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Fluorescent detection of protein kinase based on positively charged gold nanoparticles

Guoyan Lu, Penglong Tan, Chunyang Lei, Zhou Nie, Yan Huang*, Shouzhou Yao

State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, PR China

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ABSTRACT

Herein, we report a fluorometric method for monitoring the activity and inhibition of protein kinase based on positively charged gold nanoparticles, (+)AuNPs. In this assay, when the cationic substrate peptide (S-peptide) is phosphorylated by protein kinase, the resulting negatively charged product peptide (P-peptide) will be adsorbed onto (+)AuNPs through electrostatic interaction, and the fluorescence of fluorescein isothiocyanate (FITC) on the peptide will be quenched by (+)AuNPs. Thus, the fluorescence of solution can respond to the activity of protein kinase. The feasibility of this (+)AuNPs-based method has been demonstrated by sensitive measurement of the activity of cAMP-dependent protein kinase (PKA) with a low detection limit (0.5 mU μL^{-1}). Furthermore, the system is successfully applied to estimate the IC_{50} value of PKA inhibitor H-89. The fast mix-and-readout detection process as well as the simple synthesis of the unmodified (+)AuNPs makes this proposed method a promising candidate for simple and cost-effective kinase activity detection and a good potential in high-throughput screening of kinase-related drugs.

1. Introduction

Phosphorylation of proteins is carried out by protein kinases, which transfer the γ-phosphoryl from adenosine-5′-triphosphate (ATP) to a free hydroxyl group of serine, threonine, or tyrosine in a peptide or protein substrate [1]. This process plays a significant role in many vital biological processes, including cellular signal transduction [2,3], cell proliferation and differentiation [4,5]. It is also reported that aberrant phosphorylation and abnormal expression of kinases are implicated in the pathogenesis of many diseases, such as cancer, diabetes, Alzheimer's disease, immune deficiencies, endocrinological disorders, and cardiovascular diseases [6–10]. Therefore, identification of kinases activities and their potential inhibitors plays vital roles not only in basic biology to clarify molecular mechanisms of phosphorylation related processes, but also in clinical pharmacology and drug discovery [8,11].

Traditional radiometric methods for assessing kinase activities, mainly using [γ-32 P] ATP as substrate, are very sensitive [12,13]. Considering the unhealthy radioactive wastes and the complicated multistep procedures, many efforts have been devoted to the development of non-radioactive assays for monitoring protein kinase activities, such as optical [14–16], colorimetric [17–19], fluorescent [20–22], electrogenerated chemiluminescence (ECL) [23], electrochemical [24–27], and quartz-crystal-microbalance-based methods [28]. Compared with other protein kinase assays, fluorescence approaches have particular advantages, including homogeneous detection, high throughput, low sample volume, and simple operation. Among fluorescence approaches, the direct measurement of fluorescence intensity for protein kinase generally needs electrophoresis [29] or a solid microarray [30] to separate the fluorescent phosphopeptides from the unphosphorylated ones, resulting in a time-consuming and labor-intensive process. Fluorescence polarization (FP) methods, based on specific combination of fluorescent phosphopeptides with phosphoantibodies [31] or phosphopeptide-binding nanoparticles [32], are homogeneous and simple, but it has been reported that they are very sensitive to fluorescence interference [33]. On the basis of the specific recognition of phosphopeptide by a phosphoamino acid binding domain, Tsien et al. [34,35] developed fluorescence resonance energy transfer (FRET) biosensors which can detect the protein kinase activity in real-time or in living cells. However, the complicated molecular construction of the biosensors limits their development. Therefore, it still remains a great challenge to develop homogeneous, sensitive, and simple methods to detect protein kinase activity.

Herein, using positively charged gold nanoparticles ((+)AuNPs), we developed a simple and homogeneous fluorescent assay for sensing the activity and inhibition of protein kinase. Phosphorylation can transform the positively charged substrate peptide (S-peptide) into negatively charged product peptide

* Corresponding author. Tel./fax: +86 731 88821626.
E-mail address: huangyan.hnu@gmail.com (Y. Huang).

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(P-peptide), and change the interaction between the peptides and (+)AuNPs. The electrostatic attraction leads to the adsorption of P-peptide on the surface of (+)AuNPs, accompanied by fluorescence quenching of fluorescein isothiocyanate (FITC) on the peptide by (+)AuNPs. Thus, the fluorescence signal of the homogeneous solution could respond to the phosphorylation reaction catalyzed by kinase and work as a read-out. This assumption is proved by sensitive measurement of the activity and inhibition of cAMP-dependent protein kinase (PKA, a typical serine/threonine protein kinase). Compared with the existing kinases assays, using (+)AuNPs for phosphorylation recognition can detect protein kinase without expensive antibodies or binding proteins, and the materials in this assay are easily accessible for general laboratories. In addition, as a mix-and-readout detection system, common steps in above-mentioned bioanalysis, such as nanoparticle probe and several wash steps, are avoided in this method. It is worth noting that although many AuNPs-based methods have been reported for biocatalytic detection, the application of (+)AuNPs in kinase sensing is scarce. Thus this method will potentially broaden the applicability of AuNPs in bioanalysis.

2. Experimental

2.1. Materials

FITC-labeled S-peptide of PKA (FITC-LRRASLG) and control peptide (FITC-RGKGGKGLGKGGAKA) were obtained from GL Bio Chem. Ltd. (Shanghai, China). PKA was purchased from New England Bio Labs Inc. (Beverly, MA, USA). Adenosine triphosphate (ATP) was purchased from Generay Biotech Co. Ltd. (Shanghai, China). Chloroauric acid ($HAuCl_4$) and sodium borohydride were purchased from Sinopharm Chemical Reagent Company (Beijing, China). Cysteamine was purchased from Sigma-Aldrich (St. Louis, MO, USA). H-89 was obtained from EMD Biosciences (La Jolla, CA, USA). Tris and magnesium chloride were obtained from Sangon (Shanghai, China). All other chemicals were of analytical reagent grade and used without further purification. All solutions were prepared using ultrapure water (18.3 MΩ cm) from the Millipore Milli-Q system.

2.2. Apparatus

Fluorescence measurements were performed on a Synergy™ Mx multimode microplate reader (Bio-Tek, USA). Transmission electron microscopy (TEM) measurements were conducted on a JEOL JEC-3010 electron microscope (Japan). UV–vis absorption spectra of (+)AuNPs were obtained on a Beckman DU-800 spectrophotometer (CA, USA). zeta potentials and dynamic light scattering (DLS) measurements were recorded with a Nano Zetasizer ZS90 (Malvern, U.K.).

2.3. Preparation of positively charged AuNPs ((+)AuNPs)

All glasswares used in the following procedure were cleaned in a bath of freshly prepared 1: 3 HNO_3–HCl, rinsed thoroughly with water and dried in air prior to use. The positively charged AuNPs were prepared according to the published protocol [36]. Briefly, a cysteamine solution (400 μL, 213 mM) was added into 40 mL of 1.42 mM $HAuCl_4$ solution. After stirring for 20 min at room temperature, fresh sodium borohydride solution (10 μL, 10 mM in deionized water) was added to reduce gold ions, and the mixture was vigorously stirred for 10 min at room temperature in dark. Then, the mixture was further stirred until the solution turned to wine-red, and the resulting solution was kept in dark at 4 °C prior to further measurements. The as-prepared (+)AuNPs were characterized with UV–visible absorption spectra, zeta potentials, DLS, and TEM.

2.4. Detection of protein kinase activity and its inhibition

PKA was stored in a solution of 20 mM Tris–HCl buffer (pH 7.5), containing 50 mM NaCl, 1 mM EDTA, 2 mM DTT, and 50% glycerol. The PKA reaction solution was composed of FITC labeled S-peptide (10 μL, 20 μM), ATP (5 μL, 100 μM), PKA (4 μL, 0–50 U μL^{-1}), $MgCl_2$ (10 μL, 10 mM) and Tris–HCl buffer (50 mM, pH 7.5). The reaction solutions (100 μL) were incubated at 37 °C for 1 h in dark for phosphorylation, and then diluted with 300 μL Tris–HCl buffer (50 mM, pH 7.5). Then 50 μL of (+)AuNPs was added to 100 μL diluted reaction solution. The mixture was dispersed by shaking at medium intensity for 1 min, and then the fluorescence was measured by the multimode microplate reader. All samples were illuminated at an excitation wavelength of 480 nm, and the fluorescence emission was scanned from 500 to 700 nm. Except for the specific cases mentioned in the text, the fluorescence intensity at the maximum emission peak was measured as the emission intensity. The fluorescence measurements were performed three times for each sample ($n=3$), and the results showed the average of measurements with error bars, indicating the relative standard deviation.

For PKA inhibitor assay, the experiments were carried out similarly to that of PKA assay stated above, except for the involvement of 1 U μL^{-1} PKA and varied concentrations of H-89 (5 μL, 0–40 μM) in the reaction mixture.

3. Results and discussion

3.1. Discrimination of S-peptide and P-peptide based on (+)AuNPs

According to previous reports [37], negatively charged AuNPs ((−)AuNPs) can interact with positively charged peptide/protein, and quench the fluorescence of dye on the peptide/protein through FRET, we assumed that (+)AuNPs can also distinguish positively charged peptides from negatively charged ones through electrostatic attraction. To prove this assumption, first (+)AuNPs were synthesized through the reduction of $HAuCl_4$ by $NaBH_4$ in the presence of cysteamine [36]. The synthesized wine-red AuNPs solution exhibited an absorption peak at 525 nm (Fig. S1), which was ascribed to the surface plasmon resonance of AuNPs and similar to the report [38]. A transmission electron microscope (TEM) and dynamic lights scattering (DLS) showed that the particles are highly dispersed in original water solution with an average size of about 30.0 nm (Fig. 1A) and a hydrodynamic diameter of 40.21 nm (Fig. S2). Due to the adsorption of the positively charged $-NH_3^+$ group of cysteamine, AuNPs were positively charged (+26.7 mV, pH=3.6, as zeta potential shows in Fig. S3). These results suggested that the (+)AuNPs were successfully synthesized.

Since mix-and-readout detection system has several merits, such as easy manipulation and potential in high-throughput screening of drugs, we try to design such process in our method. To fit the following phosphorylation reaction, (+)AuNPs were added in Tris–HCl buffer (50 mM, pH 7.5) and characterized. Due to the electrostatic attraction between (+)AuNPs and anions in the buffer, most AuNPs aggregated in the buffer (Fig. 1B), resulting in the increase of hydrodynamic diameter from 40.21 nm to 803.7 nm (Fig. S2), the red-shift of maximum absorption peak from 525 nm to 850 nm (Fig. S1), and the color change from wine-red to blue (data not shown). Although surface charge of the aggregated particles reduced, it still remained positive (+20.6 mV) (Fig. S3).

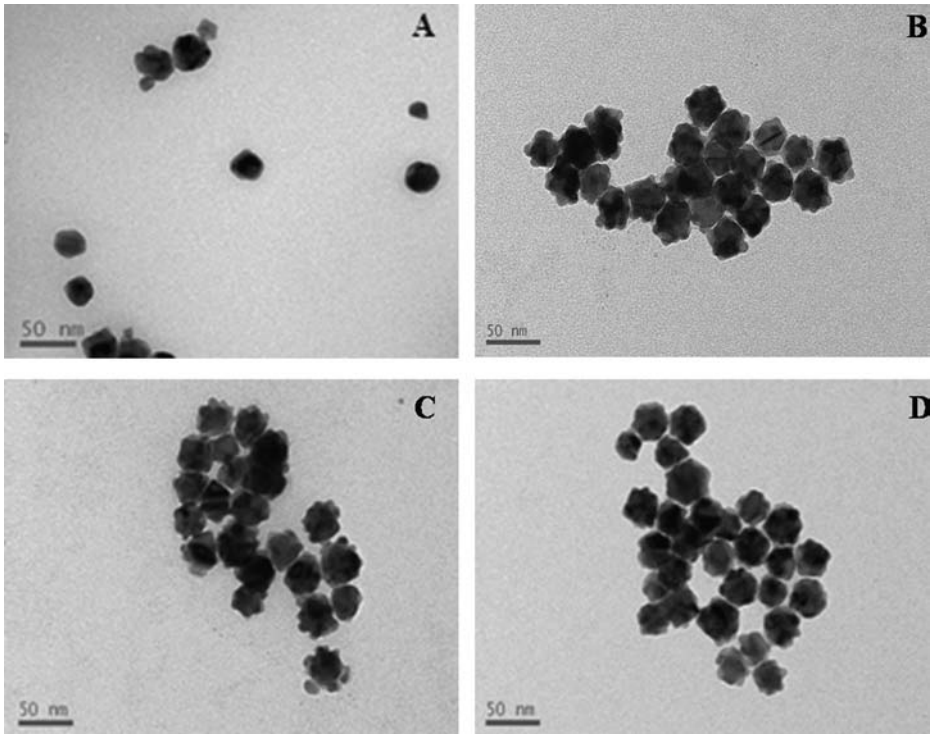


Fig. 1. TEM of the as-prepared (+)AuNPs under different conditions: (A) ultrapure water (18.25 MΩ cm); (B) 50 mM Tris–HCl (pH 7.5, 25 °C); (C) 50 mM Tris–HCl (pH 7.5, 25 °C) with S-peptide, $MgCl_2$, and ATP; and (D) 50 mM Tris–HCl (pH 7.5, 25 °C) with P-peptide, $MgCl_2$, and ATP.

Previously, reports on interaction between aggregated AuNPs and peptides are very scare. Here two peptides, PKA-specific substrate peptide (S-peptide, FITC-LRRASLG) and its phosphorylated product (P-peptide, FITC-LRRApSLG), were designed to act on the aggregated (+)AuNPs. S-peptide possesses a positive charge (+1), and P-peptide has a negative charge (−1) after introduction of a phosphate group to S-peptide (the phosphorylated reaction did not obviously change the fluorescence of the peptide (data not shown)). As we expected, the interaction between (+)AuNPs and carboxyl group on the peptide led to slight decrease (1%) of the fluorescence of S-peptide solution after adding 50 μL (+)AuNPs (the pink curve in Fig. 2). However, when P-peptide was mixed with the same volume of (+)AuNPs, the fluorescence change took place rapidly and dramatically. Within 1 min of mixture, the fluorescence intensity of the peptide solution decreased from 4008 to 895 (the black curve in Fig. 2), which was only 30% of that of the S-peptide solution. These results suggested that the negatively charged P-peptide was attracted by (+)AuNPs, followed by the fluorescence quench of FITC labeled on peptide. Comparatively, there was no obvious fluorescence change observed in the presence of a positively charged control substrate (another FITC-labeled peptide without PKA phosphorylated site, FITC-RGKGGKGLGKGGAKA, with charge +5) (the red curve in Fig. 2), no matter PKA existed or not. To elucidate the interaction between peptides and (+)AuNPs, several experiments were performed. TEM and DLS revealed that the existence of both S-peptide and P-peptide had little influence on the aggregation of (+)AuNPs (Fig. 1C and D, and Fig. S2). Although (+)AuNPs did not obviously quench the fluorescence of S-peptede, zeta potentials of S-peptide/(+)AuNPs mixture altered from +20.6 mV to 8.12 mV (Fig. S3). Comparatively, zeta potentials of P-peptide/(+)AuNPs mixtures changed from +20.6 mV to +6.98 mV, indicating that the positive charge on the surface of AuNPs was largely neutralized by P-peptide (Fig. S3). Thus, the aggregated (+)AuNPs can efficiently differentiate between P-peptide and S-peptide with different fluorescence quenching efficiency, and the fluorescence signal of this peptide/(+)AuNPs system can specifically and selectively respond to the phosphorylation reaction catalyzed by PKA.

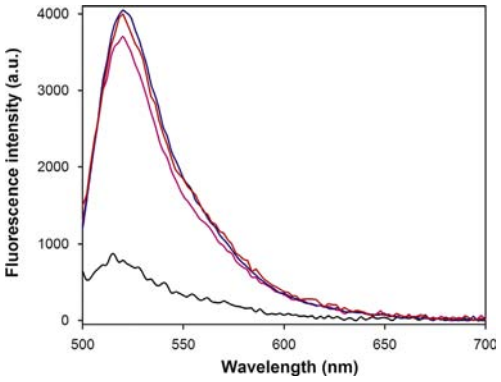
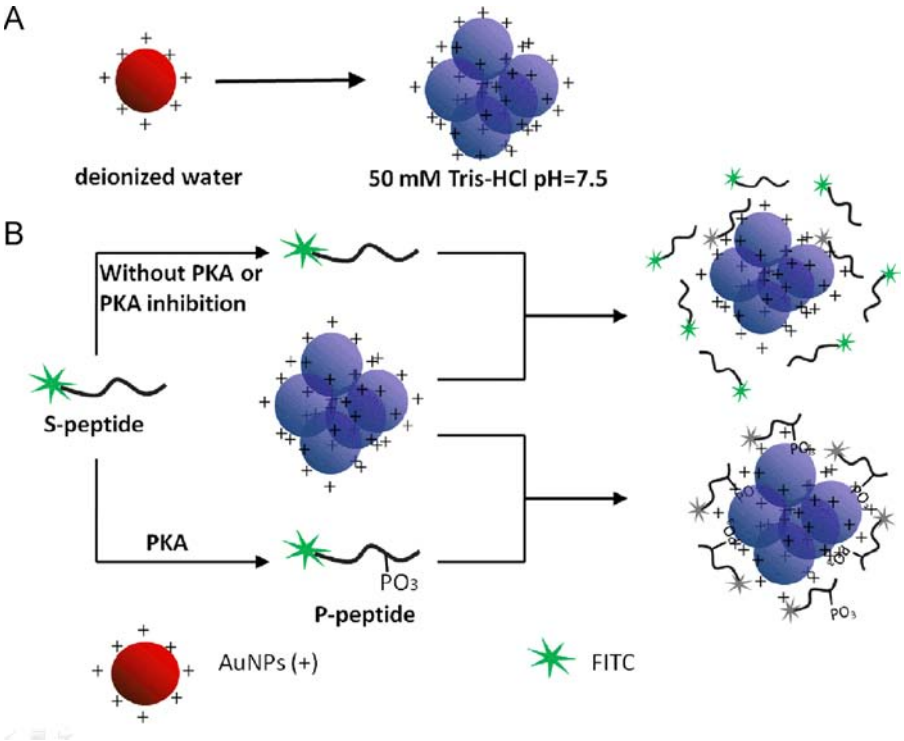


Fig. 2. Fluorescence detection of the (+)AuNPs/peptide mixtures. The blue curve: 2 μM S-peptide; the black curve: the (+)AuNPs/P-peptide mixture; the pink curve: the (+)AuNPs/S-peptide mixture; and the red curve: the (+)AuNPs/control peptide mixture. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Scheme 1 shows the detection mechanism of the kinase activity assay based on (+)AuNPs.

3.2. Optimization of experimental conditions

Considering that the interaction between (+)AuNPs and carboxyl group of the polypeptide may partly cause unspecific adsorption of S-peptide by (+)AuNPs, the concentration ratio of (+)AuNPs to peptides was investigated. The fluorescence ratio (I/I_0) was calculated based on the fluorescence intensities. Here, I is the fluorescence intensity of the P-peptide/(+)AuNPs mixture, where S-peptides was phosphorylated by 1 U μL^{-1} PKA, and I_0 is the fluorescence intensity of the S-peptide/(+)AuNPs mixture. The lower I/I_0 indicates the better ability of (+)AuNPs to distinguish these two peptides. Fig. S4 presents the relationship between (+)AuNPs concentration and the fluorescence ratio (I/I_0). It was found



Scheme 1. Concept of the fluorescence kinase activity assay based on (+)AuNPs.

that fluorescence ratio decreased with the increase of (+)AuNPs concentration up to 50 μL. When more than 50 μL (+)AuNPs were added into the solutions, the fluorescence ratio (I/I_0) leveled off and was kept at about 20% (Fig. S4). Thus, 50 μL (+)AuNPs were chosen in the following assay.

In the PKA-catalyzed phosphorylation reaction, ATP and Mg^{2+} work as co-substrate and cofactor of PKA, respectively. Such factors not only are necessary for phosphorylation reaction, but also may influence the electrostatic interaction between peptide and (+)AuNPs. Therefore the concentration of ATP and Mg^{2+} needs to be optimized. It can be seen from Fig. S5A that the fluorescence intensity of the solution decreased with the increase of concentration of ATP, suggesting that the more the ATP, the more the peptides phosphorylated by PKA and adsorbed onto (+) AuNPs. When the concentration of ATP was in excess of 5 μM, the fluorescence intensity of the solution began to increase rather than decrease, which indicated that PKA was saturated at such concentration of ATP, and the residual free ATP and the generated ADP after phosphorylation may compete with P-peptide for (+)AuNPs. The similar trend was found in the fluorescence intensity of the solution responding to the concentration of Mg^{2+} from 0 μM to 100 μM (Fig. S5B). However, the signal remained almost unchanged when the concentration of Mg^{2+} exceeded 80 μM, suggesting that excess Mg^{2+} has little effect on peptide/(+)AuNPs interaction. According to previous works [19,39], 1 mM Mg^{2+} was often chosen for phosphorylation reaction. Hence, 5 μM ATP and 1 mM Mg^{2+} were used in the following phosphorylation reactions.

3.3. Detection of the activity and inhibition of protein kinase

Detection of kinase activity was carried out under the aforementioned pre-optimized experimental conditions with different amounts of PKA (0–2 U μL^{-1}) using the proposed method. Fig. 3A shows that the fluorescence intensity of the solution decreases gradually along with the increase of PKA concentration, demonstrating that the increased amount of P-peptide was produced, and subsequently adsorbed by (+)AuNPs. When the concentration of PKA is higher than 1 U μL^{-1}, the fluorescence intensity of the solution hardly decreases. It suggests that S-peptide in the system has been fully phosphorylated. The PKA concentration dependence of the ratiometric fluorescence intensity change I/I_0 is plotted in Fig. 3B. The EC_{50} value (enzyme concentration with which 50% substrate is converted) for PKA was determined to be 215.0 mU μL^{-1}. The detection limit of PKA, 0.5 mU μL^{-1}, is not only close to that of our group's previous works, quantum dots aggregation based fluorescent PKA assay (0.47 mU μL^{-1}) [20] and fluorescent detection of protein kinase based on zirconium ions-immobilized magnetic nanoparticles (0.5 mU μL^{-1}) [40], but also highly comparable to the recently reported PKA assays [41,42].

To further demonstrate the potential application of this method in the inhibition assay, the experiments were performed in the presence of the kinase inhibitor H-89, a potent and cell-permeable inhibitor of PKA. It can be seen from Fig. 4A that the fluorescence intensity of the solution increases as the concentration of H-89 increases, with the fixed concentration of PKA and S-peptide. It indicated that the more the H-89, the higher the inhibition of PKA activity, and the lower the level of peptide phosphorylation. When the ratiometric fluorescence intensity change I/I_0 versus the H-89 concentration was plotted, a sigmoidal profile was obtained (Fig. 4B). The IC_{50} value, the half-maximal inhibitory concentration, was determined to be 55 nM, which is in good agreement with the previous reports [43]. It should be mentioned that with or without enzyme inhibitor, the fluorescence signal can be read out after just 1 min of mixture of the phosphorylation reaction solution and (+)AuNPs, demonstrating that the proposed mix-and-read assay is fast and easily operated, which has the potential to be developed as a high-throughput method for clinical diagnosis and drug discovery.

4. Conclusions

In summary, we proposed a simple, rapid, sensitive, and effective strategy for the fluorescence detection of protein kinase using positively charged gold nanoparticles ((+)AuNPs). This novel

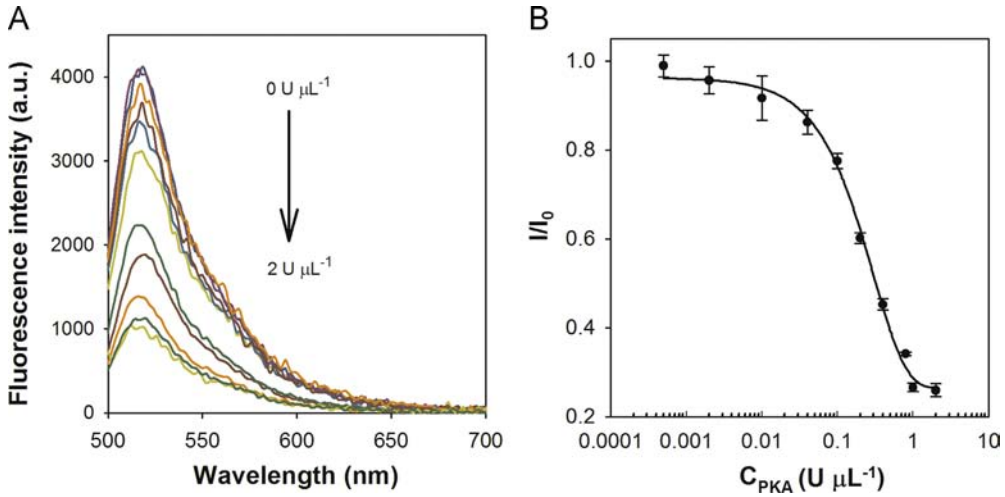


Fig. 3. (A) The effect of PKA concentration on fluorescence emission spectra. (B) The PKA concentration dependence of the ratiometric fluorescence changes I/I_0, where I is the relative fluorescence intensity of reaction solutions with different concentrations of PKA, and I_0 is that without PKA. EC_{50}=215.0 mU μL^{-1}.

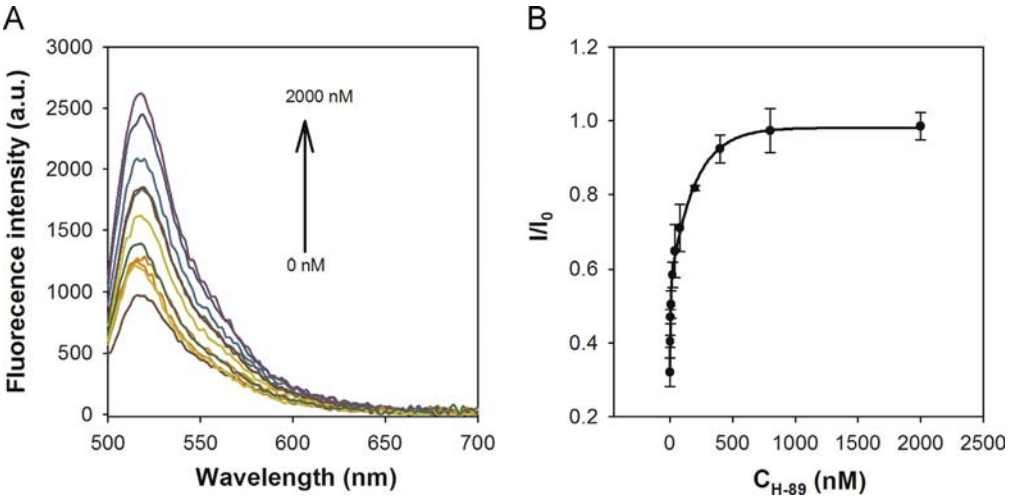


Fig. 4. (A) Fluorescence responses of the reaction solution in the presence of different concentrations of PKA inhibitor H-89 (0–2 μM) and 1 U μL^{-1} PKA. (B) Dose–response curve of H-89 inhibition of PKA activity monitored by the change of fluorescence intensity ratio.

strategy is based on the electrostatic interaction between (+)AuNPs and the negatively charged P-peptide and the fluorescence quenching ability of (+)AuNPs. Such positively charged gold nanoparticles-based method possesses several notable advantages such as simple synthesis of (+)AuNPs, no need for any (+)AuNPs modification, and easy manipulation. In addition, the fast mix-and-read assay shows great potential in high-throughput screening of kinase-related drugs.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.04.061.

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