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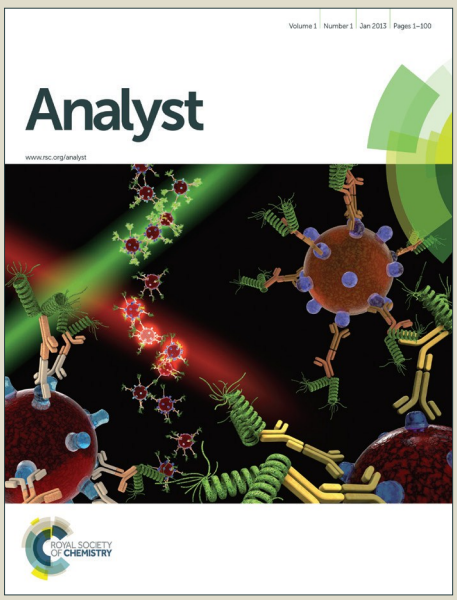

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ARTICLE TYPE www.rsc.org/xxxxxx | XXXXXXXX

A gold nanoparticle-based label free colorimetric aptasensor for adenosine deaminase detection and inhibition assay

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A novel strategy for the fabrication of colorimetric aptasensor using label free gold nanoparticles (AuNPs) is proposed in this work, and the strategy has been employed for the assay of adenosine deaminase (ADA) activity. The aptasensor consists of adenosine (AD) aptamer, AD and AuNPs. The design of the biosensor takes the advantage of the special optical properties of AuNPs and interaction between AuNPs and single-strand DNA. In the absence of ADA, the AuNPs are aggregated and are blue in color under appropriate salt concentration because of the grid structure of AD aptamer when binding to AD. While in the presence of the analyte, AuNPs remain disperse with red color under the same concentration of salt owing to ADA converting AD into inosine which has no affinity with AD aptamer, thus allowing quantitative investigation of ADA activity. The present strategy is simple, cost-effective, selective and sensitive for ADA with a detection limit of 1.526 U/L, which is about one order of magnitude lower than that of previously reported. In addition, a very low concentration of inhibitor erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) could generate distinguishable response. Therefore, the AuNPs-based colorimetric biosensor has great potential in the diagnosis of ADA-relevant diseases and drug screening.

Introduction

As an essential enzyme in purine metabolism, adenosine deaminase (ADA), which catalyzes the irreversible deamination of adenosine (deoxyadenosine) into inosine (deoxyinosine), is ubiquitously distributed in almost all tissues.[1] The expression level of ADA plays an important role in several diseases. ADA deficiency leads to impaired lymphoid development and severe combined immunodeficiency disease (SCID), which accounts for approximately 15% of all SCID cases and one-third of autosomal recessive SCID cases.[2] On the other hand, high levels of ADA in serum are connected to several diseases, including tuberculosis, sarcoidosis, acute leukemia, etc.[3] On account of the significance of ADA in pathology, it is therefore important to develop a rapid and high-throughput assay for ADA activity. Traditional methods for monitoring ADA are mainly based on fluorescence or colorimetric assays of ammonia or inosine, which are generated by deamination of adenosine[4]. Unfortunately, most of these methods require one or more other coupling enzymes to complete the assays, which make the detection process time consuming, labor-intensive and costly. Therefore, it is highly required to develop straight forward and easy operation methods for the assay of ADA activity.

Aptamers generated by an in vitro selection process termed systematic evolution of ligands by exponential enrichment (SELEX)[5] are functional DNA or RNA structures that can bind with high affinity and specificity to various targets, such as small inorganic or organic substances,[6,7] proteins,[8] and even cells[9]. Since the first description in 1990,[10-12] aptamers have been involved in numerous studies in the area of molecular genetics,[13] therapeutics,[14,15] and especially biosensors.[6-20] These artificially selected oligonucleotides are able to bind various targets with desirable selectivity, specificity, and affinity, allowing them to rival antibodies in biosensing and diagnostic applications,[12,21-25] so many aptamer-based sensors (aptasensors) have been proposed.[26-30] As one of aptamers having been screened, AD aptamer has been used in assays of ADA activity.[31-37] Li et al. have reported a typical strategy of electrochemical aptasensor.[35] In this strategy, a well-designed oligonucleotide containing three functional regions (an adenosine aptamer region, a G-quadruplex halves region, and a linker region) was adopted as the core element, and the enzymatic reaction of adenosine catalyzed by adenosine deaminase plays a key role in the regulation of the binding of the G-quadruplex halves with hemin, the electroactive probe which reflected the activity of the enzyme indirectly but the processing of electrode was complicated. As a relatively simple method, fluorescence detection was also reported. Brown et al. employed a fluorescence-signaling aptamer with high affinity for adenosine and virtually no affinity for inosine to develop a screening assay for ADA.[36] Recently, our group reported fluorescent aptasensor for simple and accurate detection of ADA activity and inhibition on the basis of graphene oxide (GO) using adenosine (AD) as the substrate.[37] However, fluorescent labeling is always required in most fluorescence methods, enhancing the time and cost for the detection.

Gold nanoparticles (AuNPs) based colorimetric assays have attracted increasing attention in bioanalysis due to their unique size and distance dependent optical properties. The extremely high extinction coefficients of AuNPs have led to high sensitivity in the AuNP-based colorimetric assays, and the color changes could be easily observed by naked eye. Used as a visual signal transducer, AuNP-based bioanalytical platforms have been successfully applied to detect metal ions, small molecules, proteins, and nucleic acids.[38] Generally,

these colorimetric sensors can be divided into two kinds according to the AuNPs used: modified AuNPs and unmodified AuNPs. Zhao *et al.* firstly developed a label-free method for AD, ADA, and ADA inhibitor assay by employing DNA-modified AuNPs conjugates. This colorimetric assay based on AuNPs has shown great advantages. However, the modification of DNA on AuNPs enhanced the time and cost for the detection.[42] Using unmodified AuNPs to detect enzymes based on the different stability of AuNPs in the presence of the enzyme's substrates or products has attracted much attention.[39] Herein, we present a label-free colorimetric assay for the convenient and sensitive detection of ADA activity and its inhibitors using unmodified AuNPs. Our strategy combines the interaction between AuNPs and single-strand DNA with enzymatic reaction, which is not only simple and sensitive, but also cost-effective.

Experimental section

Chemicals and materials

The following materials were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai): tetrachloroauric acid terahydrate ($HAuCl_4 \cdot 4H_2O$), trisodium citrate dihydrate, and sodium chloride (NaCl). Adenosine and adenosine deaminase were purchased from Amresco Co., Ltd. (USA), and used without further purification. 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) was purchased from Aladdin Reagent Co. Ltd. (Shanghai, China). Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) was purchased from Santa Cruz Biotechnology, Inc. (USA). Thrombin was purchased from Sigma (USA). Cytochrome C and lysozyme were purchased from Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). Bovine serum albumin (BSA) was purchased from Shanghai Ruji Biotechnology Development Co., Ltd (China). The AD aptamer with a sequence 5'-ACCTGGGGGAGTATTGCGGAGGAAGGT-3' used in this work was purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (China) and used without further purification. Milli-Q 126 purified water was used for the preparation of all aqueous solutions.

Apparatus

All UV-visible absorption spectra were made using a UV-Vis Spectrometer UV-2550 (Shimadzu, Japan) with a 1-cm path length quartz cell. A transmission electron microscope (TEM) JEM100CX Ⅱ (JEOL, Japan) was used to observe the size, shape and dispersity of AuNPs. The surface zeta potential of AuNPs was measured through a zetasizer NanoZSZEN3600 (Malvern, Britain). Photos of AuNPs in experiment were taken by digital camera IXUS210 (Canon, Japan).

Preparation and characterization of AuNPs

Approximately 13 nm diameter AuNPs were prepared by the citrate reduction of $HAuCl_4$.[40,41] All glassware was cleaned in nitrohydrochloric acid (3 parts HCl, 1 part HNO_3 in volume), rinsed with Nanopure H_2O, and then oven dried prior to use. Briefly, 50 mL of 1 mM $HAuCl_4$ solution was brought to boiling under continuous stirring, and then 5 mL of 38.8 mM trisodium citrate solution was added quickly. After color change from pale yellow to wine red quickly, the solution was refluxed for additional 10 min, then removed the heater and stirring for another 15 min, allowing cool to room temperature and stored in dark at 4 ℃. A typical solution of 13 nm diameter AuNPs exhibited a characteristic surface plasmon band centered at ~520 nm (Fig. S1). Then the AuNPs were characterized with a JEM100CX Ⅱ transmission electron microscopy (Fig. S2) and the size was determined to be ~13 nm. Moreover, a zetasizer NanoZSZEN3600 was used to characterize the surface charge of as-prepared AuNPs, and the zeta potential was -30~-40 mV (Fig. S3).

Analytical procedure

For quantitative measurement of ADA, 40 μL of 10 μM AD was mixed with different concentration of ADA in 10 mM HEPES (pH 7.5) buffer at 37 ℃ for a appropriate time, then 40 μL of 1 μM aptamer was added and incubated at room temperature. Subsequently, 60 μL AuNPs was added to the solution and shaken gently for 20 min. Then UV-Vis absorption spectra were recorded between 400 nm~800 nm or photos were taken after 20 μL of 0.7 M NaCl (a final concentration of 70 mM) was added to the AuNPs system in an appropriate time. For inhibition assay, ADA was firstly mixed with EHNA and incubated at 37 ℃ for 20 min, the following steps were as the same as described above.

Results and discussion

Design Strategy

The colloidal solution of gold nanoparticles (AuNPs) with diameter of ~13 nm exhibits a red color, owing to the specific optical absorption peak at around 520 nm caused by surface plasmon resonance[27].The color of gold colloid would change according to the aggregation degree of AuNPs in solution. Generally, the as-prepared AuNPs of ~13 nm diameter were well dispersed against aggregation by a negatively charged coating of citrate ions (Fig. S3). However, AuNPs aggregated under 40 mmol L^{-1} NaCl, which screened the electrostatic repulsion between the ion-coated AuNPs, resulting in a broad absorption spectrum with a blue color. This phenomenon could be simply monitored by a UV-Vis spectrophotometer or observed by naked eyes. Interestingly, in the presence of short single-strand DNA (ssDNA), AuNPs kept stable in red even against the same concentration of NaCl, that is, short strand ssDNA could protect AuNPs from aggregating under a relatively high concentration of salt.

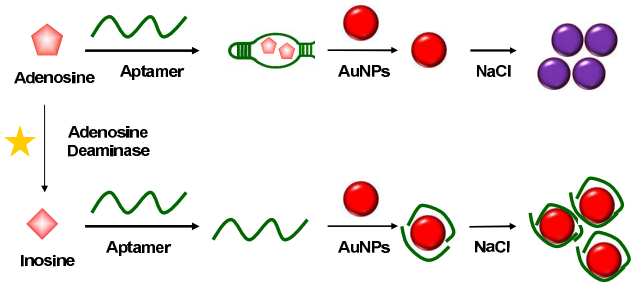


Fig.1 Scheme for the principle of AuNPs-based colorimetric aptasensor for ADA detection.

The principle for adenosine deamianse (ADA) assay based on AuNPs and enzyme catalyzed reaction is depicted in Fig. 1. When ADA is absent, aptamers fold into grid structure from the result of binding with adenosine (AD), leading to an aggregation of AuNPs under an appropriate concentration of NaCl and a blue color change at the same time. While in the presence of ADA, aptamers maintain a free single strand due to the conversion of AD into inosine by ADA and the product inosine has no affinity with aptamers, which protects AuNPs from aggregating and keeps a red color. Therefore, ADA can be detected quantitatively according to the degree of aggregation or color change of AuNPs using a UV-Vis spectrometer or even naked eyes.

Feasibility validation of the method

In order to realize the detection of ADA using the proposed method, firstly we validated the feasibility. As shown in Fig. 2, the absorption of AuNPs solution at 520 nm (curve 0) disappeared, while the absorption peak at about 700 nm appeared (curve 1) and the solution became blue when NaCl was added. In the presence of aptamer, the absorption at 520 nm remained and the solution kept red at the same concentration of NaCl (curve 2). However, when both aptamer and AD were present, it perfomed (curve 3) in a similar way to curve 1. Interestingly, the absorption at 520 nm appeared again with a red color when ADA was added besides the addition of aptamer and AD (curve 4). Therefore, the system clearly shows the feasibility of the assay for ADA.

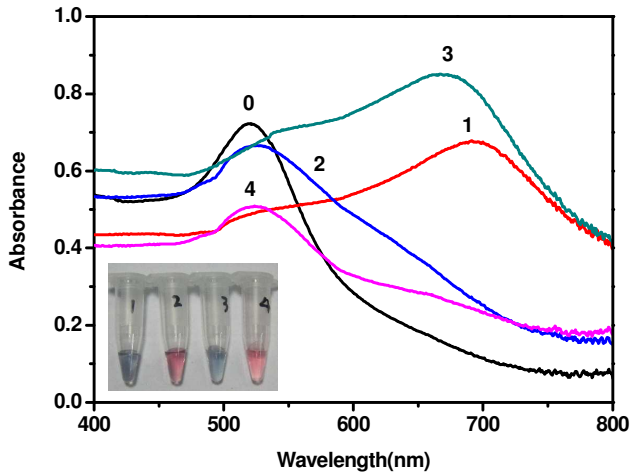


Fig. 2 UV-Vis spectra of AuNPs solution after addition of (0) H_2O, (1) NaCl, (2) aptamer + NaCl, (3) aptamer + AD + NaCl, (4) aptamer + AD + ADA + NaCl. Inset: photograph of AuNPs solutions numbered 1 to 4.

Optimization of detection strategy

A series of experiments were performed to optimize the conditions with acceptable signal response. The amounts of NaCl, AD aptamer and AD are critical in the process of ADA detection. Previously, it is necessary to choose an appropriate NaCl concentration. As the color change of AuNPs system upon the addition of NaCl solution is a dynamic process, we ascertained the testing time point firstly. Fig. S4 shows the change of the ratio of absorbance at 700 nm to 522 nm (A_{700}/A_{522}) following the time, which increases rapidly in the first 5 minutes, and relatively slow from 5 to 10 minutes, and slower after 10 minutes until reaching equilibrium gradually. Thus, we chose 20 mimutes as the testing time point. Then we optimized the concentration of NaCl. When added a series of NaCl solution with different concentration, the AuNPs system exhibited color change in different degree with various absorption spectra (Fig. S5). As shown in Fig. S6, the ratio of absorbance at 700 nm to 522 nm (A_{700}/A_{522}) increased as the increase of the concentration of NaCl is below 70 mM, and then it became stable. Thus, we chose 70 mM as the optimum NaCl concentration.

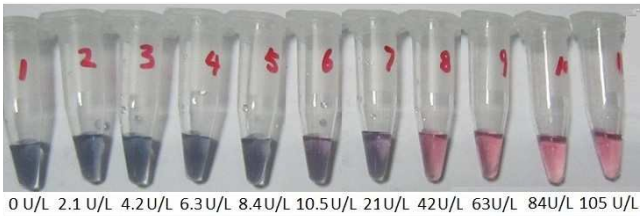
In order to achieve the best sensing performance, the concentration of AD aptamer was also optimized. When added a series of different concentrations of aptamer solution, AuNPs system exhibited color change in different degree with various absorption spectra (Fig. S7). As shown in Fig. S8, the ratio A_{522}/A_{700} increased as the increase of the concentration of aptamer is below 200 nM, and then it became stable. Thus, we chose 200 nM as the optimum aptamer concentration.

We also optimized the concentration of AD as 2 μM, as shown in Fig. S9. Theoretically, an AD aptamer molecule can bind two AD molecules, so when the concentration of AD aptamer is 200 nM, only 400 nM AD is needed. In fact, in this experiment 2 μM AD was required to reach the equilibrium. The reason is probably that the strong coordination interaction between the nitrogen atoms of unfolded ssDNA and AuNPs resists the binding between AD aptamer and AD, and thus more AD was needed to pull aptamers from the surface of AuNPs, leaving AuNPs out of protection and aggregated.

The detection of ADA

ADA assay was performed under the optimal conditions as confirmed above. ADA concentration ranged 0-105 U/L were investigated in quantitative detection of ADA. As the increase of ADA, the AuNPs aptasensor system presents different colors from blue gradually to red (Fig. 3A), while the absorption peak at about 650 nm disappeared by degree and absorption peak at about 522 nm emerged (Fig. 3B). When the concentration of ADA was 21 U/L (No.7 in Fig. 3A), the value of A_{522}/A_{650} reached equilibrium (Fig. 3C). In addition, a linear correlation between A_{522}/A_{650} and ADA concentration was obtained in the range from 0 to 21 U/L (shown in the inset of Fig. 3C). The regression equation is $y = 0.5861 + 0.0536x$ with a correlation coefficient of 0.9898. The detection limit was estimated to be 1.526 U/L in terms of the rule of three times standard deviation over the blank, which is two order of magnitude lower than the reported modified AuNPs method.[42] The high sensitivity may be attributed to extremely high extinction coefficient of AuNPs and unmodified AuNPs show higher sensitivity to the change of the surrounding environment.

A



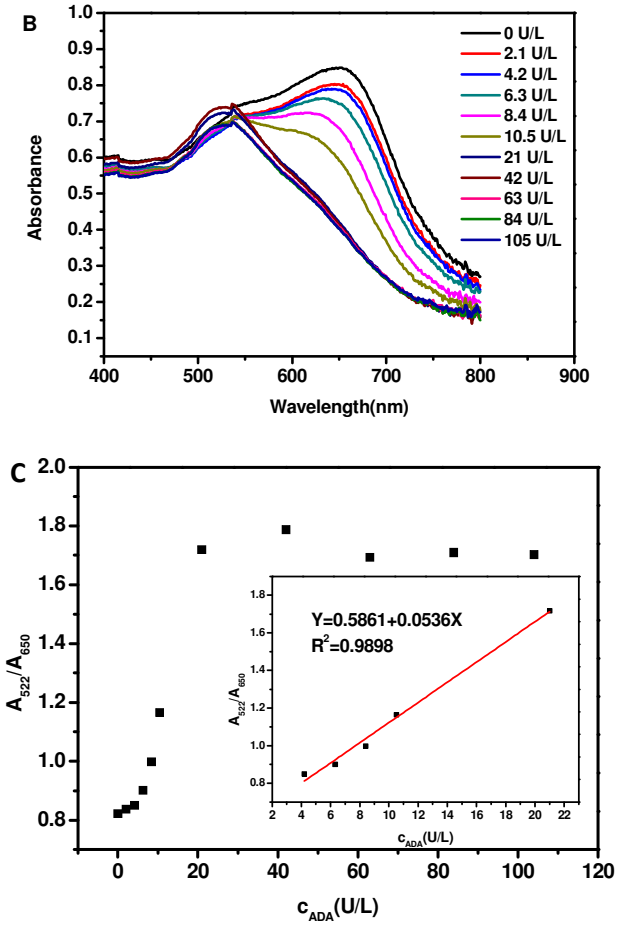


Fig. 3 (A) Photograph of AuNPs aptasensor system solutions with different concentrations of ADA. (B) Absorption spectra of the colorimetric sensing system for different concentrations of ADA as in (A). (C) Plot of ratio of A_{522}/A_{650} versus concentration of ADA (U/L). Inset: calibration curve of ratio A_{522}/A_{650} versus concentration of ADA.

Inhibition of the ADA activity

Since the importance of excess ADA in pathology, inhibitors of ADA may have potential clinical applications.[43] To demonstrate the feasibility of this method for screening ADA inhibitors, erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), a well known inhibitor of ADA, was selected as a model inhibitor for this study.[35] Fig. 4 depicts the absorption spectral responses in the assay of EHNA. The ratio of A_{650}/A_{522} increased with increasing EHNA concentration, and at least down to 2.5 nM EHNA could achieve successful inhibition. This value is approximate to that of previous reports.[35-36,44] These results indicated that this AuNP-based colorimetric strategy could provide a convenient, sensitive method for quantitatively screening the inhibitors of ADA.

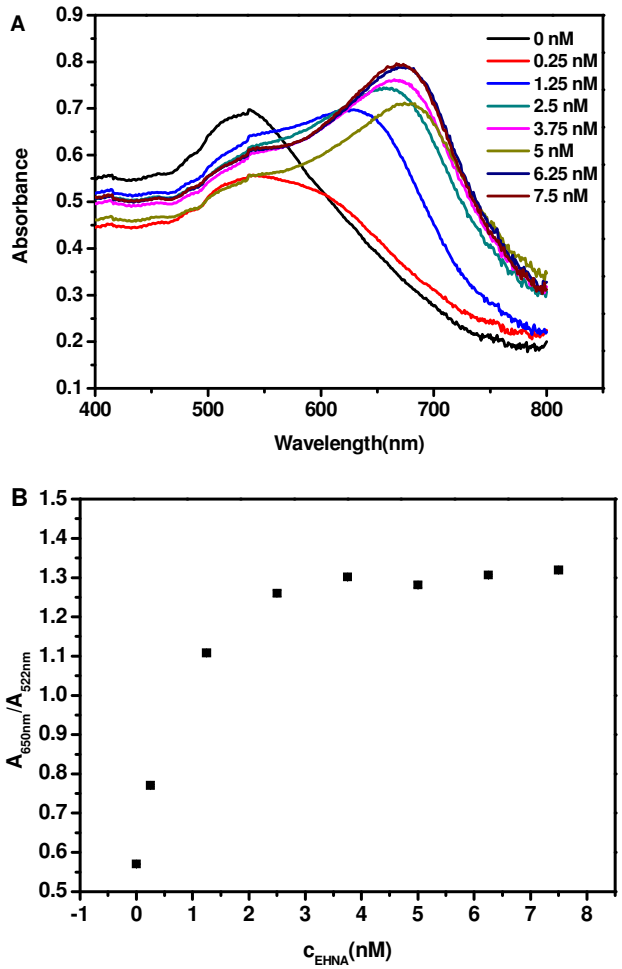


Fig. 4 (A) Absorption spectra of AuNPs sensing system upon addition of different concentrations of EHNA. (B) Plot of ratio of A_{650}/A_{522} versus concentration of EHNA (nM).

Specificity of the colorimetric sensor

The AuNPs system shows excellent specific response to ADA. Here, we selected four other enzymes or proteins, thrombin, cytochrome C, lysozyme and bovine serum albumin (BSA), to study the specificity of the method under the condition for ADA detection. We found that the response generated by ADA was more sensitive than that of the analogs which only show as low background as the blank (Fig. 5). The result indicates that only ADA causes remarkable response, thus, the method can be applied for highly sensitive detection of ADA with high specificity.

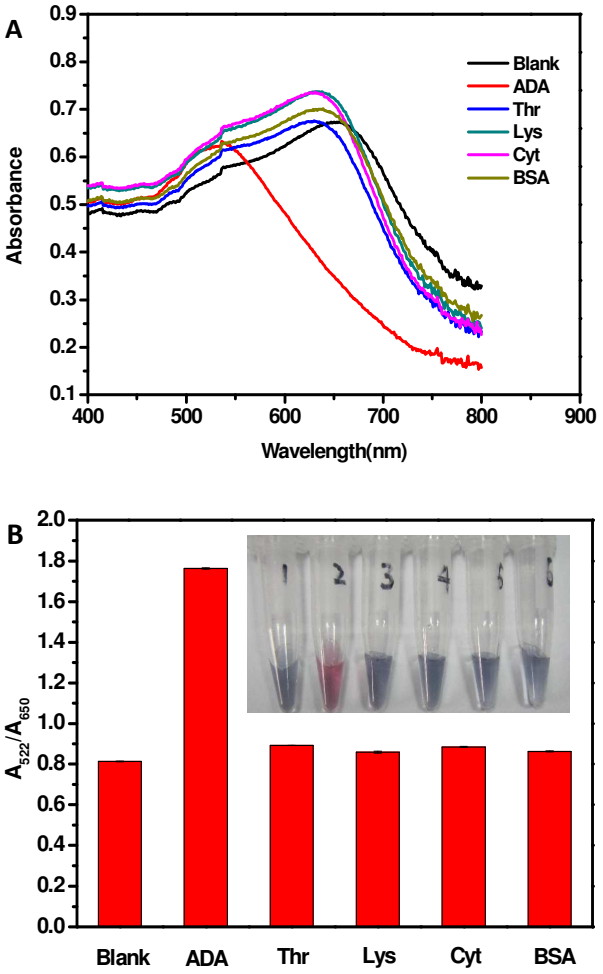


Fig. 5 (A) Absorption spectra of AuNPs aptasensor system responding to ADA (105 U/L or 18 nM approximately), Thrombin (150 U/L), Cytochrome C (350 U/L), lysozyme (20 nM) and BSA (17.6 nM), respectively. (B) Histogram of A_{522}/A_{650} of ADA, the analogs of enzyme or protein and blank. Insert: Photograph of the AuNPs system with different target.

Conclusions

In conclusion, we have developed a simple, rapid, sensitive and selective colorimetric assay for adenosine deaminase (ADA) activity and inhibition based on the special optical properties of AuNPs and interaction between AuNPs and single-strand DNA. Single-strand DNA (AD aptamer) released from the complex of AD-AD aptamer due to deamination of adenosine by ADA greatly increases the stability of citrate-capped AuNPs through the coordination interaction between the nitrogen atom of ssDNA and Au atom of AuNPs. This method allowed a visual and homogeneous assay of ADA activity without any other coupling enzymes or additional modifications, making the proposed strategy simple and cost-effective. Furthermore, this method also exhibits high sensitivity with a detection limit of 1.526 U/L, which is about one order of magnitude lower than that of previously reported.[31-37] The evaluation of inhibition of ADA activity using this colorimetric method has been successfully demonstrated. Therefore, this method holds great potential for applications in clinical diagnostics and drug screening.

Acknowledgements

This work was supported by the National Basic Research Program of China (973 Program, Nos. 2011CB933600 and 2006CB933100), the National Natural Science Foundation of China (21275110, 21275111), and the Fundamental Research Funds for the Central Universities (2042014kf0246).

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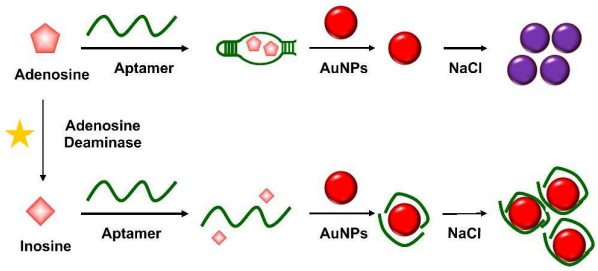

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