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Multiple signal amplification electrogenerated chemiluminescence biosensors for sensitive protein kinase activity analysis and inhibition

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ABSTRACT

A novel electrogenerated chemiluminescence (ECL) biosensor was built for the detection of kinase activity based on multiple signal amplification nanoprobes. In this strategy, the Xanthine oxidase (XOD) and 5′-phosphate group end DNA conjugated AuNPs was integrated with the phosphorylated peptide by Zr^{4+}. The XOD on gold nanoparticles can catalyze dissolved oxygen to produce H_2O_2 in the presence of hypoxanthine (HA) which acts as a coreactor for luminol ECL reaction. In addition, due to the excellent catalytic activity of gold nanoparticle toward luminol ECL reaction and its large surface area that can accommodate large number of XOD and DNA on the surface, the ECL signal of luminol was significantly amplified, affording a highly sensitive ECL analysis of kinase activity. The as-proposed biosensor presents a low detection limit of 0.09 U mL^{-1} for protein kinase A (PKA) activity, wide linear range (from 0.1 to 10 U mL^{-1}) and excellent stability even in serum samples. This biosensor can also be applied for quantitative kinase inhibitor evaluation. The robust ECL biosensor provides a valuable tool for the high throughput assay in the applications of clinic diagnostic and therapeutic.

1. Introduction

Kinase catalyzed protein phosphorylation plays a critical regulatory role in a majority of biological processes including metabolism, cell growth, cellular signal communications and survival differentiation (Cohen, 2002; Kalume et al., 2003; Manning et al., 2002). The exceptional protein phosphorylation and kinase activity are coupled with many diseases, such as various of cancers (Ji et al., 2009; Xu et al., 2009) and Alzheimer's diseases (Bertoncello and Forster, 2009; Chong et al., 2005). Over 500 proteins kinase genes are contained in human genes, and about 25% of drug development efforts are now focused on protein kinase inhibitors discovery. Therefore, accurate measurement of protein kinases activity and their potential inhibitors is not only meritorious to the protein kinase-targeted drug discovery and molecular-target therapies but also essential to provide the insights regarding the fundamental biochemical process of signal transduction.

Recently, electrochemical measurements of kinase activity have been received much attention. Comparing to the methods such as radioactive (Juskowiak, 2006), fluorescence (Rothman et al., 2005; Stulz et al., 2011) and surface-plasma resonance (Burge et al., 2006) analysis systems, electrochemical methods are simple, cost-effective and sensitive. A lot of electrochemical biosensors have been designed lately for the detection of kinase activity by measuring the current and charge responses of redox probes conjugated during the phosphorylation processes (Ji et al., 2009; Kerman and Kraatz, 2007, 2009; Kerman et al., 2008′, 2007b; Wieckowska et al., 2008; Xu et al., 2009). Electrogenerated chemiluminescence (ECL) is a light emission process in a redox reaction of electrogenerated reactants, which combines the electrochemical and luminescent techniques (Bertoncello and Forster, 2009; Marquette and Blum, 2009; Marquette et al., 2003; Richter, 2004). The ECL technique not only exhibits high sensitivity and wide dynamic concentration response range, and but also is potential- and spatial-controlled, and is widely used in immunoassay (Ala-Kleme et al., 2006; Jie et al., 2008; Liu and Ju, 2008; Marschall et al., 1995), DNA analysis (Duan et al., 2010; Hu et al., 2009; Li et al., 2007; Pinijsuwan et al., 2008; Zhang et al., 2009), environmental detection and clinic diagnostics (Fahnrich et al., 2001; Lu et al., 2007; Miao and Bard, 2004; Wang et al., 2009). Recently, the ECL biosensors have been also developed for kinase activity analysis (Chen et al., 2013; Zhao et al., 2012), and show a great potential in the sensitive, and rapid measurements of kinase activity and inhibition.

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The rapid developments of nanomaterials facilitate the evolution of ECL biosensor with high-performances. Owing to their good biocompatibility, fascinating electrocatalytic activity, large surface area, excellent conductivity and stability, gold nanoparticles have been widely used in the ECL biosensor developments (Liu and Lin, 2007; Rusling et al., 2009; Wang et al., 2005', 2003', 2004', 2011', 2006). An ECL biosensor for kinase activity detection has been design based on the excellent catalytic activity toward luminol ECL reaction of gold nanoparticles. In an alternative way, a DNA conjugated gold nanoparticles adsorbed with tris(2,2′-bipyridyl)ruthenium(II)[Ru(II)] was reported as an ECL signal amplifier for protein kinase activity analysis, in which the gold nanoparticle can also act as both of conductive element and carrier of electroactive species (Zhao et al., 2012). Even though the enzyme probe for signal amplification has been proved to be high efficient and biocompatible in ECL biosensor design, less work has been reported on the analysis of kinase activity and inhibition.

In this work, a novel enzyme coated gold nanoparticle-based biocatalyzed ECL biosensor was built for the detection of kinase activity employing the Xanthine oxidase (XOD) and 5′-phosphate group end DNA conjugated gold nanoparticles (AuNPs) (XOD–DNA@AuNPs) as multiple signal amplification nanoprobes. After the peptide phosphorylation in the presence of kinase, the XOD–DNA@AuNPs nanoprobes were integrated with the phosphorylated peptide by the coordination between the phosphate group of DNA and Zr^{4+}. The gold nanoparticles are not only the catalyst toward luminol ECL reaction, and are also the large carrier to XOD enzyme and DNA capture probes. Furthermore, the one XOD could biocatalytically produce improved amount of H_2O_2, leading to the significant enhancement of luminol ECL signal. This strategy affords a simple, sensitive, selective and universal platform for kinase activity assay and inhibitor screening.

2. Material and methods

2.1. Materials and reagents

PKA (catalytic subunit) was obtained from New England Biolabs, 4,4,5,5,6,6-hexahydroxydiphenic acid 2,6,2,6-dilactone (Ellagic acid), luminol and hypoxanthine (HA) were purchased from Sigma. Cysteine-terminated kemptide (CLRRASLG) was obtained from GL Biochem (Shanghai, China). 1-Hexanethiol was obtained from J&K Co. (Beijing, China). DNA (5′-P-GCTTGTAGTAGTCTG-C6-SH-3′) were synthesized and purified by Sangon Inc. (Shanghai, China). ATP disodium salt (ATP) was obtained from Dingguo Biological Products Company (China). $HAuCl_4 \cdot 3H_2O$ (48% w/w) was obtained from Shanghai Reagent (Shanghai, China). Xanthine oxidase (XOD) was from Yuanye Biological Company (Shanghai, China). Other regents of analytical grade were provided from Beijing Chemical Company (China).

2.2. Characterization

UV–vis experiments were performed with a UV-3900 spectrophotometer (Hitachi, Japan). TEM images were obtained with a Hitachi model H-800 (Hitachi, Japan). Electrochemical impedance spectroscopy (EIS) was conducted on SP-150 (Bio-Logic, France) in a solution containing 5 mM of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ probes and 0.1 M KCl with the frequency range from 0.01 Hz to 10^5 Hz. The ECL measurements were carried out on a MPI-B multifunctional electrochemical analytical instrument (Xi'an Remex Analytical Instrument Ltd. Co., China). The voltage of PMT was maintained at 600 V.

2.3. Preparation of XOD–DNA@AuNPs nanoprobes

The gold nanoparticles were fabricated as reported previously (Grabar et al., 1995). In brief, 100 mL of 0.01% (w/v) $HAuCl_4$ solution was boiled with vigorous stirring, and 2.5 mL of 1% (w/v) trisodium citrate solution was quickly added to the boiling solution. Wine red solution was obtained, indicating the formation of AuNPs which was characterized by transmission electron microscopy (TEM) and UV–vis spectra. The XOD–DNA@AuNPs nanoprobe was prepared by adding 20 μL of XOD into 1 mL of gold colloidal solution. After stirring for 2 h, 150 μL of DNA was added into above XOD–AuNPs solution, and was incubated for 12 h at 4 °C. Then, 100 μL of 1 M NaCl was added dropwise. Finally, the mixture was centrifuged at 12,000 rpm for 10 min twice and redispersed in the buffer solution (100 mM NaCl, 50 mM Tris–HCl, pH 7.4). The as-prepared DNA and XOD conjugated AuNPs were characterized and used for kinase activity detection.

2.4. Assembly and phosphorylation of peptides on gold electrode

A gold electrode (diameter of 2 mm) was polished carefully first with 0.3 μM Al_2O_3 powder on fine abrasive paper, and was washed with distilled water and ethanol, respectively. Then, the gold electrode was activated in 0.5 M H_2SO_4 between 0.2 and 1.65 V (vs Ag/AgCl) before modification. After drying with N_2, the gold electrode was immersed into a PBS (10 mM, pH 7.4) solution containing 500 μM cysteine terminated kemptide at room temperature for 12 h. After thoroughly washing by PBS solution, the electrode was incubated in 1 mM hexanethiol solution for 30 min to block the electrode, and then washed thoroughly with PBS solution. PKA catalyzed phosphorylation reaction was performed by incubating the modified electrode in the buffer solution (50 mM Tris–HCl and 20 mM $MgCl_2$, pH 7.4) containing a desired amount of PKA and ATP at 37 °C for 1 h. For PKA inhibitor assay, the procedures were similar as above except that inhibitors with different concentrations were added in the PKA reaction solution.

2.5. ECL characterization and kinase activity analysis

The phosphorylated kemptide modified electrode was treated with 0.5 mM Zr^{4+} at room temperature for 1 h, and was rinsed with PBS solution and distill water. After drying with N_2, the electrodes were incubated in the solution containing XOD–DNA@AuNPs nanoprobes, and were thoroughly washed and nitrogen flow dried. Finally, the modified electrode was immersed in 0.1 M PBS (pH 8.0) solution containing 100 μM luminol and 0.1 M HA for ECL characterization.

2.6. Kinase activity assay in MCF-7 lysates

MCF-7 cells (1×10^5 cells) were supplemented with 10% fetal bovine serum, MEM nonessential amino acid solution (0.1 mM), 1% insulin-transferrin-selenium-A supplement, penicillin (100 U mL^{-1}), streptomycin (100 mg mL^{-1}), and amphotericin B (0.25 mg mL^{-1}). The cells were incubated under a humidified atmosphere containing 5% CO_2 at 37 °C. The culture medium was replaced by serum-free medium and the cells staved for 4 h before stimulation. Then the cells were treated with adenylate cyclase activator, forskolin and PKA inhibitors, ellagic acid, respectively with final concentration at 25 uM for 30 min before harvesting. The cultured cells were removed by scraping and lysed and the cell lysates were clarified by centrifugation at 12, 000 rpm for 4 min at 4 °C. Finally, the clarified lysates were ready for the activity and inhibition PKA detections.

3. Results and discussion

3.1. Multiple signal amplification strategy for ECL kinase activity detection

The proposed multiple signal amplification ECL strategy for kinase activity detection is demonstrated in Scheme 1. The cysteine-terminated kemptides were assembled on the gold electrode through the Au–S bond. Then, the peptide modified electrode was treated with hexanethiol to block blank binding sites. In the present of PKA and ATP, the serine of substrate peptide was phosphorylated and conjugated with XOD–DNA@AuNPs by the coordination interaction between Zr^{4+} and the 5′-phosphate end of DNA. The XOD on gold nanoparticles can catalyze the reaction between hypoxanthine and dissolved oxygen to produce uric acid and H_2O_2 which significantly improves the ECL signal of luminol as a coreactor. In addition, the introduction of gold nanoparticle by immobilizing XOD on the surface can further enhance the ECL signal owing to the excellent catalytic activity of gold nanoparticle toward luminol ECL reaction and its large surface area that can accommodate large number of XOD on the surface (Xu et al., 2011). As a result, a significant ECL signal amplification can be obtained for sensitive detection of phosphorylation and kinase activity.

3.2. Characterization of the XOD–DNA@AuNPs nanoprobes

The gold nanoparticles and the XOD–DNA@AuNPs nanoprobes were characterized by TEM and UV–vis spectra, as shown in Fig. S1. An absorption peak at 520 nm (curve a) was observed for the as prepared gold nanoparticles, and the diameter of the gold nanoparticles was ca. 15 nm which was confirmed by TEM pattern (the inset in Fig. S1). After the modification of XOD and DNA, the absorption peak of gold nanoparticles was red-shifted to 524 nm due to the surrounding environment change, indicating the formation of XOD–DNA@AuNPs. To further conform the nanoprobes, the enzymatic activity of the XOD–DNA@AuNPs nanoprobes were characterized based on the color reaction of ABTS and H_2O_2 as substrates. Using HRP as catalyst and H_2O_2 as coreactor, ABTS was changed to its oxidation product ($ABTS^{\bullet+}$), a blue–green radical cation, which shows three characteristic absorption peaks centered at 645 nm, 734 nm and 815 nm. As shown in Fig. 1, There is no obvious absorption peak in the visible region in the mixture of ABTS, HRP and HA (curve a). In addition, the mixed solution containing ABTS, HRP, HA and AuNPs only shows a absorption peaks centered at 520 nm, which is the characteristic peak of AuNPs (curve b). As a comparison, three obvious absorbance peaks in the visible region was observed (curve c) when the XOD–DNA@AuNPs probes was added in the mixture of ABTS, HRP and HA. The phenomenon is similar to that of the mixture of ABTS, HRP and H_2O_2 (curve d), which indicates the successful immobilization of XOD with high activity on the gold nanoparticle.

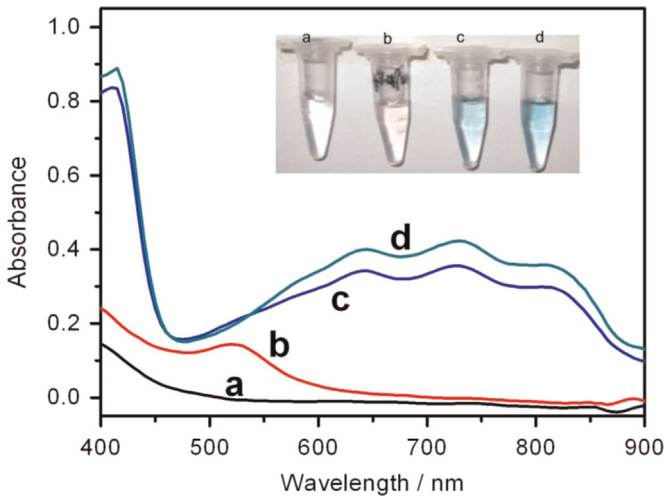
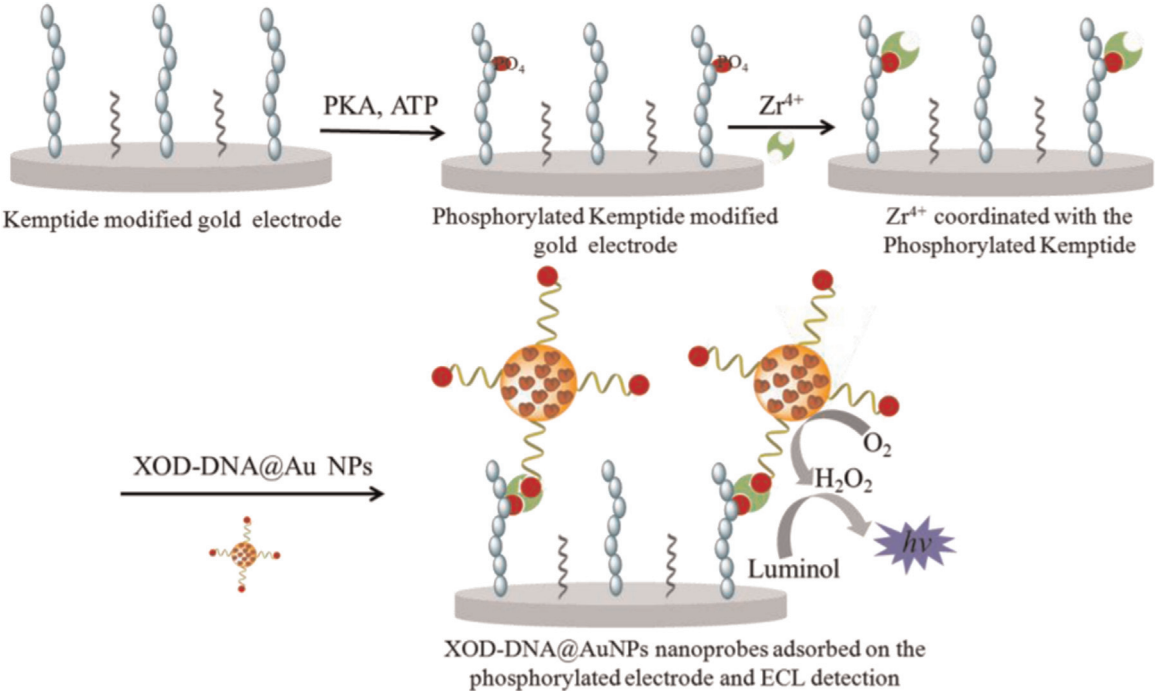


Fig. 1. The UV–vis spectra of the mixture of ABTS, HRP and HA (a), ABTS, HRP, HA and AuNPs (b), ABTS , HRP, HA and XOD–DNA@AuNPs nanoprobe (c), and ABTS, HRP and H_2O_2 (d). The inset is the corresponding photographs of the mixture.



Scheme 1. The configuration of multiple signal amplified ECL biosensor for PKA activity analysis including the procedures of phosphorylation of kemptide in the presense of PKA and ATP, coodinative adsorption of Zr^{4+} on the electrode and the assembly of XOD–DNA@AuNPs nanoprobe.

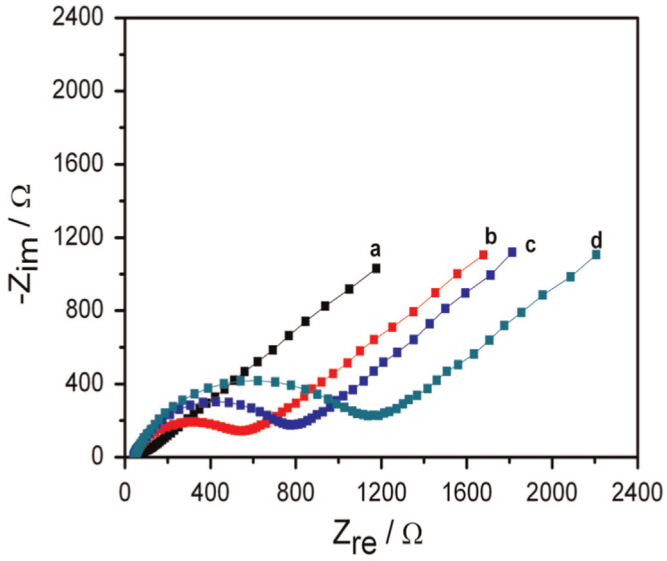


Fig. 2. Electrochemical impedance spectra of bare Au electrode (a), kemptide modified Au electrode blocked by hexanethiol (b), the kemptide modified Au electrode after phosphorylation by PKA (c), and P-kemptide modified Au electrode bound with XOD–DNA@AuNPs by Zr^{4+} (d).

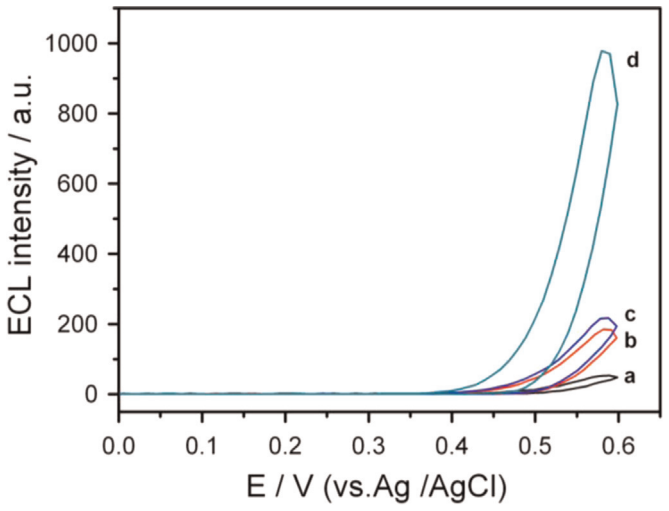


Fig. 3. The ECL intensity–potential curves of kemptide modified gold electrode (a), phosphorylated kemptide modified gold electrode with the treatment of XOD–DNA@AuNPs in the absence of Zr^{4+}(b), the phosphorylated kemptide modified electrode without the treatment of XOD–DNA@AuNPs in the presence of Zr^{4+}(c), and the phosphorylated kemptide modified electrode after treatments with both XOD–DNA@AuNPs and Zr^{4+}(d). The curves were recorded at scan rate of 100 mV s^{-1} in a 0.1 M PBS solution (pH 8.0) containing 100 μM luminol and 0.1 M HA. The voltage of PMT was maintained at 600 V. The concentration of PKA is 100 U mL^{-1}.

3.3. Electrochemical characterization of the biosensor

EIS is a powerful tool to evaluate interfacial properties of the electrode, and was used to characterize the assembly processes of the gold electrode using $[Fe(CN)_6]^{4-/3-}$ as electroactive probes. Fig. 2 presents the Nyquist plot of the assembled electrodes. In the Nyquist plot, the diameter of the semi-circle at high frequency region equals to the electron transfer resistance of the electrode interface. It was obvious that the diameter of semi-circle after the assembly of kemptide (curve b) was larger than that of bare gold electrode (curve a), indicating an increase of the electron transfer resistance after the assembly of kemptide on the gold electrode. This is on account to the electron inert feature of kemptide that blocks the electron transfer on the electrode interface. The electron transfer resistance was further decreased after the phosphorylation by kinase (curve c) which was ascribed to the electrostatic repulsion between the negative charged phosphate groups and electroactive probes. After the conjugation of Zr^{4+} and XOD–DNA@AuNPs nanoprobes (curve d), the electron transfer resistance also increase because of the negative charge and electron inert characteristic of the DNA and XOD on the gold nanoprobes.

3.4. ECL characterization and kinase activity analysis

In the presence of HA, XOD on gold nanoparticles can catalyze the reaction to produce H_2O_2 that significantly improves the ECL signal of luminol as a coreactor. In addition, AuNPs can not only increase the loading capacity of XOD on the modified electrode to amplify luminol ECL signals but also catalyze the ECL reaction of luminol. Thus, the XOD–DNA@AuNPs nanoprobes can mediate the ECL reaction even at a low content, allowing highly efficient ECL signal amplification. Fig. 3 shows the ECL response of the kemptide modified gold electrode before (curve c) and after (curve d) phosphorylation by PKA with the treatments of XOD–DNA@AuNPs mediated by Zr^{4+}. As it is shown, the kemptide modified electrode before phosphorylation exhibits a weak ECL signal. After the phosphorylation by kinase, a strong ECL signal was observed due to the XOD–DNA@AuNPs nanoprobes can largely amplify the ECL signal of luminol. In addition, nearly no ECL signal was observed on the peptide modified electrode without treatment (curve a). As a control, the ECL signal of the phosphorylated peptide modified electrode with nanoprobes was measured without the treatment of Zr^{4+} (curve b), and only a weak ECL signal was observed. It is indicated that the nanoprobes are conjugated on the electrode through the coordination interaction between Zr^{4+} and phosphate groups. The facts demonstrate that the multiple XOD–DNA@AuNPs nanoprobes signal amplification strategy can be applied for sensitive ECL detection of kinase activity and inhibition.

3.5. Optimization of the experimental Conditions

The phosphorylation reaction plays a critical role on the properties of the ECL biosensor for kinase activity detection. It's known that the pH has a great influence on the enzymatic catalysis reaction. The extreme pH of the solution may make the enzyme inactivated. Fig. S2A shows the ECL signal intensity at different pH of the reaction solution. The ECL signal of the electrode increased when the pH increase from 6.5 to 8.0, and then it decreased (Fig. S2A). Therefore, the optimal pH for the phosphorylation reaction was 8.0. In addition, the ECL performance of the biosensor was closely related to effect of probe incubation time. The ECL signal quickly increase and then reached a plateau in 60 min (Fig. S2B), indicating a completely phosphorylation of kemptide. After that, the increasing time nearly don't affect the ECL intensity. Therefore, the optimal phosphorylation time was 60 min and was used in the following experiments.

3.6. ECL detection of protein kinase activity

On the basis of the optimal conditions, the activity of protein kinase was analyzed with different concentrations of PKA. Fig. 4 displays the ECL signals of the ECL biosensor corresponding to different PKA concentrations. The ECL signal increased accordingly with the increase of PKA concentration, and reached platform value after 50 U mL^{-1}. A linear relationship between the ECL signal and the concentrations of PKA was obtained from 0.1 to 10 U mL^{-1}, and can be represented as $I_{ECL(a.u.)}=58.8\times C_{PKA}$ (U mL^{-1})+212.8, $R=0.997$, where I is the ECL intensity and c is the kinase activity. The detection limit of PKA was 0.09 U/mL ($S/N=3$), which was lower than that of previous reported

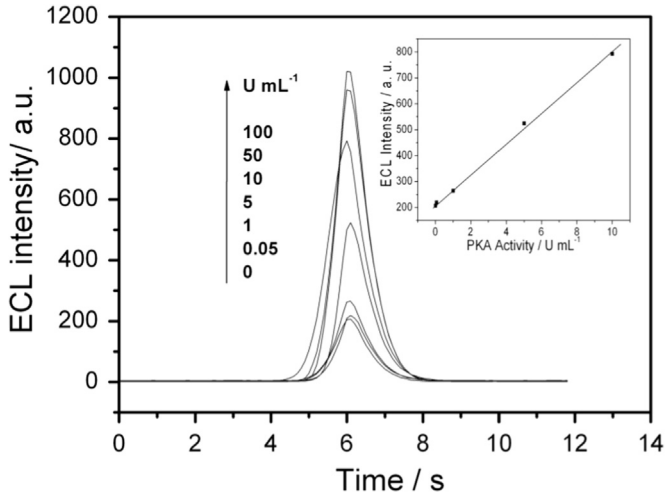


Fig. 4. The ECL intensity–time curves in the 0.1 M PBS solution (pH 8.0) containing 100 μM luminol and 0.1 M HA and different concentrations of PKA. The inset is the linear relationship between ECL intensity and the concentrations of PKA. The scan rate is 100 mV s^{-1}. The voltage of PMT was maintained at 600 V. All experiments were carried out in 0.1 M PBS solution (pH 7.6) containing 100 μM luminol and 0.1 M HA.

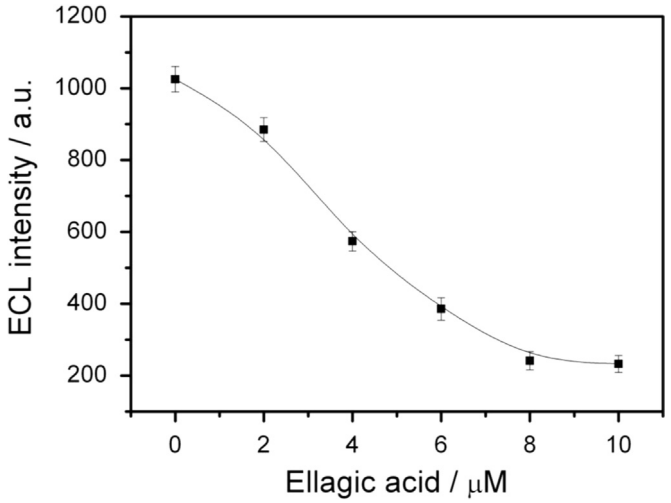


Fig. 5. The ECL intensity as a function of concentration of ellagic acid. Error bars were obtained from parallel experiments ($n=3$). The phosphorylation of kemptide was carried out with 100 U mL^{-1} PKA.

electrochemical assays (Ji et al., 2009; Kerman et al., 2007a; Xu et al., 2009). This value was higher than that reported ECL biosensor based on the magnetic beads (MB) technology (Zhao et al., 2012) and signal enhancement of gold nanoparticles (Chen et al., 2013), which could be ascribed to ECL background of luminol solution and the nonspecific adsorption of the XOD and DNA conjugated gold nanoparticle probes. Otherwise, the sample separation was avoided in this biosensor.

Operational stability was evaluated by the successive potential scanning. Fig. S3 displays the ECL emission of the biosensor under continuous potential scans in 0.1 M PBS in the presence of 100 μM Luminol and 0.1 M HA. Stable and high ECL signals were observed with a relative standard deviation of 4.7%, indicating the excellent potential cycling stability of the ECL biosensor. Meanwhile, the reproducibility of the ECL biosensor was also studied by determining the same level kinase activity with five electrodes, and the relative standard deviation was obtained to be 4.6% at the kinase activity of 100 U mL^{-1}. Thus, the as-proposed biosensor exhibited good performance in the detection of PKA activity.

3.7. Kinase activity inhibition evaluation

The screening of kinase inhibitors for kinase activity regulation is important in the applications of disease diagnosis and clinic therapy. The multiple amplified ECL biosensor was also used to quantitatively evaluate the inhibition efficiency of kinase using ellagic acid as a model, which is a cell-permeable antioxidant with anti-mutagenic and anti-carcinogenic characteristics. In the inhibition assay, the PKA activities were evaluated in the presence of ellagic acid at different concentrations, and the half-maximal inhibition value IC50 was calculated. Fig. 5 shows the ECL responses of the biosensor with different concentration of ellagic acid. The ECL signal decreased along with the increasing concentrations of ellagic acid, and then reached a stable level when the concentrations of ellagic acid were over 8 μM. Based on the above results, the IC_{50} of ellagic acid was measured to be 3.65 μM, which is in agreement with that reported in literatures obtained with conventional kinase assay.(Cozza et al., 2006; Xu et al., 2010) These facts imply that the as designed multiple signal amplified ECL biosensor has great potential in quantitative kinase inhibitors screening.

3.8. PKA assay in biological Fluids

To further demonstrate the application of the as-designed biosensor in complex biological samples, we applied this sensor in FBS for kinase activity detection. The PKA in FBS samples were conducted by adding certain concentrations of PKA in ten times diluted FBS solutions, and the PKA activity were measured. Table S1 (in Supporting information) shows the PKA activity in the FBS solution with three parallel measurements by the ECL biosensor, and was compared with the reference values. The relative deviation between the ECL biosensor and reference value was ranged from −4.7% to 1.1%, suggesting the excellent accuracy, and can be used as a reliable technique for the kinase activity detection in serum samples. In addition, the activation and inhibition of the intracellular PKA from MCF-7 Cell line were also measured by the treatment of forskolin and ellagic acid respectively as shown in Fig. S4. It was observed that the MCF-7 cells treated with ellagic acid exhibited lowest PKA activity of ca. 120 U, and it was lower than that of the control cell line without stimulations of 140 U. The MCF-7 cell line treated with forskolin expressed exhibited the highest level of PKA among these samples. The results were also compared with those by ELISA measurements, and similar results were obtained for the cell line with forskolin treatment. The results measured by ECL biosensor for the controlled cell line and that treated with ellagic acid were slightly higher than that by ELISA, which may be resulted from the nonspecific adsorption on the proteins from the cell lysis. These facts demonstrated that the ECL biosensor could be used for kinase activity detection in complex biological samples with high sensitivity.

4. Conclusion

In conclusion, a novel multiple signal amplification ECL biosensor has been developed for kinase activity and inhibition assay, which integrates the synergetic catalysis of both gold nanoparticles and the in situ generated coreactant H_2O_2 by XOD and the high loading of DNA capture probes and XOD on gold nanoparticles to amplify luminol ECL signal. As a result, the as-proposed ECL biosensor offers a highly sensitive method for PKA activity analysis with a low detection limit of 0.09 U mL^{-1}, wide linear range, and excellent stability. The as designed ECL biosensor also offers a highly sensitive strategy for PKA kinase activity monitoring even in the complex biological samples. In addition,

the biosensor also shows great potential in the application of accurate and quantitative kinase inhibitor assay. The robust biosensor can also be ready for high through analysis of kinase activity and inhibition in clinic diagnostics and drug discovery applications.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.02.006.

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