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Sensitive and versatile electrogenerated chemiluminescence biosensing platform for protein kinase based on $Ru(bpy)_3^{2+}$ functionalized gold nanoparticles mediated signal transduction

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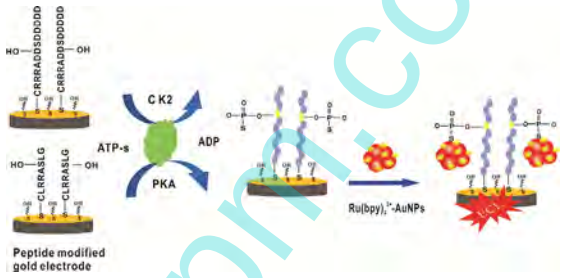
HIGHLIGHTS

- A sensitive and versatile ECL biosensing platform is developed for monitoring protein kinase activity and inhibition.
- $Ru(bpy)_3^{2+}$ functionalized gold nanoparticles are used as thiol-versatile signal probe.
- The strategy exhibits unique advantages of high sensitivity, good selectivity and versatility.
- The strategy is promising for multiple protein kinase assay and kinase inhibitor profiling.

GRAPHICAL ABSTRACT



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ABSTRACT

A novel, sensitive and versatile electrogenerated chemiluminescence biosensing platform is developed for monitoring activity and inhibition of protein kinase based on $Ru(bpy)_3^{2+}$ functionalized gold nanoparticles ($Ru(bpy)_3^{2+}$-AuNPs) mediated signal transduction. $Ru(bpy)_3^{2+}$-AuNPs were formed by functionalizing AuNPs with $Ru(bpy)_3^{2+}$ through electrostatic interactions and were used as thiol-versatile signal probe. Casein kinase II (CK2) and cAMP-dependent protein kinase (PKA), two classical protein kinase implicated in disease, were chosen as model protein kinases while a CK2-specific peptide (CRRRADDSDDDDD) and a PKA-specific peptide (CLRRASLG) were employed as molecular substrate for CK2 and PKA, respectively. The specific peptide was self-assembled onto the gold electrode via Au–S bond to form ECL biosensor. Upon thiophosphorylation of the peptide on the electrode in the presence of protein kinase and co-substrate adenosine-5'-(γ-thio)-triphosphate, $Ru(bpy)_3^{2+}$-AuNPs was assembled onto the thiophosphorylated peptides via Au–S bond. The $Ru(bpy)_3^{2+}$-AuNPs attached on electrode surface produce detectable ECL signal in the presence of coreactant tripropylamine. This strategy is promising for multiple protein kinase assay and kinase inhibitor profiling with high sensitivity, good selectivity and versatility. The ECL intensity is proportional to the activity of CK2 in the range of 0.01–0.5 unit/mL with a low detection limit of 0.008 unit/mL and to the activity of PKA in the range of 0.01–0.4 unit/mL with a detection limit of 0.005 unit/mL. Additionally, this assay was applied to the detection of CK2 in serum samples and the inhibition of CK2 and PKA. This work demonstrates that the developed

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ECL method can provide a sensitive and versatile platform for the detection of kinase activity and drug-screening.

1. Introduction

Protein kinases have been shown to play very important roles in many signal transduction pathways [1]. The over-expression of protein kinases is reported to cause various diseases such as diabetes, aggressive tumor, or Alzheimer's disease [2–4]. The identification of kinase activities and their potential inhibitors is not only necessary for understanding many fundamental biological metabolism processes, but also valuable for the research of protein–kinase-targeted drug discovery [5.6]. Currently, the gold standard for monitoring protein kinase activities and inhibition is the [γ-^{32}P] ATP radiometric assay [7–9] with an obvious drawback. An alternative to radiolabeling is an immunoassay approach to using non-labeled or fluorescently labeled anti-phospho antibodies (phospho-Ab) for the detection of specific phosphorylation products based on sequence-specific antibodies [10]. Although these methods have made great advances, most of them generally require labor-intensive operation, sophisticated preparation, or expensive instrumentation because they typically rely on transfer of radioactive phosphate or phospho-specific antibodies. Other kinds of analytical techniques such as colorimetric [11], electrochemistry [12], and fluorescence [13] employing peptide as molecule recognition elements are arising in the protein kinase analysis with high sensitivity. However, challenges related to complexity and sensitivity are still present. Therefore, it is still a challenge in developing sensitive, accurate, and simple methods for monitoring the activity and inhibition of protein kinases.

Electrogenerated chemiluminescence (electrochemiluminescence, ECL) is the generation of light through electrochemical processes and has been proved to be a useful tool for bioassay method due to its high versatility, simplified optical setup, good temporal and spatial control, fast sample analysis, and a very low background signal [14.15]. In spite of its excellent properties, few studies were conducted concerning the detection of kinase activity and inhibition by ECL methods. Recent reports described a few intriguing kinase assays based on ECL signal labeled phospho-specific antibodies bound to the phosphorylated proteins specifically [16.17], introducing ECL probes by the coordination effect of phosphate group with some metallic ions such as Ti^{4+} and Zr^{4+} [18–21] or using gold nanoparticles (AuNPs) as signal probe for the kinase activity and inhibition analysis [22]. These works proved that the ECL sensing system is a promising way to detect kinase with its high sensitivity. Though the above ECL methods could obtain good results, sophisticated procedures such as the complicated synthesis of ECL probe are needed and sometimes, the performances such as linear range, sensitivity, etc. were also limited. Additionally, these methods are typically designed using one kind of ECL labels such as enzymes, metal complex, and nanoparticles for one analyte. This approach is limited to the application of multiple bioassays since different labels for different analytes are required to be detected.

In this work, a versatile and sensitive ECL biosensing platform was developed for monitoring protein kinase activity and inhibition based on $Ru(bpy)_3^{2+}$ functionalized gold nanoparticles ($Ru(bpy)_3^{2+}$-AuNPs) mediated signal transduction. $Ru(bpy)_3^{2+}$-AuNPs was synthesized by functionalizing AuNPs with $Ru(bpy)_3^{2+}$ through electrostatic interactions in aqueous medium and was used as thiol-versatile signal probe [23]. The schematic diagram of the ECL detection of protein kinase is shown in Fig. 1. A specific peptide substrate was self-assembled onto the surface of gold electrode, and then thiophosphorylated in the presence of target protein kinase and co-substrate adenosine-5'-(γ-thio)-triphosphate (ATP-s). When the thiophosphorylated substrate peptide was exposed to $Ru(bpy)_3^{2+}$-AuNPs, the $Ru(bpy)_3^{2+}$-AuNPs were assembled onto the electrode based on the affinity between the AuNPs and thiol group on the substrate peptide. The ECL response obtained from the $Ru(bpy)_3^{2+}$-AuNPs in the presence of coreactant tripropylamine made it possible to monitor the activity of protein kinase. This platform avoided labeling different peptides with different ECL emitters as signal probes. Simply changing the peptide substrate, this versatile method could detect different target protein kinase. As a proof-of-principle, casein kinase II (CK2) and cAMP-dependent protein kinase (PKA), two classical protein kinases implicated in disease [24.25], are chosen as model protein kinases. A CK2-specific peptide (CRRRADDSDDDDD, Fig. S1) is designed according to Wieckowska et al. [26] as a molecular substrate for CK2; A PKA-specific peptide (CLRRASLG, Fig. S2) is designed according to Bai et al. [27] as a molecular substrate for PKA. Both of two peptides contained a cysteine residue at the terminal of peptide to facilitate self-assembly on the surface of the gold electrode. The characteristics of the versatile and sensitive biosensing platform and the analytical performance of CK2 and PKA are investigated.

2. Experimental

2.1. Reagents and apparatus

Casein kinase II (CK2, P6010L) and cAMP-dependent protein kinase catalytic subunit (PKA, P6000L) were obtained from New England Biolabs (Beverly, MA, USA). Cysteine-terminated peptides CRRRADDSDDDDD and CLRRASLG were synthesized by Shanghai apeptide co., ltd (Shanghai, China) and purified by HPLC and analyzed by MALDI-TOF. Alkaline phosphatase (ALP), Adenosine 5′-[γ-thio] triphosphate tetralithium salt (ATP-γ-s, ATP-s), N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89), 4,5,6,7-Tetrabromobenzotriazole (TBB), Tris(2,2′-ripyridine) dichlororuthenium(II) ($Ru(bpy)_3^{2+}$), 6-mercapto-1-hexanol (MCH) and Tween-20 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Gold(III) chloride trihydrate ($HAuCl_4 \bullet 3H_2O$), Triton X-100, tripropylamine (TPA), sodium citrate, tris(hydroxymethyl)aminomethane (Tris) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). The CK2 storage buffer was 20 mM Tris–HCl (pH 7.4) containing 350 mM NaCl, 1 mM Na_2EDTA, 2 mM DTT and 0.1% Triton X-100. 20 mM Tris–HCl (pH 7.4) containing 50 mM KCl, 10 mM $MgCl_2$ was used as reaction buffer solution. 10 mM Tris–HCl buffer (pH 7.4) containing 0.05% tween-20 was used as the washing buffer. Other reagents were of analytical grade and millipore Milli-Q water (18.2 MΩ cm) was used in this work.

All electrochemical measurements were performed on a CHI 660D electrochemical workstation (Shanghai Chenhua Instrument Co. Ltd., China). ECL measurements were performed with a MPI-A ECL detector (Xi'an Remax Analysis Instruments Co.Ltd, China). A commercial cylindroid glass cell was used as an ECL cell, which

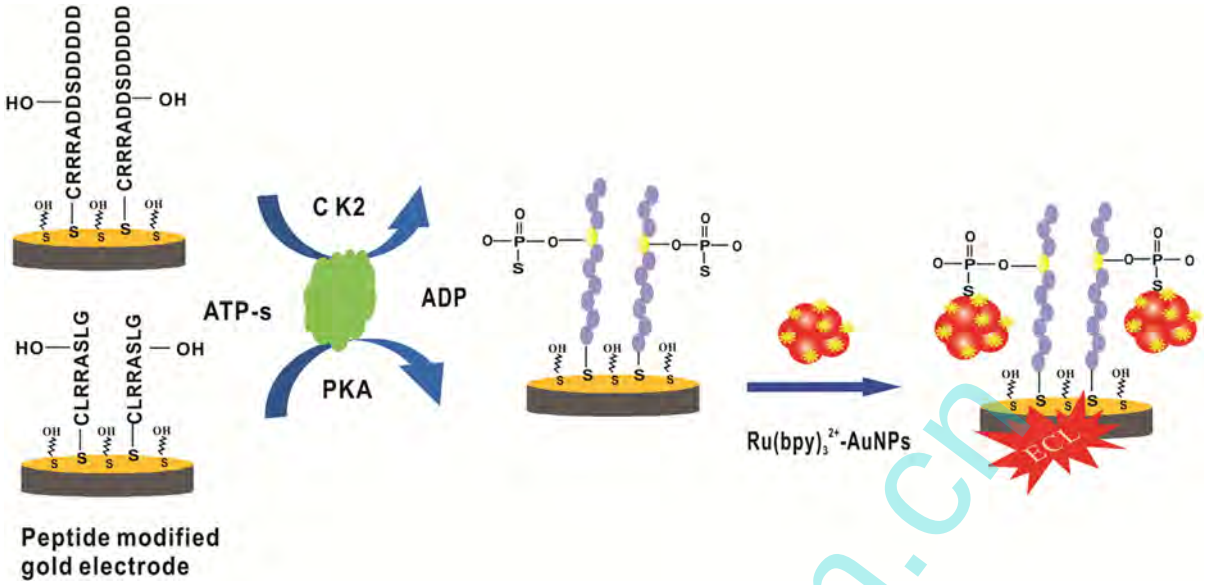

Fig. 1. The schematic diagram of the ECL detection of protein kinase using $Ru(bpy)_3^{2+}$-AuNPs as thiol-versatile signal probe.

contained a conventional three-electrode system that consisted of a gold electrode (2.0 mm diameter) or peptide modified electrode as the working electrode, a platinum wire as the counter electrode, and an Ag/AgCl (saturated KCl) as the reference electrode. All potentials in this work were referred to the reference electrode. The ECL cell was placed directly in front of a photomultiplier tube (PMT) that was biased at 900 V unless otherwise stated, and the PMT window was only opened towards the working electrode to eliminate the blank CL and the blank ECL from the counter-electrode. No any filtering was used in this work because the ECL emission was only come from $Ru(bpy)_3^{2+*}$.

A JEM-2100 transmission electron microscope (JEOL, Japan) was used to obtain Transmission electron micrograph (TEM) images. Atomic force micrograph (AFM) images were obtained with a CSPM5500 Scanning Probe Microscope (Being Nano-Instruments, ltd. China). Static contact-angle measurements were performed on the modified Au slides with Video-based, contact angle measuring device OCA 20 (Dataphysics, Germany).

2.2. Preparation of $Ru(bpy)_3^{2+}$-AuNPs

AuNPs with a diameter of ~12 nm were prepared by citrate reduction of $HAuCl_4$ in aqueous solution according to Reference [28]. According to the process in the previous works [23.29], 100 μL of 10 μM $Ru(bpy)_3Cl_2$ aqueous solution was slowly added into 1.0 mL of AuNPs solution under vigorous stirring at room temperature. The resulting precipitates were collected by centrifugation and re-suspension in 1.0 mL water with sonication to form $Ru(bpy)_3^{2+}$-AuNPs aggregates. The formed $Ru(bpy)_3^{2+}$-AuNPs were characterized by UV–Vis spectrum and TEM (Fig. S-3).

2.3. Immobilization of peptide on gold electrode

The ECL biosensor was fabricated by self-assembling peptide on gold electrode via thiol–gold bond between cysteine residue in peptide and gold electrode as our previous work [30]. Prior to the experiment, a gold electrode was polished with 0.3 μm, 0.05 μm and 0.01 μm alumina slurry and then ultra-sonicated in water for 5 min, and then cleaned electrochemically by a linear scanning potential between 0 and + 1.5 V in 0.10 M H_2SO_4 until a stable cyclic voltammogram was obtained [31]. After being dried with nitrogen, the cleaned gold electrode was immersed into 1.0×10^{-5} M peptide solution (dissolved in 20 mM Tris–HCl, containing 10 mM $MgCl_2$, 50 mM KCl, pH 7.4) at room temperature in darkness for 1 h. Following this treatment, the surfaces were rinsed several times with washing buffer and dried under nitrogen. After that, the resulting electrode was immersed into 1 mM MCH solution at ambient temperature for 30 min to block the nonspecific binding sites on the electrode and washed with washing buffer to get the peptide-modified electrode as ECL biosensor.

2.4. ECL measurements

CK2 and PKA were assayed as following process. Firstly, CK2-catalyzed phosphorylation or PKA-catalyzed phosphorylation were performed by incubating the peptide-modified electrode into a reaction buffer solution containing different activities of target CK2 or target PKA and fixed concentration of ATP-s (75 μM) at 30 °C for 1 h. After thiophosphorylation, 10 μL 5-fold dilution of $Ru(bpy)_3^{2+}$-AuNPs was dropped onto the thiophosphorylated peptide-modified electrode for 1 h. Then the electrode was rinsed thoroughly with 10 mM Tris–HCl (pH 7.4) containing 0.05% Tween-20 to remove adsorption components. ECL measurement was performed at a constant potential of +1.20 V in 1.0 mL of 50 mM Tris–HCl (pH 7.4) containing 50 mM TPA. The initial cycle data was recorded for assay. The activity of CK2 or PKA was quantified by the increased ECL intensity ($\Delta I = I_s - I_0$), where I_0 is the ECL peak in the absence of target protein kinase and I_S is the ECL peak in the presence of target protein kinase. Error bars were obtained from three times parallel experiments. In the inhibition experiment, the procedures were similar as above, except for the reaction buffer solutions containing 0.3 unit/mL CK2 or 0.3 unit/mL PKA and different concentrations of inhibitors.

3. Results and discussion

3.1. Fabrication and characterization of the biosensor

Firstly, CK2 was chosen as model target protein kinase to characterize the fabrication processes and the feasibility of the designed

strategy for the detection of protein kinase. The fabrication processes of the ECL biosensor and ECL detection of CK2 were characterized using surface coverage for the peptide, AFM image for the topography of surfaces, contact angle images for surface charge, electrochemical impedance spectroscopy and cyclic voltammogram for the interfacial electron-transfer behaviors, and ECL method for luminescence properties. The surface coverage of the CK2-specific peptide on the gold electrode surface is estimated on basis of the amount of peptide and the electrode surface area. The amount of the peptide was related with the charge associated with the electrode desorption reaction arising from the one-electron reduction of cysteine-terminated peptide layer on gold surface [32]. The surface coverage of the CK2-specific peptide was calculated to be 3.1×10^{-11} mol cm^{-2} when the electrode area of gold electrode is 0.030 cm^2 (Fig. S-4).

The different electrodes in the fabrication of the ECL biosensor were characterized by cyclic voltammetry and electrochemical impedance spectrometry in the presence of the negatively charged redox couple ferri/ferrocyanide as redox probe (Fig. S-5). It can be seen that the electron-transfer resistance ($R_{et,}$ 4450 Ω) at the peptide-modified electrode was bigger than that (~200 Ω) at a bare electrode. The positive shift of oxidation potential and the negative shift of the reduction potential, and the increase in the peak-to-peak separation ($\triangle E_p$ = 213 mV) and a decrease of peak current ($\triangle i_p$ = 15.6 μA) occurs after the self-assembly of CK2-specific peptide on gold electrode. This is mainly attributed to the fact that the CK2-specific peptide immobilized on the surface of the gold electrode prohibits the mass transfer of $[Fe(CN)_6]^{4-/3-}$ ions from the solution to the electrode surface and partially blocks the electron transfer of $Fe(CN)_6^{4-/3-}$ on the surface of gold electrode [33.34]. The R_{et} at the MCH blocked electrode decreased to 2411 Ω and the current increased to 25.8 μA. A possible reason of the decrease in R_{et} and the increase in current is that nonspecifically adsorbed peptide is removed from the surface by MCH [35.36]. These results supported that the gold electrode is successfully self-assembled with the CK2-specific peptide.

Fig. 2 shows AFM images of different gold plate surfaces. From Fig. 2, it can be seen that plenty of hillocks ascribed to peptide are presented and peptide is adsorbed onto the gold plate as randomly oriented monolayers with rms = 1.42 ± 0.05 nm (b). At the view scale, there is no obvious topography difference between the peptide-modified gold plate and the thiophosphorylated peptide-modified gold plate (c). Upon thiophosphorylation with CK2 and ATP-s and then incubation with $Ru(bpy)_3^{2+}$-AuNPs, it can be seen

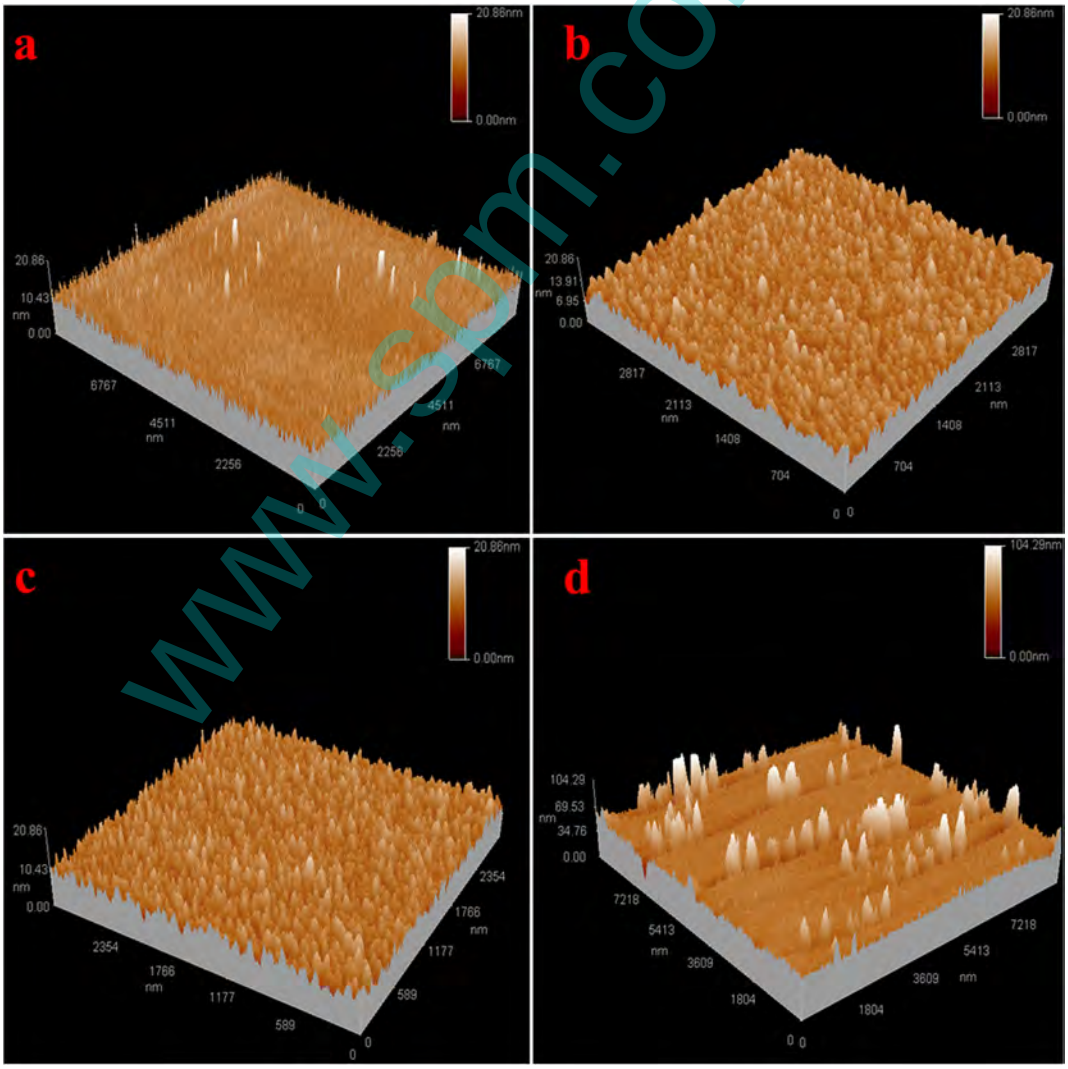

Fig. 2. AFM images on different surfaces. (a) bare gold plate, (b) CK2-specific peptide-modified gold plate, (c) thiophosphorylated peptide-modified gold plate and (d) $Ru(bpy)_3^{2+}$-AuNPs assembled onto thiophosphorylated peptide-modified gold plate.

that $Ru(bpy)_3^{2+}$-AuNPs presenting as islandlike prominences are distributed on the surface of the gold plate surface with diameters of 54.95 ± 0.58 nm(d). This is ascribed to the fact that after the CK2-catalyzed reaction in the presence of ATP-s, the thiophosphate groups were transferred to the serine residues of substrate peptide and $Ru(bpy)_3^{2+}$-AuNPs could then be conjugated onto the thiol-phosphorylated peptide though Au—S bond.

Fig. 3 depicts the shape of an aqueous droplet deposited on different gold plate surfaces. The water droplet positioned on the bare gold surface exhibited a contact angle with 67.3° ± 4.6° (a). The modification of CK2-specific peptide on the gold surface decreased the contact angle to 45.6° ± 4.5°(b), consistent with the formation of peptide layer on gold surface with enhanced hydrophilicity. After treatment of the CK2-specific peptide-modified surface with CK2/ATP-s, the thiol-phosphorylated surface revealed a contact angle of 14.7° ± 1.1°(c). This is attributed to the fact that phosphorylation of the serine unit yields an anionic site that enhances the hydrophilic nature of the CK2-specific peptide-modified surface. The binding of $Ru(bpy)_3^{2+}$-AuNPs to the thiophosphorylated monolayer-modified surface altered the contact angle to 35.5° ± 2.7°(d). The decrease in the hydrophilicity of the surface is attributed to the fact that the $Ru(bpy)_3^{2+}$-AuNPs-thiol-phosphate bond masks the ionic nature of the phosphate groups [37].

The feasibility of the designed strategy for the detection of CK2 was examined by measuring ECL signal at different electrodes and the results were shown in Fig. 4. A very low ECL signal (101 a.u., curve a) at 0.97 V vs Ag/AgCl was obtained at the CK2-specific peptide-modified electrode in 0.1 M PBS containing 50 mM TPA, attributed to the oxidation of TPA at gold electrode [38]. Two weak peaks appeared at 0.95 V and 1.15 V at the CK2-specific peptide-modified electrode treated with $Ru(bpy)_3^{2+}$-AuNPs (330 a.u., curve b), which may be due to the slightly physical absorption of $Ru(bpy)_3^{2+}$-AuNPs. The ECL peak at 0.95 V is mainly attributed to $Ru(bpy)_3^{2+*}$ produced by the reaction between of $TPA^{\cdot+}$ and $Ru(bpy)_3^{+}$ and ECL peak at 1.17 V is generated from the reaction between radical TPA and $Ru(bpy)_3^{3+}$ [39]. A broad and relative obvious ECL signal at 1.0 V (2199 a.u., curve c) was observed at the peptide-modified electrode treated with ATP-s and $Ru(bpy)_3^{2+}$-AuNPs but without CK2. The relatively high ECL signal suggests the relatively strong physical absorption of $Ru(bpy)_3^{2+}$-AuNPs in the presence of ATP-s. Fortunately, it was found that a broad and high ECL signal (6454 a.u., curve d) at 1.0 V was obtained at the CK2-specific peptide-modified electrode after thiophosphorylation with 0.1 unit/mL CK2 and ATP-s and then incubation with $Ru(bpy)_3^{2+}$-AuNPs. When the activity of CK2 was increased from 0.1 unit/mL to 0.3 unit/mL, the ECL intensity was increased correspondingly from 6454 to 11280 (curve e). One broad ECL peak may be ascribed to the overlap of two ECL peaks of $Ru(bpy)_3^{2+}$-TPA due to the change of topography and hydrophobicity of the electrode surface [40,41]. The results reveal that the CK2-specific peptide immobilized onto electrode is thiophosphorylated in the presence of CK2 and co-substrate ATP-s, and then $Ru(bpy)_3^{2+}$-AuNPs is attached onto the thiophosphorylated peptide. At higher kinase activity, there are more thiophosphorylated peptide on the electrode, and then more $Ru(bpy)_3^{2+}$-AuNPs on the electrode, resulting in higher ECL intensity. $Ru(bpy)_3^{2+}$-AuNPs can be used as both signal reporters and signal transduction probe to monitor the CK2 activity. All of above-mentioned results indicate that the assembly process is successful and the as-designed ECL biosensor can be used for monitoring the activity of protein kinase through the strategy of $Ru(bpy)_3^{2+}$-AuNPs report and transduction.

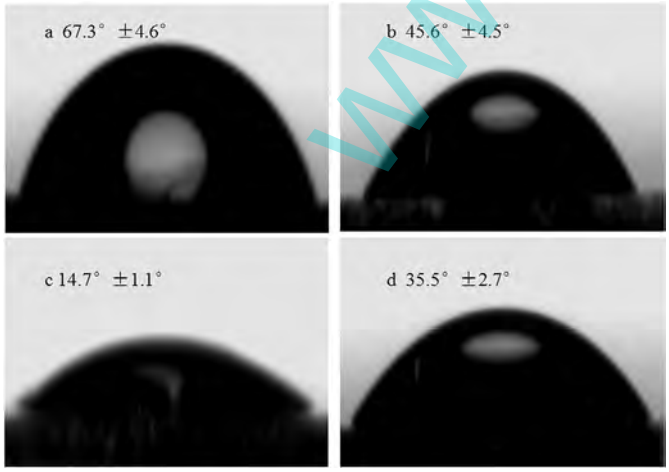

Fig. 3. Contact angle images of aqueous droplets on different surfaces. (a) bare gold plate, (b) CK2-specific peptide-modified gold plate, (c) thiophosphorylated peptide-modified gold plate and (d) $Ru(bpy)_3^{2+}$-AuNPs assembled onto thiophosphorylated peptide-modified gold plate.

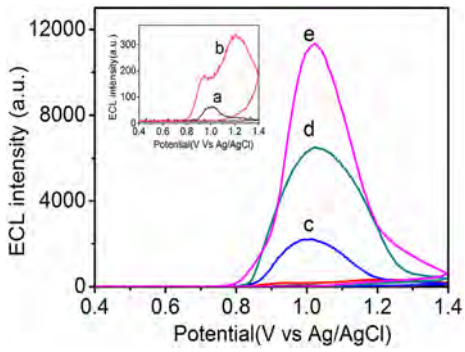

Fig. 4. ECL intensity-potential profiles obtained at different electrode in 50 mM Tris—HCl (pH 7.4) containing 50 mM TPA at a scan rate of 50 mV/s (a) CK2-specific peptide-modified gold electrode; (b) CK2-specific peptide-modified gold electrode treated with $Ru(bpy)_3^{2+}$-AuNPs; (c) CK2-specific peptide-modified gold electrode treated with ATP-s and $Ru(bpy)_3^{2+}$-AuNPs; (d) CK2-specific peptide-modified gold electrode treated with 0.1 unit/mL CK2, ATP-s and $Ru(bpy)_3^{2+}$-AuNPs; (e) CK2-specific peptide-modified gold electrode treated with 0.3 unit/mL CK2, ATP-s and $Ru(bpy)_3^{2+}$-AuNPs.

3.2. Versatile biosensing platform for the detection of CK2 and PKA

Applied potential and phosphorylation time were examined in this work to optimize the detect conditions. +1.20 V vs Ag/AgCl of constant potential and 60 min of phosphorylation time were chosen as optimized conditions for the detection of CK2 (Fig. S-6). It's known that Au—S bond is not stable at a potential as high as ca. 1.0 V [42], therefore, we only recorded the initial cycle data in the ECL detection. The problem can be solved by covalent coupling immobilized peptide onto glassy carbon electrode [43] or by immobilizing peptide onto Nafion-AuNPs modified electrode [44].

The activity of CK2 was evaluated by assay different activities of CK2 under the optimized condition. Fig. 5 shows the ECL intensity vs time profiles for different activities of CK2. The increased ECL intensity ΔI is directly proportional to the CK2 activity in the range from 0.01 unit/mL to 0.5 unit/mL (Fig. 5). The linear regression equation is $\Delta I = 24115C + 1343$ (unit of C is unit/mL) with the correlation coefficient of 0.9884. The detection limit of 0.008 unit/mL was calculated according to the signal which was equivalent to three times the standard deviation of the blanks. The detection limit of CK2 is lower than that of the previous assays (Table S1). High sensitivity and low detection limit were achieved as a result of the signal amplifying strategy of AuNPs. The relative standard

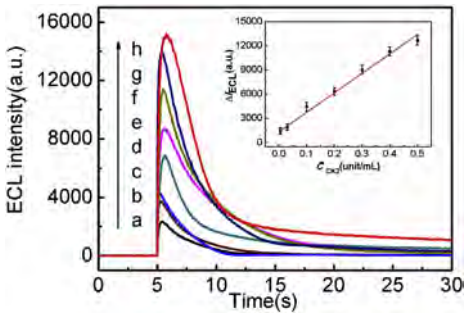

Fig. 5. ECL intensity vs time profiles of the CK2-specific peptide-modified electrode for different concentrations of CK2. Insert, the calibration curve of CK2. Concentrations of CK2: (a) blank, (b) 0.01 unit/mL, (c) 0.03 unit/mL, (d) 0.1 unit/mL, (e) 0.2 unit/mL, (f) 0.3 unit/mL, (g) 0.4 unit/mL, (h) 0.5 unit/mL. The measurement conditions: 50 mM Tris–HCl (pH 7.4) containing 50 mM TPA, applied potential, 1.20 V.

deviation (RSD) was 4.8% obtained for six measurements for 0.1 unit/mL CK2.

The universal of the designed biosensing platform based on $Ru(bpy)_3^{2+}$ functionalized gold nanoparticles ($Ru(bpy)_3^{2+}$-AuNPs) mediated signal transduction is demonstrated for the determination of PKA. The increased ECL intensity ΔI is directly proportional to the PKA activity in the range from 0.01 unit/mL to 0.4 unit/mL (Fig. S-7). The linear regression equation is $\Delta I = 23920C + 2405$ (unit of C is unit/mL) with the correlation coefficient of 0.9919. The detection limit was calculated to be 0.005 unit/mL. The relative standard deviation (RSD) was 3.8% obtained for six measurements for 0.1 unit/mL PKA.

The specificity of the ECL biosensor fabricated by self-assembly specific peptide was tested by examining PKA, ALP and CK2. For CK2-specific peptide modified electrode, a remarkable change in the ECL signal was observed for 0.1 unit/mL CK2 ($\Delta I = 4445$) while negligible changes in the ECL intensity were done in the presence of 2-fold higher activity of other proteins ($\Delta I = 292$ for ALP and $\Delta I = 345$ for PKA). For PKA-specific peptide modified electrode, a remarkable change in the ECL signal was observed for 0.1 unit/mL PKA ($\Delta I = 6000$) while a very slight change in the ECL signal was found for 2-fold higher activity of CK2 ($\Delta I = 580$) and ALP ($\Delta I = 159$) (Fig. S-8). These results indicate the specificity of specific phosphorylation of CRRRADDSDDDDD by CK2 and the specificity of specific phosphorylation of CLRRASLG by PKA.

The potential application of the proposed ECL assay in human serum samples was examined for the detection of CK2 as example. The phosphorylation reaction solution was prepared by adding a desired amount of CK2 in the 20-fold dilution of human serum. Good recoveries are obtained for the investigated human serum in the range from 96.1% to 120%, indicating the feasibility of the ECL method for the determination of CK2 in serum samples (seen Table S-2).

3.3. Evaluation of kinase inhibition

To further demonstrate the potential application of this ECL method in the inhibition assay, the experiments were performed under a fixed CK2 or PKA activity in the presence of different concentrations of protein kinase inhibitors, such as H-89 or TBB (the structure of H-89 and TBB were shown in Figs. S-9 and S-10). Firstly, the effects of H-89 and TBB on the kinase activity of CK2 were examined (Fig. 6). As a result, an inhibitor concentration-dependent decrease of ECL intensity is clearly observed and the ECL signal decreased with the increasing concentrations of H-89 or TBB. The ECL intensity in the inhibition plateau is ca. 2100, which is the blank ECL intensity of the CK2-specific peptide-modified gold electrode treated with ATP-s and $Ru(bpy)_3^{2+}$-AuNPs. Further effort is going on in our lab to reduce the high blank signal. The IC_{50} (inhibitor concentration producing 50% inhibition) of H-89 is estimated to be 5.7 μM, which is slightly higher than that reported in the literature (IC_{50} = 1.18 μM) [13] and lower that reported in the

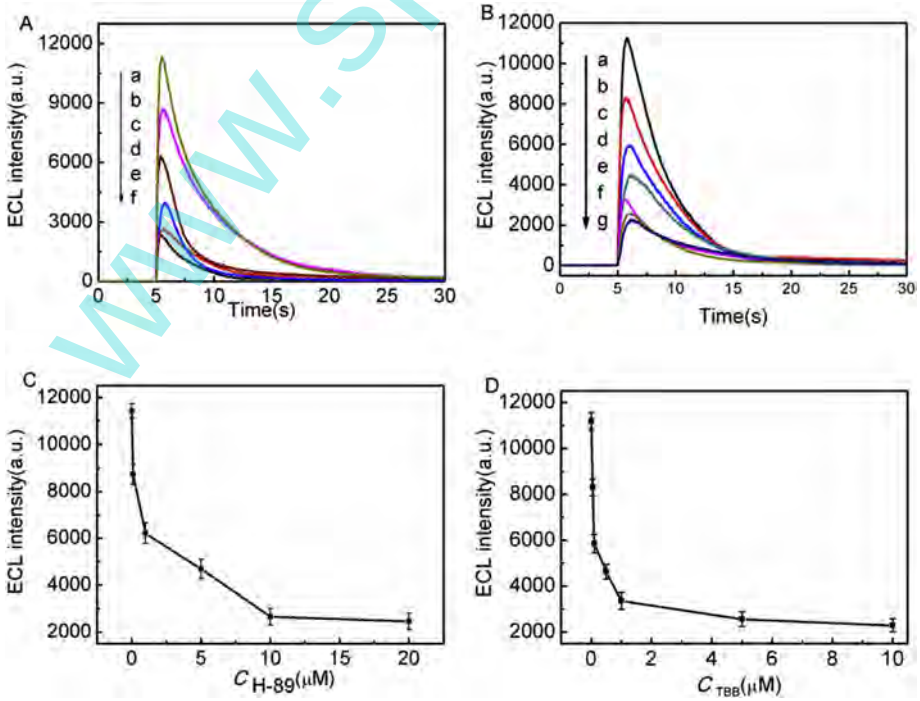

Fig. 6. ECL intensity vs time profiles of the CK2-specific peptide-modified electrode for different concentrations of H-89 (A) and TBB (C); The dependence of ECL intensity on H-89 (B) and TBB (D) concentrations in the presence of 0.3 unit/mL CK2. Concentrations of H-89: (a) 0, (b) 0.1 μM, (c) 1 μM, (d) 5 μM, (e) 10 μM, and (f) 20 μM (C); Concentrations of TBB: (a) 0, (b) 0.05 μM, (c) 0.1 μM, (d) 0.5 μM, (e) 1 μM, (f) 5 μM, and (g) 10 μM (E). The measurement conditions were same as Fig. 5.

literature (IC_{50} = 136.7 μM) [45]. The IC_{50} of TBB is estimated to be 0.58 μM, which is in agreement with that reported in the literature (IC_{50} = 0.50 μM) [46]. Although there are slightly different IC_{50} value, which may rise from the different method and different commercial reagent, it is true that the IC_{50} value of TBB for CK2 is lower than that of H-89. This indicates that TBB is much more potent for CK2 than that of H-89, which is in accordance with previously reported results [45.47].

Secondly, the effect of H-89 on the activity of PKA was examined and the ECL intensities also decreased gradually with the increase of concentration of H-89, indicating the inhibition of PKA activity by H-89 (see Fig. S-11 in supporting information). The IC_{50} value is calculated to be 52 nM, which is slightly higher than that reported in the literature (IC_{50} = 39.5 nM) [27]. As reported in Refs. [48], H-89 inhibits PKA in competitive fashion against ATP. In order to further illustrate the inhibition effect, the inhibition constant K_i was determined according to the equation $IC_{50}/[Substrate] = K_i/K_m$ [49], in which K_m is determined in the absence of H-89 from Lineweaver–Burk double-reciprocal plots (Figs. S-12 and S-13) [48]. K_m is determined to be 61.5 μM and K_i is determined to be 42.6 nM for H-89, which is similar with that of K_i reported in Ref (48 nM) [48]. This suggests that the proposed method has the potential to qualitatively screen the kinase inhibitors.

4. Conclusion

In summary, a sensitive and versatile biosensing platform for monitoring protein kinase activity and inhibition was developed on the basis of $Ru(bpy)_3^{2+}$-AuNPs mediated signal transduction strategy. AuNPs not only can capture numerous signal-generating molecules, resulting in high ECL intensity, but also can recognize thiophosphorylated peptide, providing a universal detection model for protein kinase catalyzed processes using one single $Ru(bpy)_3^{2+}$-AuNPs. Compared with conventional ECL methods with one label per recognition element, the strategy show high sensitivity on basis of signal amplification of $Ru(bpy)_3^{2+}$-AuNPs and the intrinsic sensitivity of ECL method. Compared with these bioassays using one ECL probe for one analyte, this approach can be employed for the bioassay using one ECL probe for multiple targets by simply changing the peptide substrate. This assay was also successfully applied to the detection of CK2 in serum samples and the inhibition of CK2 and PKA, indicating that the developed method have the potential applications in clinical diagnosis. The strategy presented here could be easily extended to develop other ECL and electrochemical biosensing methods for other disease-related protein kinases, and it is promising for multiplex bioassay of protein kinase and can be employed for kinase inhibitor profiling.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.aca.2015.11.047.

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