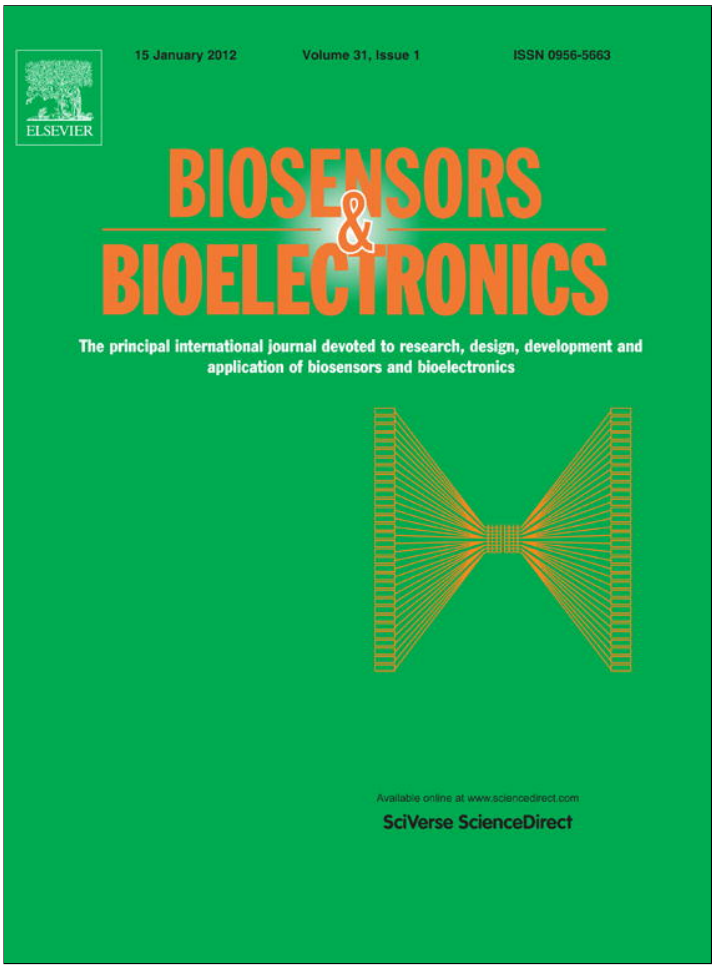

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Highly sensitive protein kinase activity assay based on electrochemiluminescence nanoprobes

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ABSTRACT

Herein, we describe a novel electrochemiluminescence (ECL) biosensor for protein kinase activities and inhibition monitoring based on the magnetic beads (MB) technology and signal enhancement of gold nanoparticles (GNP). In this design, ECL nanoprobes were prepared by conjugating GNP with phosphorylated DNA capture probes and tris-(2,2′-bipyridyl) ruthenium (TBR)–cysteamine. Zirconium cations, a specific bridging agent, mediate the linkage between biotin modified phosphorylated peptides and ECL nanoprobes. The complexes were then captured and enriched on the electrode surface by streptavidin-coated MB for ECL reaction. To confirm the feasibility of this biosensor, we employed protein kinase A (PKA) as the model kinase to validate the assay and a satisfactory detection limit of 0.005 U/mL was achieved. The combination of ECL and GNP lays a solid foundation for highly sensitive assay, meanwhile, the coupling of MB surfaces used for separation and capture with unmodified ECL electrode detection results in a greatly simplified and reusable protocol. Thus, our biosensor offers great promise for a highly sensitive and simple assay for protein kinase activity. Furthermore, the inhibition of PKA activity was monitored on the basis of the ECL signals change in response to the concentration of PKA inhibitor.

1. Introduction

The phosphorylation of proteins catalyzed by kinases is known as a control mechanism with most aspects of physiology functions. Previous studies have demonstrated that protein kinases play crucial regulatory roles in most metabolic pathways and in regulating many cellular processes, such as cell cycle regulation, cell migration, and survival differentiation (Bertolotto et al., 1998; June et al., 1990; Manning et al., 2002). Meanwhile, the over-expression of protein kinases is reported to indicate various diseases such as diabetes, aggressive tumor or Alzheimer's disease (Sebolt-Leopold and English, 2006). As a result, monitoring and analyzing kinase activity have become a critical topic, which is not only necessary for elucidation of signal transduction mechanisms in basic biology, but also very important for clinical pharmacology, drug discovery and treatment (Chong et al., 2005; Cohen, 2002; de Jonge and Verweij, 2006).

The generally used techniques for the measurement of protein kinase activities mostly relied on radioisotopic methods which used radioactive ATP for monitoring phosphorylation reactions (Lehel et al., 1997; Macala et al., 1998) and phosphor-specific antibodies bound to the phosphorylated proteins specifically (Sato et al., 2002; Umezawa, 2005). These methods had wide applications on diagnosing disease. However, radio-isotope based assays may damage human cells and antibodies, besides, these methods need sophisticated preparation of recognition protein, which is laborious and expensive.

With the purpose of eliminating those drawbacks and achieving high sensitivity, many novel detection strategies were developed such as mass spectroscopy (Beausoleil et al., 2006), surface plasmon resonance based systems (Moon et al., 2007; Stenlund et al., 2006), fluorescence-based assays (Shults and Imperiali, 2003; Shults et al., 2005; Xu et al., 2011) and electrochemical methods (Wang et al., 2011).

Among them, the electrochemical sensing system attracted substantial research interest for its distinctive features such as high sensitivity and no need for complicated equipments. This system was associated with other signal amplification technologies such as horseradish peroxidase, TiO_2-assisted silver enhancement, and GNP, which gained widespread acceptance to perform electrochemical assay better (Ji et al., 2009; Wieckowska et al., 2008; Xu et al., 2009). Broadly speaking, the electrochemical systems were the promising approaches of protein kinase activity monitoring because of their high sensitivity. However, their applicability is limited due to the indispensable operation process of immobilizating substrate on electrode surface. It was complicated and laborious, and made the electrode be reused hardly. Also, ultrahigh sensitivity is needed in the fields of clinical diagnosis and pharmacological study, but detection limit of those assays cannot meet the requirement. Thus, it is still challenging in developing a more sensitive,

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rapid and simple assay for monitoring the activity and inhibition of protein kinases.

Electrochemiluminescence is a marriage between electrochemical and chemiluminescent methods and exhibits the merits of both such as high sensitivity and selectivity, good temporal and spatial control, and versatility. Hitherto, ECL has been employed as a powerful technique for immunoassays (Jie et al., 2010; Kijek et al., 2000; Niu et al., 2011; Yu et al., 2000), DNA analyses (Bergen et al., 1996; Qian et al., 2010; Zhang et al., 2009), environmental pollution monitoring (Li et al., 2010, 2011; Qiu et al., 2011; Rusling, 2009; Zhu et al., 2009, 2010), capillary electrophoresis (Deng et al., 2009; Du and Wang, 2007; Gao et al., 2006; Huang et al., 2007), medical diagnosis (Han et al., 2011; Hu and Xu, 2010; Rider and Newton, 2002; Su et al., 2010) and so on. However, only few ECL biosensors for protein kinases activity assay have been reported recently, which are potential systems for highly sensing but strongly neglected (Xiao et al., 2007; Xu et al., 2010).

Aspiring after higher sensitivity is one of the main aims in biological assays and the higher the sensitivity of detection technologies are, the better, and the more delighted we shall be. For this purpose, lots of research works have focus on exploration of novel tools to improve the detection sensitivity. Among them, GNP come to the avant-courier and are widely applied in bioassay (Peng et al., 2010; Oishi et al., 2007; Sawada et al., 2011). One of the latest important applications of GNP is signals amplification associated with self-assembled GNP-biomolecules structures, which provide more binding sites or carry more target signals. These designs open the door to ultrasensitive ECL detections with polymerase chain reaction (PCR)-like sensitivity for DNA, RNA, protein targets and so on.

However, the combination of ECL and GNP cannot settle problems which are long-standing in electrochemical or ordinary ECL biosensors, such as the needing of a complex series of washing steps and a laborious surface-pretreated process for substrates immobilization on the electrode. Fortunately, the development of magnetic materials and technology effectively overcomes most of weaknesses previously mentioned. The coupling of MB surfaces used for separation and reaction with unmodified ECL electrode detection results in a greatly simplified and reusable protocol.

Herein, we present a simple and highly sensitive ECL method for protein kinase activity assay and inhibition monitoring based on the MB technology and signal enhancement of GNP. In this ECL-based strategy, zirconium cations (Zr^{4+}) are used for linking ECL nanoprobes with peptides and result in the capture of ECL nanoprobes on MB surface. Zr^{4+} can easily recognize phosphates and rapidly link two biomolecules containing phosphate groups without the need for chemical activation steps, thus our strategy would be simple and rapid (Dong et al., 2007; Monot et al., 2008; Nonglaton et al., 2004). Taken as a whole, the couple of ECL and GNP provides the possibility for highly sensitive assay and the application of MB and Zr^{4+} results in a greatly simplified and reusable protocol. To the best of our knowledge, we are integrating these strategies into one assay system for the first time. Moreover, this assay system will be further applied as universal platform for clinical diagnosis.

2. Materials and methods

2.1. Materials

PKA (catalytic subunit from bovine heart), adenosine 5′-triphosphate (ATP) disodium salt hydrate, tris (2-carboxyethyl) phosphine hydrochloride (TCEP), tripropylamine (TPA) and other chemicals to synthesize the tris-(2,2′-bipyridyl) ruthenium N-hydroxysuccinimide ester (TBR-NHS ester) were purchased from Sigma (St. Louis, MO). Biotinylated Kemptide (biotin-LRRASLG) and DNA capture probes (5′-HPO_4^--CCTGAGACGTTTTTTTT-SH-3′) were synthesized and HPLC-purified by Sangon Inc. (Shanghai, China). Sodium Citrate and hydrogen tetrachloroaurate(III) tetrahydrate ($HAuCl_4{\cdot}4H_2O$) were both from Sinopharm Group Chemical Regent Co., Ltd. (Shanghai, China). Streptavidin-coated magnetic beads (2.8 μm in diameter) were obtained from Dynal Biotech (Lake Success, NY). $ZrOCl_2$, cysteamine and ellagic acid were purchased from TCI (shanghai) Development Co., Ltd. Other chemicals were of analytical reagent grade and were used as received. In all instances, high-purity deionized water (>18 MΩ) was used throughout.

2.2. Preparation of ECL nanoprobes

GNP (13 nm) were prepared according to literature (Grabar et al., 1995). Ruthenium bis (2,2′-bipyridine) (2,2′-bipyridine-4,4′-dicarboxylic acid) N-hydroxysuccinimide ester (TBR-NHS ester) was synthesized and activated by our laboratory in term of previously published papers (Zhou et al., 2009). The procedure of synthesizing ECL nanoprobes was as follows. The mixture of DNA capture probes and cysteamine (both 7.2 μL, 100 μM) was activated in Tris acetate buffer (pH 8.2) with 6 μL of 10 mM TCEP for 30 min, then 1 mL of GNP was added and incubated for 6 h. Followed by adding 120 μL of sodium boric acid buffer (0.1 M, pH 9.0) and 8 μL TBR (11.2 mM), and incubating under shake overnight in dark. Then the solution was centrifuged at 12,000 rpm for 30 min and resuspended in 800 μL of washing buffer (100 mM NaCl, 25 mM Tris acetate, pH 8.2). Note that well-synthesized ECL nanoprobes should be in the same color as the unmodified GNP with no visible aggregates.

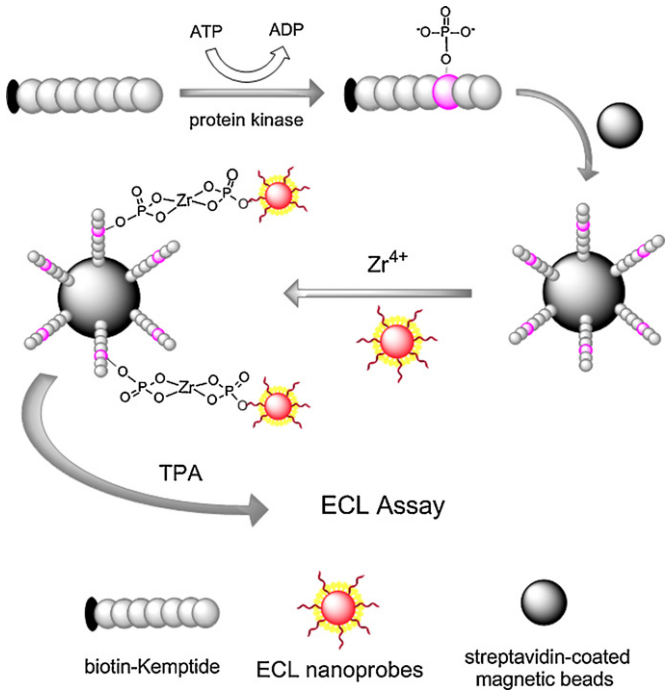
2.3. PKA-catalyzed phosphorylation and its inhibition

PKA-catalyzed phosphorylation was performed by the following protocols. First, PKA was diluted in the supplied solution (20 mM Tris–HCl, 50 mM NaCl, 2 mM DTT, and 1 mM EDTA, 50% glycerol, pH 7.5). Then, different amount of PKA were respectively added into an reaction buffer (40 mM Tris–HCl and 20 mM $MgCl_2$, pH 7.4) containing a desired amount of substrate Kemptide (20 μM) and ATP (40 μM). The mixture was incubated at 30 °C for 1 h with gently stirred. After this step, 15 μL (10 μg/μL) of streptavidin coated magnetic beads were added to the mixture with gentle stirring for 15 min. Finally, magnetic beads capturing phosphorylated Kemptide were resuspended in 100 μL reaction buffer after 3 times washing to effectively remove unwanted constituents. In this strategy, Tris–HCl buffer was used instead of the PKA sample solution for the control.

For PKA inhibition experiments, the procedures were similar as above, except for involving the reaction between 100 U/mL PKA and different concentrations of inhibitors (0–20 μM) in the reaction buffer.

2.4. ECL detection

ECL assays were accomplished with our custom-built ECL detection system (Zhou et al., 2009). Detailed operating procedures were as follows: The above mentioned magnetic beads were treated with 10 μL of ECL nanoprobes and 10 μL of Zr^{4+} solution (20 μM) at room temperature for 60 min. Via magnetic separation, they were then washed three times with reaction buffer to remove all unbounded ECL nanoprobes and resuspended in 100 μL of ECL assay buffer (100 mM TPA, 40 mM Tris–HCl, 20 mM $MgCl_2$, 50 μM NaCl, 7 mM NaN_3, 0.8 μM TritonX-100 and 0.4 mM Tween20, pH 8.0). Well-processed assay buffer was injected to the ECL reaction cell where the MB–Zr^{4+}–ECL nanoprobes complexes were captured on, and temporarily immobilized on the working electrode by a magnet



Scheme 1. Schematic representation of protein kinase activity assay based on Electrochemiluminescence nanoprobes. Step (1) Biotin modified Kemptide were phosphorylated by protein kinase; (2) MB enriched phosphorylated Kemptide; (3) ECL nanoprobes were specifically immobilized on MB surface; (4) MB carried ECL nanoprobes to unmodified electrode for ECL detection.

under it. A voltage of 1.25 V was applied across the electrode and the photon signal was measured.

3. Results and discussion

3.1. Protein kinase activity assay based on ECL nanoprobes and MB capture and enrichment

The principle of current protein kinase assay was illustrated in Scheme 1. Protein kinase activity was estimated by analyzing the kinase-catalyzed phosphorylation of synthetic special substrate peptides, Kemptide. Kemptide, a well defined substrate for PKA, possesses the phosphorylation site to accept phosphate groups from ATP, besides, its activity toward PKA is 1.5- to 45-fold greater than that of other commonly used substrates such as histone fractions (de la Houssaye and Masaracchia, 1983). Thus, it was widely used for PKA activity analysis.

In this study, PKA was used as a model. First, TBR was labeled to GNP through the bridging of cysteamine which contains sulfide group and amino-group at two ends (Duan et al., 2010). TBR, a crucial ECL reagent, can react with tripropylamine (TPA) and produce high ECL signals. Meanwhile, the intrinsic phosphate groups modified DNA were bound to GNP via a sulfur–gold linkage. Then, these nanoprobes with extremely efficient signals amplification were applied for ECL. In the presence of PKA and ATP, substrate peptides were phosphorylated and then conjugated to streptavidin-coated magnetic beads via the biotin–streptavidin interaction. After simple wash steps, reaction disruptors such as ATP and PKA were washed out and then ECL nanoprobes with Zr^{4+} were added. With the help of Zr^{4+} mediating linkage between the phosphorylated peptides and phosphorylated DNA probes, MB captured ECL nanoprobes to the electrode surface. When a voltage was applied, ECL readouts were given with correspond to kinase activity. Zr^{4+} was chosen for linking two phosphorylated components since it can afford several obvious advantages. For example, Zr^{4+} could

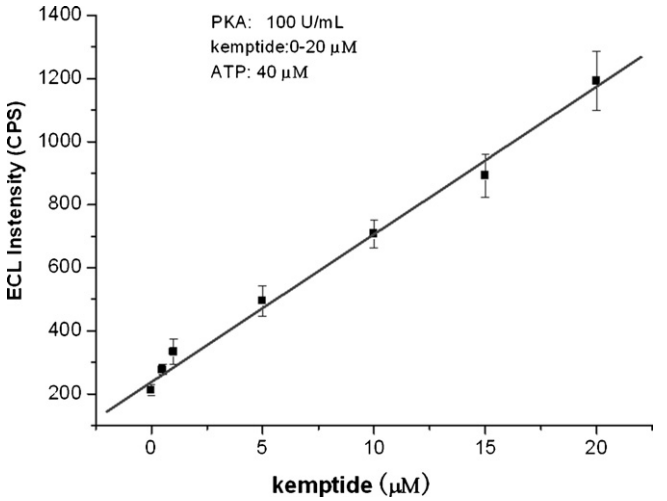


Fig. 1. The response of ECL intensity changes to the substrate peptides at different concentrations. Here 0, 0.5, 1, 5, 10, 15 and 20 μM substrate peptides were phosphorylated by 100 U/mL PKA in reaction buffer (40 μM ATP, 40 mM Tris–HCl and 20 mM $MgCl_2$, pH 7.4) for 1 h at 30 °C. The error bars indicate the relative standard deviation of measurements performed in triplicate.

fastly link two biomolecules containing phosphate groups without the need for chemical activation steps and specific chemical modifications. Furthermore, the zirconium–phosphonate linkage was very robust from pH 1 to pH 10 and kept stable regardless of the highly changing reaction condition. Another momentous improvement of our ECL biosensor was the application of MB. In this strategy, MB served not only for separation but also as a platform for enhanced target capture. The coupling of MB with renewable biomolecules labeled surfaces of capture and label-free electrode of ECL detection eliminated the need for a complex series of pretreatment and washing steps for electrode surface modification. These prominent characteristics resulted in a greatly simplified and high-efficiency protocol. And it was in great contrast with ordinary ECL designs which were laborious to perform. Moreover, other laboratories without more practical operating experiences may suffer from several technical limitations in terms of repeatability and reproducibility in ordinary ECL designs.

3.2. Optimization of ECL reaction

To obtain the best sensing and efficient performance of this detection system, two of the most important parameters for the efficiency of kinase-catalyzed reaction were investigated. One was the Kempeptide concentration and the other was reaction time of PKA catalyzing phosphorylation.

Influences of Kempeptide concentrations on catalytic reaction were assessed by incubating variety concentrations of peptides (0–20 μM) with a certain amount of PKA in the reaction buffer containing other elements necessary to reaction. The choice of this range of peptides concentrations was deliberated based on two aspects. One was that there was only a small quantity of substrate for enzyme catalysis in wild environment and this choice was close to the practice more. Also, using MB greatly eliminated waste and 20 μM of substrate basically met the demands of catalysis reaction in term of some previous research and our preliminary experiments (Sun et al., 2005; Xu et al., 2011).

To demonstrate whether the ECL intensity was affected by the degree of phosphorylation of substrate peptides, we used high concentration of PKA to ensure that all the substrates contained in the reaction buffer were phosphorylated. As shown in Fig. 1, the ECL intensities corresponding with different concentrations of Kempeptides were recorded and data came from PKA activity assay

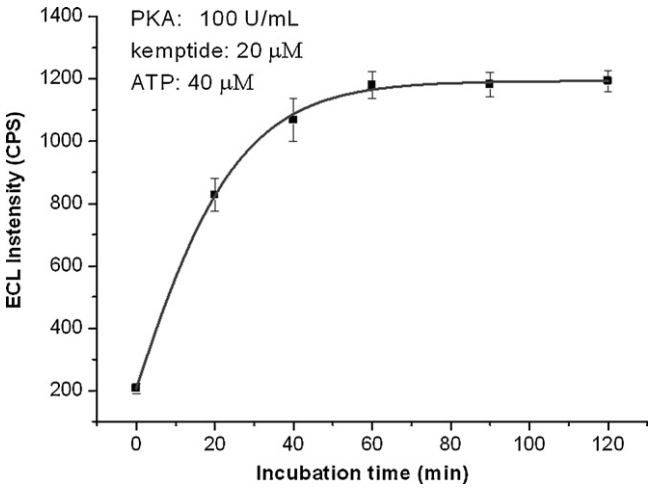


Fig. 2. Effect of incubation time on the ECL intensity responses. All the experiments were carried out in reaction buffer containing required PKA, and substrate peptides. The error bars indicate the relative standard deviation of measurements performed in triplicate.

performed in triplicate. It was clear that the ECL intensity grew noticeably with amount of Kempeptides increased and the results exhibited an approximately linear relationship with the concentrations of Kempeptides from 0 to 20 μM. Moreover, the ECL intensity increased about 6 times at last, which indicated that kinase performed effectively on this condition.

Based on all the data analysis, it was evident that the increase in ECL intensity observed can be attributed to the degree of phosphorylation of the substrate peptides. And thus, this assay was allowed to be used to monitor substrate phosphorylation quantitatively. Based on experimental results, we reasonably concluded that PKA had a capacity for effectively performing in catalytic reaction under the chosen condition and the choice of this range of peptides concentrations was scientific and rational. Hence, subsequent work employed 20 μM as the concentration of reaction substrate.

The effect of reaction time of PKA catalyzing substrate peptides was also studied by stopping the catalyzing reaction at different time intervals and analyzing ECL signals output correspondingly. The phosphorylation reactions were stopped after 20, 40, 60, 90 and 120 min respectively and the reactions were in the presence of PKA of 100 U/mL and 20 μM Kemptide. As shown in Fig. 2, with the increasing phosphorylation time, the ECL intensity shows an initial quick increase but then followed by a slow enhancement and ECL intensity began to level off. In addition, no obvious change could be observed when the reaction was performed after 60 min and the ECL intensity reached a plateau, which meant the phosphorylation was completed. From these results, we gave the conclusion that 60 min was the optimal phosphorylation time and hence applied in the subsequent experiments.

3.3. Performance of ECL biosensor in protein kinase activity monitoring

The activity of protein kinase was assessed using different concentrations of PKA (0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 100 U/mL) based on the above-mentioned optimal condition. Fig. 3A shows that the ECL intensity increased with promoting PKA activity units accordingly and only a very low ECL response could be observed without PKA, which was possibly due to non-specific adsorption of ECL nanoprobes. The limit of detection (LOD) of 0.005 U/mL was achieved and estimated from formula $I_{LOD} = I_{control} + 3I_{stdev(con)}$, where $I_{control}$ was the ECL intensity in the absence of PKA and $I_{stdev(con)}$ represented the standard deviation of

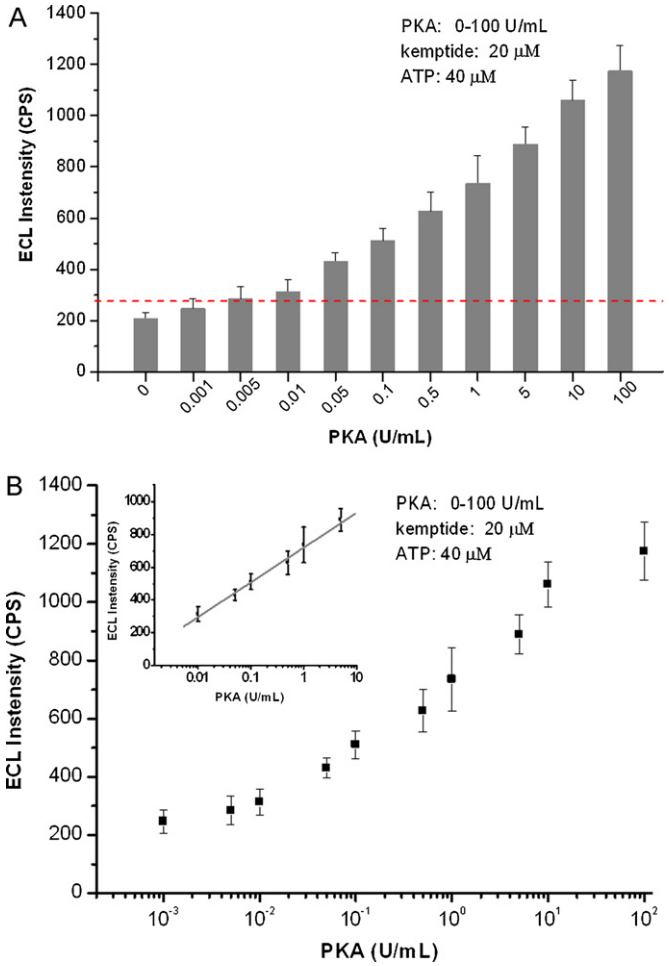


Fig. 3. (A) Quantification of the ECL intensity in response to different concentrations of PKA. (B) Plot for the dependence of ECL intensity on the logarithm of PKA concentrations. The inset is the linear correlation between ECL intensity and PKA concentrations. All reactions are carried out on above optimized conditions. The error bars indicate the relative standard deviation of measurements performed in triplicate.

$I_{control}$. To the best of our knowledge, this detection limit is about two orders of magnitude lower than the previous reports of fluorescent (Shults and Imperiali, 2003), electrical (Ji et al., 2009) and colorimetric assays (Sawada et al., 2011). Fig. 3B is the plot for the dependence of ECL intensity on the logarithm of PKA concentrations. Inset presented the linear correlation between ECL intensity and different PKA concentrations. The obtained linear range was from 0.01 U/mL to 50 U/mL with the correlation coefficient of $R^2 = 0.9951$. The characteristics of high sensitivity and wide dynamic range of our ECL biosensors are mainly attributed to three aspects. First, ECL assay is a promising tool exhibiting the merits of both electrochemical and chemiluminescent methods and inherently have the potential to produce high sensitivity. Secondly, GNP with lots of ECL labels (TBR) were applied to construct ECL nanoprobes and thus the ECL signal was extremely amplified, which resulted in raising sensitivity. Thirdly, the application of MB brings about revolutionary improvements on the biosensor, which not only achieves a remarkable circumvention of signal interference from unreacted constituents, but also efficiently enriches targets. These advanced characteristics enable the method with low background signals and high assay sensitivity.

Furthermore, the stability of our ECL biosensor was also assessed. It was found that the ECL nanoprobe, the most important component in our ECL assay, still retained its ECL activity after

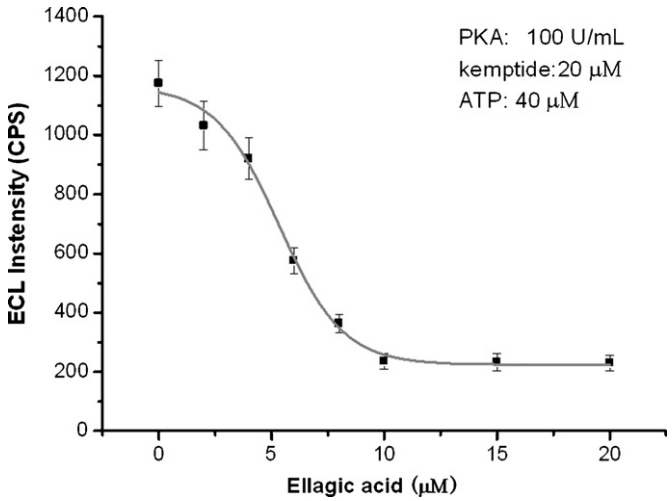


Fig. 4. Relationship between ECL intensity and kinase reaction inhibitors. All reactions are carried out on above optimized conditions except for the addition of different concentrations of inhibitors (0, 2, 4, 6, 8, 10, 15 and 20 μM). The error bars indicate the relative standard deviation of measurements performed in triplicate.

having been continuously stored at 4 °C for no less than a month (data not shown).

3.4. The assessment of protein kinase activity inhibition

To further validate our ECL biosensors for kinase assay, we quantitatively characterized the inhibition of PKA activity in the presence of small molecule kinase inhibitors at different concentrations. It is well established that kinases such as PKA play the regulation and functional roles in cellular signaling cascades, and their overexpression is causative of neoplastic growth. Thus, the application of kinase inhibitors for regulating kinase activity will provide insights into the connection of the regulation and dynamics of kinase activity to functional feedbacks of living properties such as cell life and tumor suppressor genes expression. Moreover, the study of kinase inhibitors is very helpful to disease diagnosis and drug development. Therefore, quantitative inhibition assay of kinases activity is necessary and implemented in this program.

In these quantitative inhibition assays, ECL intensities corresponding with different kinase inhibitor concentrations were tested and the IC_{50} value (the concentration of inhibitor in agreed with the half-maximal inhibition) was also estimated. Herein, Ellagic acid (4,4′,5,5′,6,6′-hexahydroxydiphenic acid 2,6,2′,6′-dilactone), which possesses anti-mutagenic and anti-carcinogenic properties as a potent and cell-permeable antioxidant, was employed for the model inhibitor in our research. All reactions were carried out under the above optimized conditions except for the addition of different concentrations of inhibitor (0, 2, 4, 6, 8, 10, 15 and 20 μM). As was illustrated in Fig. 4, the ECL intensity decreased along with the increased concentration of ellagic acid and then reached a plateau. It was observed that the ECL intensity came about no obvious changes when the concentration of ellagic acid reached 10 μM. And the IC_{50} value was assessed as 5.33 μM which was comparable with the previous reports (Ji et al., 2009; Wang et al., 2011). These results were due to that less PKA activities leaded to less production of phosphorylated Kemptides, which brought about less ECL nanoprobes captured and lower ECL intensity. According to the work mentioned above, it is convincing that current ECL biosensor has the potential ability of screening the kinase inhibitors quantitatively.

4. Conclusions

In conclusion, we have described a novel ECL biosensor for protein kinase activity analysis based on MB technology and signals amplification of GNP using the linkage of zirconium–phosphonate. Moreover, this biosensor has been employed to quantitatively analyze the inhibition of PKA. The coupling of GNP carrying lots of ECL labels and ECL exhibiting the merits of both electrochemical and chemiluminescent methods greatly improves the quality of kinase activity assay. Based on this coupling, a detection limit of 0.005 U/mL was achieved, which is lower than those of previous works. Meanwhile, the applications of MB technology for easy bio-separation and zirconium conjunction without the need for chemical activation steps result in a greatly simplified protocol. As a result, this ECL biosensor demonstrates numerous of prominent advantages such as high sensitivity, simplicity, long-term stability, the enhanced availability of reusing and wide assay range. Based on these characteristics, our biosensor is promising for an effective assay of protein kinases as well as other enzymes for clinic diagnostics and drug therapeutics in the further research.

Acknowledgments

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