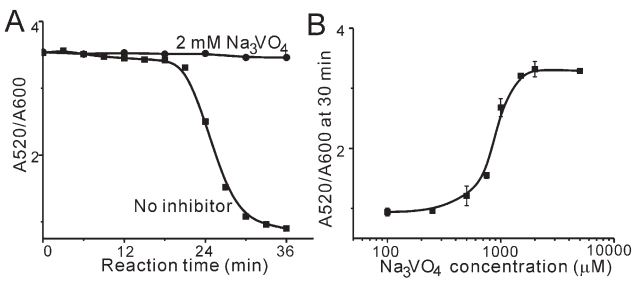

Fig. 3 (A) *A*520/*A*600 calculated from UV-Vis spectra in the AuNP test as a function of enzymatic reaction time. The enzymatic reaction was conduct with 2 mM Na_3VO_4 inhibitor and without inhibitor. (B) *A*520/*A*600 of the sample which underwent 30 min enzymatic reaction time is plotted as a function of Na_3VO_4 concentration.

enzymatic reaction known as "rolling circle amplification" where dNTPs (dATP, dTTP, dGTP and dCTP) are converted to a long single-stranded DNA (ssDNA) by phi29 DNA polymerase.[15] We anticipated that dNTPs would bind AuNP more effectively than the long ssDNA product because of the steric hindrance and large secondary structures formed in long ssDNA,[8b] and that dNTPs would stabilize AuNP more effectively than the long ssDNA. Since dNTPs and the long ssDNA product both stabilize AuNPs (although to different extents), the assay was performed at a specific salt concentration where dNTP/AuNP is stable whereas long ssDNA/AuNP would aggregate.[16] The results are shown in Fig. S5 in ESI.† Briefly, the AuNP solution mixed with the reaction mixture that either excluded or contained DNA polymerase was red and blue in color, respectively. The red-to-blue color change indicated that AuNP aggregation occurred as dNTPs were made into long ssDNA by phi29 DNA polymerase.

In conclusion, we have demonstrated simple and rapid enzyme-sensing assays using the principle of non-crosslinking AuNP aggregation. The use of AuNP is advantageous, particularly for the enzymatic reactions that cannot be easily monitored by traditional spectroscopic techniques, due to its simplicity and the colorimetric detection nature. Moreover, compared to the cross-linking AuNP aggregation, this non-crosslinking aggregation induced by a change of the electrophoretic properties of AuNPs is generally faster (the AuNP test assay in the present study was completed in 1 min).[8] In the non-crosslinking system, the aggregation is driven by the London/van der Waals attractive force between the nanoparticles.[8c] Once the electrostatic repulsion is significantly reduced due to the loss (or screen) of surface charges, the attractive forces dominate, leading to a rapid aggregation. In contrast, in the crosslinking system, the aggregation is mainly driven by random collisions between nanoparticles with relatively slow Brownian motion.[8c] Furthermore, this approach is conceptually simpler than the crosslinking system and, in principle, can be applied to any target molecules which can affect the electrophoretic properties of AuNPs, whereas in the crosslinking aggregation process, the crosslinker has to carry at least two binding tags in order to bring AuNP into close proximity. We speculate that our assay can be adapted to other charged substrates such as DNA oligonucleotides, amino acids and peptides, and that similar assays could be easily developed to detect and quantify other enzymes including ligases, endonucleases, proteases, *etc.* Nevertheless, nonspecific aggregation of bare AuNP could be a potential issue for the use of this assay in more complex matrices (rather than pure buffers) and therefore a pre-purification step might be required in some specific cases. Finally, since the interaction between nucleobases and AuNP is quite complicated and has been the subject of extensive debate,[10] we hope this work will provide insight into the nature of the interaction between DNA (or nucleotides, nucleosides) and AuNPs.

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11 The initial purpose of this dilution is to minimize the effect of local salt concentration increase on the AuNP stability when reaction sample solution is added into AuNP solution. However, our subsequent study showed that the undiluted reaction solution could also be directly added to AuNP solution without causing significant AuNP stability change (see ESI,† Fig. S2).

12 The lower CIAP concentration (*e.g.* 0.05 units/20 μL) could also be detectable, but a longer reaction time (*e.g.* 12 h) was required to ensure enough ATP had been converted into adenosine to get a quick color change (1 min) in the subsequent AuNP test.

13 Control experiments where the AuNP stability with or without CIAP were accessed by gradually adding NaCl solution (1 M) to AuNP solution showed that CIAP did not stabilize AuNP either under studied conditions.

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16 Theoretically, there is a possibility that AuNPs are bridged by the RCA product (or H-bonds between DNA molecule-adsorbed AuNPs). However, we think this is a minor contribution to AuNP aggregation in our assay given the fact that this type of inter-particle crosslinking process is known as a relatively slow process (for example, see ref. 1*a* and 8*c*). Considering the AuNP aggregation (or color change) in our assay is completed in 1 min, we believe this aggregation is induced by the loss (or screen) of surface charges.