



Electrochemical strategy for detection of phosphorylation based on enzyme-linked electrocatalysis

Jing Wang^a, Ya Cao^a, Yun Li^a, Zhiqiang Liang^b, Genxi Li^{a,b,*}

^a Department of Biochemistry and National Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, 210093 Nanjing, China

^b Laboratory of Biosensing Technology, School of Life Science, Shanghai University, 200444 Shanghai, China

ARTICLE INFO

Article history:

Received 14 July 2010

Received in revised form 8 October 2010

Accepted 13 October 2010

Available online 20 October 2010

Keywords:

Electroanalysis

Phosphorylation

Kinase

Gold nanoparticles

ABSTRACT

In this paper, we report an electrochemical strategy for the detection of phosphorylation based on enzyme-linked electrocatalysis. In this strategy, the substrate peptides modified on a gold electrode surface are firstly phosphorylated by protein kinase, and then biotinylated via the recognition and combination with the specific biotin-labeled antibody. After that, gold nanoparticles (Au-NPs) which are modified with horseradish peroxidase (HRP) loaded streptavidin are immobilized on the surface of the electrode through biotin-avidin interaction. As a result, HRP on the electrode surface may take catalytic reaction towards the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB), and the generated electrochemical signals can be used to monitor phosphorylation. This proposed electrochemical strategy can be employed to detect kinase activity with a satisfactory detection limit of 1×10^{-3} unit/mL and linear range from 1×10^{-3} to 1×10^{-2} unit/mL. Furthermore, the inhibition of protein kinase has been also studied by using this strategy.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

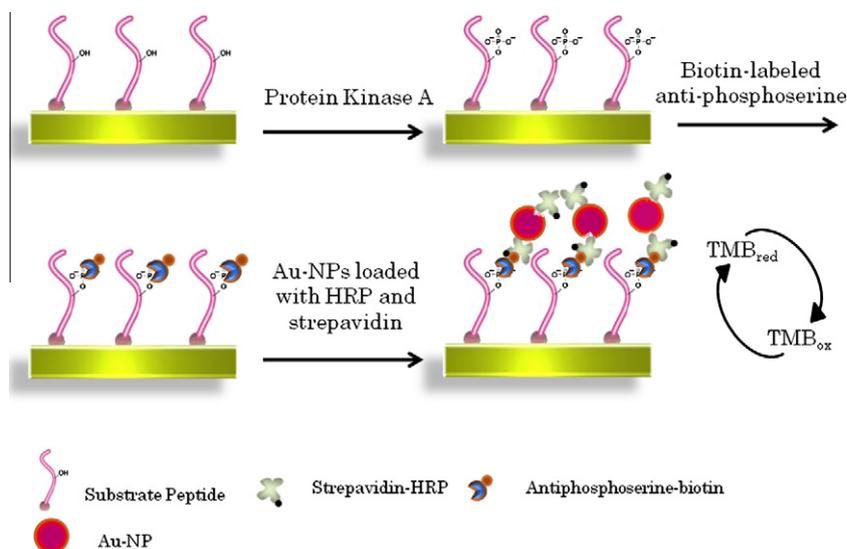
Protein kinase-catalyzed phosphorylation plays a significant role in many vital biological processes, including cellular signal transduction [1,2], cell proliferation and differentiation [3,4]. Meanwhile, in clinical applications, de-regulation of phosphorylation may indicate aggressive tumor biology or predict poor response to anti-cancer therapy in patients [5,6]. Therefore, the detection of phosphorylation and the assay of protein kinase activity is not only necessary for understanding many fundamental biological metabolism processes, but also very important for the research of cancer development, diagnosis and treatment [7–11].

The currently used techniques to detect phosphorylation include radio-isotope method, fluorescent method and mass spectroscopy [12–17]. In recent years, with the development of nanomaterials, which have excellent biocompatibility, convenient surface modification and unique optical properties, many novel detection strategies were proposed [18–30]. For instance, Katayama and co-workers have reported a colorimetric method by designing substrate peptides with different charges which may induce the aggregation of gold nanoparticles (Au-NPs) and

* Corresponding author at: Department of Biochemistry and National Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, 210093 Nanjing, China. Tel.: +86 25 83593596; fax: +86 25 83592510.

E-mail address: genxili@nju.edu.cn (G. Li).

then monitor the activity of kinase by detecting the color change of Au-NPs even with naked eyes [22,23]. This method is simple and convenient, while it has low detection sensitivity, and requires the specific charge of substrate peptides. Kerman et al. have synthesized ATP molecules linked to biotin or thiol groups, and combined them with Au-NPs. Once the nanoparticles are modified on the electrode surface, they can be used as labels through Cl^- redox chemistry for the detection of tyrosine kinase [25–27]. This method has low detection limit, though it needs complex chemical synthesis. Due to the fact that there are various kinds of kinases and the detection of phosphorylation is so important for biological and clinical applications, more and more studies are still focused on this topic. In this paper, we propose an enzyme-responsive electrochemical strategy for the detection of phosphorylation. As is illustrated by Scheme 1, after the substrate peptides modified on a gold electrode surface are phosphorylated by protein kinase A (PKA), phosphoserine is recognized by its biotinylated antibody. Furthermore, due to the biotin-avidin interaction, Au-NPs are immobilized onto the electrode surface. Since Au-NPs are also loaded with multiple HRP molecules, the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) catalyzed by HRP can be then employed for electrochemical measurement. This enzyme-responsive method can be used to monitor the activity of PKA with a detection limit as low as 1×10^{-3} unit/mL (≈ 2 ng/mL), which is at least two orders lower than the previous reports [22–30]. Furthermore, the inhibition of PKA has been also studied by using this strategy.



Scheme 1. Schematic illustration of the enzyme-responsive electrochemical strategy for the detection of PKA-catalyzed phosphorylation.

2. Experimental

2.1. Reagents and solutions

The substrate peptide (H-CALRRASLGW-NH₂) and the control peptide (H-CGRRRAGLGW-NH₂), HPLC purified, were purchased from Shanghai Science Peptide Biological Technology Co., Ltd. PKA (from bovine heart, specific activity of 0.5 unit/μg), bovine serum albumin (BSA), adenosine 5'-triphosphate (ATP) disodium salt hydrate, TMB and biotinylated monoclonal *anti*-phosphoserine were obtained from Sigma. HRP-modified streptavidin (streptavidin-HRP) was purchased from Vector Laboratories, Inc. (California, USA). Adenosine-3', 5'-cyclophosphoric acid (cAMP), mercaptohexanol (MCH) and ellagic acid were purchased from Fluka. Hydrogen tetrachloroaurate (III) trihydrate (HAuCl₄·3H₂O, 99.9%), trisodium citrate and tris (2-carboxyethyl) phosphine hydrochloride (TCEP) were from Shanghai Sangon Biological Engineering and Technology and Service Company. Other reagents were of analytical-grade. The PKA reaction buffer was 10 mM HEPES containing 10 mM MgCl₂, pH 6.5. The washing buffer was 20 mM HEPES containing 0.05% tween-20, pH 6.0. Water was purified with a Milli-Q purification system to a specific resistance (>18 MΩ cm) and used to prepare all the solutions.

2.2. Immobilization of substrate peptide on gold electrode surface

The gold electrode (diameter 2.0 mm) was firstly soaked in *piranha* solution (H₂SO₄:30% H₂O₂ = 3:1) for 2 min (*Caution! Piranha solution reacts violently with organic materials and should be handled with extreme care!*) to eliminate the adsorbed organic matter, and then rinsed with water. After that, the electrode was abraded with successively finer grades sand papers and polished to a mirror smoothness with alumina powder (Al₂O₃) of various particle sizes (1.0, 0.3 and 0.05 μm) on silk. Finally, it was sonicated for 5 min in both ethanol and water, respectively.

To prepare self-assembled monolayer (SAM) with the substrate peptide onto the surface of the above pretreated gold electrode, the electrode was firstly incubated with 100 μL of peptide solution (100 μg/mL, dissolved in 20 mM HEPES, containing 10 mM TCEP, pH 6.0) for 16 h at room temperature. TCEP was used here to prevent terminal cysteine of the substrate peptides from forming disulphide bonds. The unbound peptide was removed by rinsing

thoroughly with the washing buffer for 3 min. Next, the peptide-modified gold electrode was immersed in 100 μL of MCH solution (1 mM) for 1 h. Finally, it was immersed in the washing buffer for 1 h, so that tween-20 in the buffer blocked the adsorption of free PKA enzyme and replaced non-specific proteins by occupying the spare sites of the electrode [31].

2.3. Preparation of Au-NPs loaded with streptavidin-HRP

Au-NPs were synthesized by reducing HAuCl₄ with trisodium citrate [32]. Briefly, a 100 mL aqueous solution of 0.01% (w/v) HAuCl₄ was added into a round-bottom flask and stirred to boil. Then 3.5 mL 1% trisodium citrate was added rapidly into the boiling solution, the color of which became wine red from colorless after boiling for another 15 min with vigorous stirring. The size of the nanoparticles was 16.9 ± 1.8 nm, determined by TEM. The concentration of Au-NPs was 2.3 nM, which was calculated from the quantity of starting material (HAuCl₄) and the size of Au-NPs at the wavelength of 520 nm [33].

Au-NPs loaded with streptavidin-HRP were prepared as follows: Firstly, 80 μL of streptavidin-HRP solution (1 mg/mL, dissolved in 0.05 M NaHCO₃, pH 8.3) was added into 1 mL of freshly prepared Au-NPs solution, the pH of which was 6.0. Then, the mixed solution was incubated for 30 min at 37 °C with gentle shaking, and concentrated to 0.2 mL by centrifuging (12,000 rpm, 30 min, 4 °C). After that, 20 μL of 10% (w/v) BSA solution, which was employed as non-specific blocker, was added into the above solution to passivate the Au-NPs [34]. After a 30 min duration, the mixed solution was then centrifuged (12,000 rpm, 30 min, 4 °C). The supernatant was subsequently decanted and rinsed with 0.2 mL of washing buffer. The centrifuging/rinsing procedure was repeated for three times to remove the unbound antibodies and BSA.

2.4. Phosphorylation and modification of the electrode surface

The PKA-catalyzed phosphorylation was performed by adapting the manufacturer's protocol. Firstly, different amount of PKA was dissolved in the PKA reaction solution (10 mM HEPES containing 200 μM ATP, 600 μM cAMP, 5 mM TCEP, 10 mM MgCl₂, pH 6.5). Then, the substrate peptide-modified electrode was immersed in 100 μL of the above solution for 30 min at 37 °C in a humidified

chamber. After that, the reaction was terminated by rinsing the electrode thoroughly with the washing buffer.

The further modification of the electrode surface was carried out as follows. Firstly, the phosphorylated peptide-modified electrode was immersed into the biotinylated *anti*-phosphoserine solution at 37 °C for 1 h. Then the electrode was rinsed thoroughly using the washing buffer for 3 min to avoid non-specific adsorption. Finally, the electrode was incubated in the solution of Au-NPs loaded with streptavidin–HRP at 37 °C for 30 min, and the washing process was repeated.

For the HRP-based catalytic reaction, 20 mM TMB solution should be prepared with HEPES. The solution containing 0.2 mM TMB and 0.1 mM H₂O₂, pH 6.0, had to be freshly prepared before use [35]. To perform the catalytic reaction, the above electrode modified with Au-NPs which were loaded with streptavidin–HRP was firstly immersed in the TMB reaction solution for 30 min, and then measured with electrochemical instruments.

For the inhibition experiment, different concentration of ellagic acid was dissolved in the above PKA reaction solution. After 4 h of incubation at 30 °C, the reaction was terminated and the electrode was rinsed thoroughly by using the washing buffer [24].

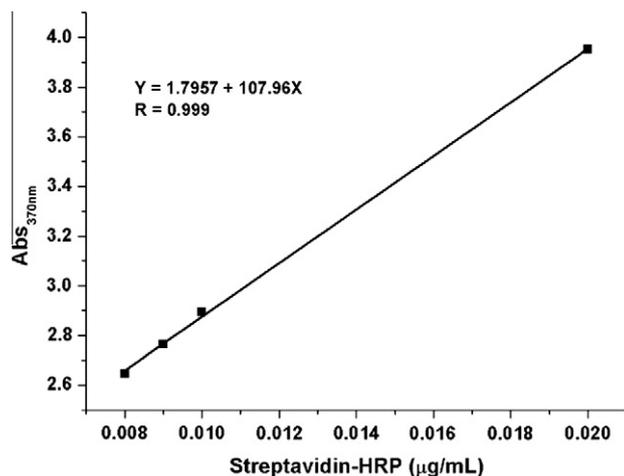


Fig. 1. Standard curve between the concentration of streptavidin–HRP and the absorbance value of the catalytic product at the wavelength of 370 nm.

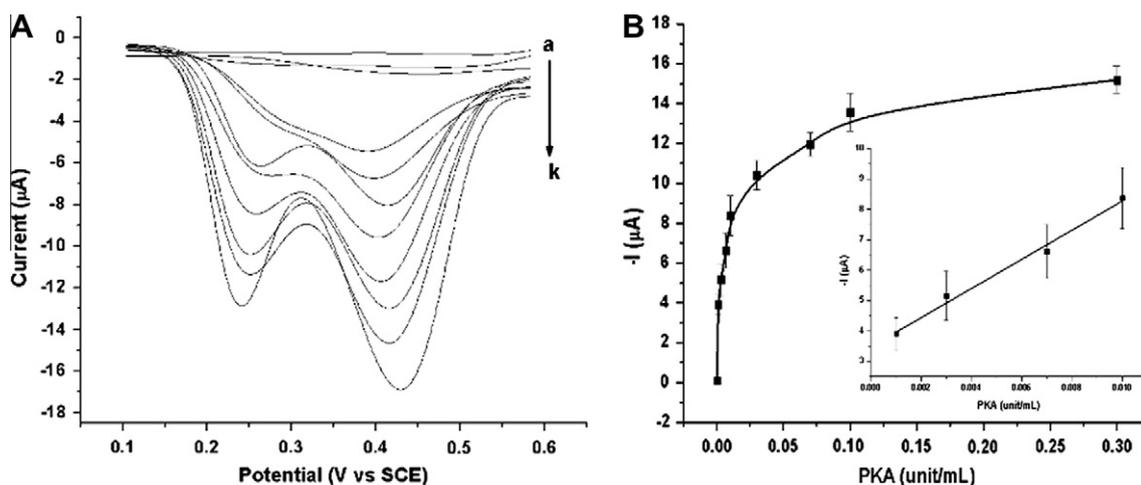


Fig. 2. (A) SWVs of TMB oxidation product quinonedimine obtained at the modified electrode in the presence of (a) 0 unit/mL PKA, (b) 10 μg/mL BSA, (d) 1×10^{-3} , (e) 3×10^{-3} , (f) 7×10^{-3} , (g) 1×10^{-2} , (h) 3×10^{-2} , (i) 7×10^{-2} , (j) 1×10^{-1} , (k) 3×10^{-1} unit/mL PKA. Curve c is the case that Au-NPs are not involved for 1×10^{-3} unit/mL PKA. (B) The relationship between the peak current of quinonedimine and the concentration of PKA in the range from 1×10^{-3} to 3×10^{-1} unit/mL. Inset is the calibration curve, and the error bars represent relative standard deviations for three independent measurements.

2.5. Apparatus and electrochemical measurements

The measurements by using the electrochemical techniques of square wave voltammetry were performed on a model 660a Electrochemical Analyzer (CH Instruments) with a conventional three-electrode cell at room temperature. A saturated calomel electrode (SCE) was used as the reference electrode, and a platinum wire electrode as the counter electrode. Square wave voltammograms (SWVs) were obtained by scanning from 0.1 V to 0.6 V with a step potential of 4 mV, pulse amplitude of 25 mV, and frequency of 15 Hz. UV–visible spectroscopy was performed by using a UV-2450 UV–visible spectrophotometer (Shimadzu Co., Japan). Transmission electron micrographs (TEM) were taken with a Jeol JEM-2000EX (Jeol Ltd., Japan). All the incubation processes during the preparation of protein-loaded nanoparticles were performed in a SHZ-C ThermoShaker (Boxun Industry Co., China).

3. Results and discussion

Scheme 1 may illustrate the mechanism of this proposed enzyme-responsive electrochemical strategy for the detection of phosphorylation, where PKA is used as the model enzyme. Here, the biotinylated *anti*-phosphoserine is employed for the recognition of phosphorylated peptide, while the involvement of nanoparticles provides signal amplification for the detection. To quantify the number of streptavidin–HRP loaded on each Au-NP, standard spectrophotometric assay has been performed. From the standard curve shown in Fig. 1, we may calculate the concentration of streptavidin–HRP modified on the surfaces of Au-NPs and estimate that each Au-NP is loaded with four streptavidin–HRP molecules. Since the ratio of HRP to streptavidin is 1.5:1, we can conclude that there are at least six HRP molecules immobilized on each Au-NP. Therefore, once one substrate peptide is phosphorylated, there will be at least six HRP molecules loaded on the surface of the electrode for signal producing. Furthermore, since the TMB oxidation product quinonedimine generated by the catalysis of HRP may have a high electrochemical signal, the sensitivity of the detection proposed in this work can be very high. Fig. 2 may show the experimental results. In the absence of PKA, no signal can be observed (curve a). And the non-specific protein cannot give any electrochemical signal either (curve b). Nevertheless, in the presence of as low as 1×10^{-3} unit/mL PKA, due to the phosphorylation of the peptide

and the subsequent modification of Au-NPs on the electrode surface, HRP molecules are immobilized on the electrode surface. Consequently, the catalytic reaction towards TMB takes place, thus the signal of quinonediimine can be clearly observed (curve d). To the best of our knowledge, this detection limit of 1×10^{-3} unit/mL is at least two orders of magnitude lower than the previous reports [22–30]. Different from the reports to monitor the quantity of Au-NPs for the detection of kinase activity [25,27], this strategy makes use of the enzyme-linked electrocatalysis and the above mentioned amplification of the electrochemical signal, which contribute to the high detection sensitivity. We have also examined the case that Au-NPs are not involved. As is shown by curve c in Fig. 2A, without the participation of Au-NPs, since only limited HRP molecules are immobilized on the electrode surface, the signal is much smaller, thus the sensitivity cannot be very high.

We have further optimized the experimental conditions so as to obtain a better sensitivity and efficiency of the detection system. Firstly, the amount of biotinylated *anti*-phosphoserine used for the recognition of phosphorylated peptide is studied. As is shown in Fig. 3, when the concentration of antibody is 5 μ g/mL, the electrochemical signal of TMB oxidation product may give a maximum

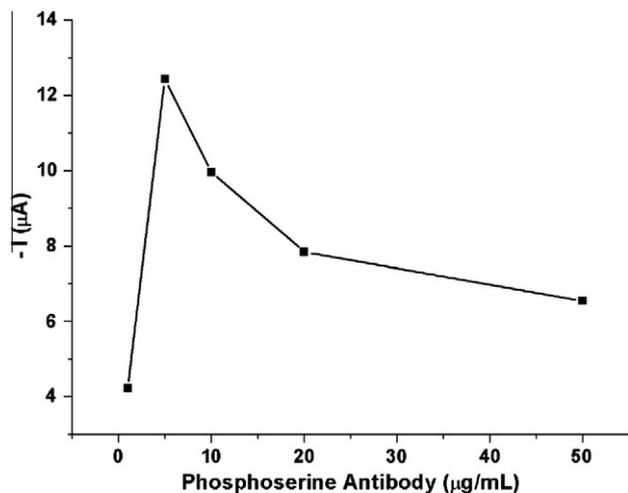


Fig. 3. Optimization of the concentration of biotinylated *anti*-phosphoserine. [PKA] = 10 unit/mL. Others same as in Fig. 2.

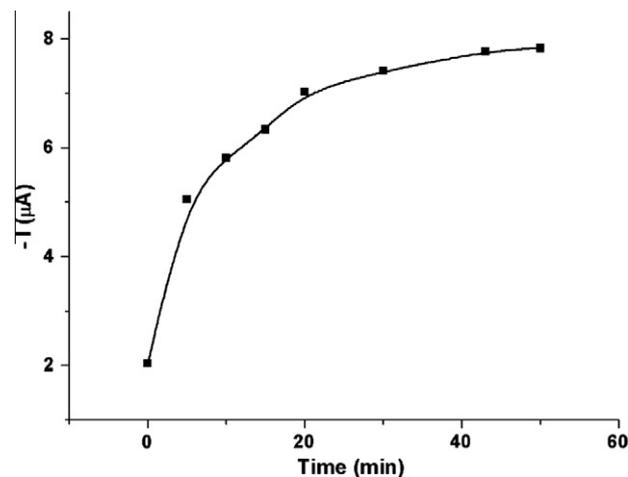


Fig. 4. Kinetic curve of the TMB oxidation reaction catalyzed by HRP on the electrode surface. [Biotinylated *anti*-phosphoserine] = 5 μ g/mL. Others same as in Fig. 3.

value. Secondly, the kinetic curve of the TMB oxidation reaction catalyzed by HRP on the electrode surface has been obtained. As is shown in Fig. 4, the optimal catalytic reaction time should be 30 min. Thirdly, the salt-induced Au-NP aggregation test has been carried out to determine the appropriate concentration of streptavidin–HRP used for the modification of the nanoparticles. It has been known that NaCl is able to cause the aggregation of Au-NPs, which will result in the shift of their absorbance peak from 520 nm to 580 nm, while proteins adsorbed on the surface of Au-NPs may prevent the nanoparticles from aggregation. So, the optimal concentration of the antibody that may best stabilize Au-NPs has been determined by measuring the difference between absorbances at 520 nm and 580 nm with the help of NaCl. From the results shown in Fig. 5, it can be known that the minimum concentration of streptavidin–HRP needed to keep the stability of Au-NPs is about 8 μ g/mL. Finally, the pH value of Au-NP solution has been optimized. As is shown in Fig. 6, the best pH value should be around 5.5–6.0.

From Scheme 1, we can also know that more PKA in the detection system will make more peptides phosphorylated, thus more HRP molecules will be ultimately immobilized on the electrode surface to catalyze the oxidation of TMB in the test solution, and consequently, higher electrochemical signal of quinonediimine

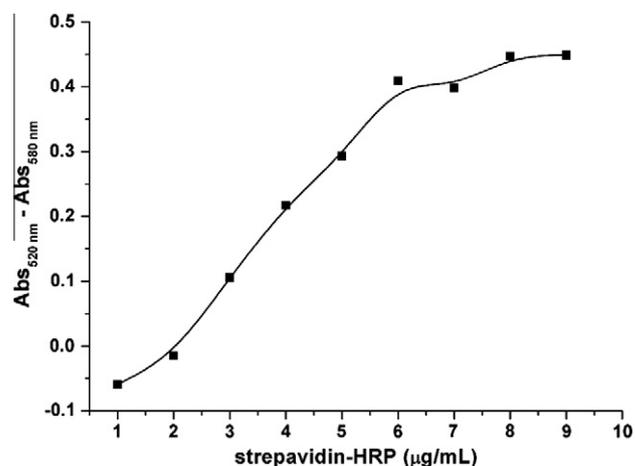


Fig. 5. Salt-induced Au-NP aggregation test to determine the optimal concentration of streptavidin–HRP used for the modification of Au-NPs.

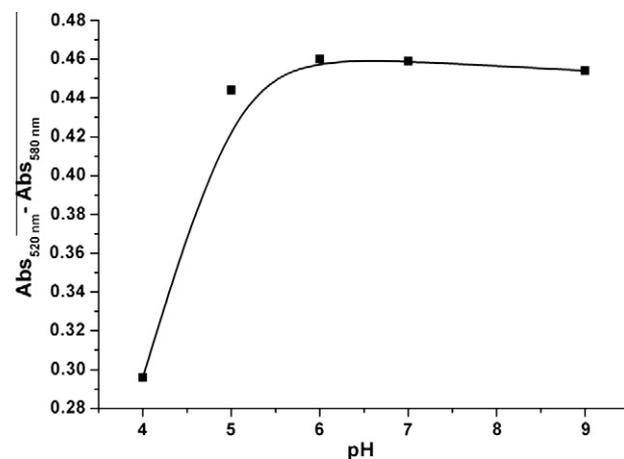


Fig. 6. Optimization of pH value of the Au-NPs solution for the modification of Au-NPs with streptavidin–HRP.

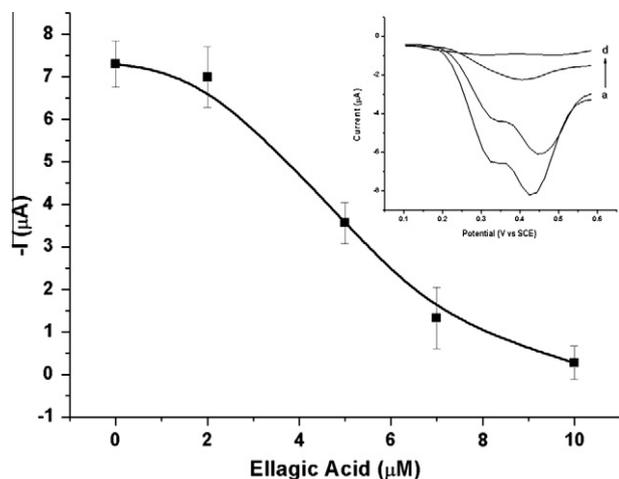


Fig. 7. Relationship between the peak current of HRP-catalyzed TMB oxidation and the concentration of ellagic acid. The error bars represent relative standard deviations for three independent measurements. Inset is the SWVs for (a) 2, (b) 5, (c) 7, (d) 10 μM ellagic acid in the presence of 1 unit/mL PKA. Others same as in Fig. 2.

can be obtained. In fact, as is shown by curves d–k in Fig. 2A, the peak current of quinonediimine increases significantly with the increase of PKA concentration. Further studies reveal that the detectable range is from 1×10^{-3} unit/mL to 3×10^{-1} unit/mL, and the linear relationship can be obtained from 1×10^{-3} unit/mL to 1×10^{-2} unit/mL, $R = 0.985$, $P < 0.05$ (Fig. 2B). Compared with the previous studies, both of the detection range and linear relationship are better than the other reports.

This proposed enzyme-linked electrochemical strategy for the detection of phosphorylation can also be employed to quantitatively characterize the inhibition of PKA activity. Ellagic acid (4,4',5,5',6,6'-hexahydroxydiphenic acid 2,6,2',6'-dilactone), which has been reported as a cell-permeable and potent antioxidant, is used as the model inhibitor in this study [36]. Its inhibition effect has been examined by comparing the electrochemical signals of the TMB oxidation product in the presence of 1 unit/mL PKA and different concentrations of ellagic acid. As is shown in Fig. 7, the signal of TMB product quinonediimine decreases with the concentration of ellagic acid. Meanwhile, curve d in the inset of Fig. 7 indicates that 10 μM ellagic acid for 1 unit/mL PKA solution can make the PKA activity to be completely inhibited. Furthermore, based on the data in Fig. 7, the IC_{50} value (half-maximal inhibition) can be estimated as 5.2 μM, which is in agreement with the previous reports [28].

4. Conclusion

In conclusion, we have proposed an electrochemical strategy to detect phosphorylation and to assay the activity of protein kinase. This approach is based on HRP-linked catalysis, while Au-NPs are employed for the amplification of the detected signal. The HRP-linked electrocatalysis may avoid the use of soluble electroactive labels such as RuHex ($[Ru(NH_3)_6]^{3+}$) which will result in high background signal [24]. Moreover, this proposed strategy makes use of the high affinity between phosphorylated amino acids and antibodies, not limited to the sequence or modification of substrate peptides. Therefore, we anticipate that this method may provide a

general platform to monitor the activity of kinases. Furthermore, the involvement of Au-NPs may make this strategy to be extended into some other detection approaches, such as surface-enhanced raman scattering (SERS) and fourier-transform infrared (FTIR) spectroscopy which makes use of the plasmon resonance and surface characteristics of nanoparticles [37].

Acknowledgements

This work is supported by the National Science Fund for Distinguished Young Scholars (Grant No. 20925520) and Shanghai Science and Technology Committee (Grant No. 09DZ2271800).

References

- [1] S.S. Taylor, D.R. Knighton, J. Zheng, L.F.T. Eyck, J.M. Sowardski, *Annu. Rev. Cell Biol.* 8 (1992) 429–462.
- [2] C.H. June, M.C. Fletcher, J.A. Ledbetter, G.L. Schieven, J.N. Siegel, A.F. Phillips, L.E. Samelson, *Proc. Natl. Acad. Sci. USA* 87 (1990) 7722–7726.
- [3] P.P. Roger, D. Christophe, J.E. Dumont, I. Pirson, *Eur. J. Endocrinol.* 137 (1997) 579–598.
- [4] C. Bertolotto, P. Abbe, T.J. Hemesath, K. Bille, D.E. Fisher, J.P. Ortonne, R. Ballotti, *J. Cell Biol.* 142 (1998) 827–835.
- [5] P. Blume-Jensen, T. Hunter, *Nature* 411 (2001) 355–365.
- [6] T. Hunter, *Philos. Trans. Biol. Sci.* 353 (1998) 583–605.
- [7] G. Werlen, B. Hausmann, D. Naeher, E. Palmer, *Science* 299 (2003) 1859–1863.
- [8] M. Koresawa, T. Okabe, *Assay Drug Dev. Technol.* 2 (2004) 153–160.
- [9] M. Uttamchandani, J. Wang, S.Q. Yao, *Mol. Biosyst.* 2 (2006) 58–68.
- [10] B.T. Houseman, J.H. Huh, S.J. Kron, M. Mrksich, *Nat. Biotechnol.* 20 (2002) 270–274.
- [11] P. Cohen, *Nat. Rev. Drug Discov.* 6 (2002) 309–315.
- [12] G. Manning, D.B. Whyte, R. Martinez, T. Hunter, S. Sudarsanam, *Science* 298 (2002) 1912–1934.
- [13] M. Schutkowski, U. Reineke, U. Reimer, *ChemBioChem* 6 (2005) 513–521.
- [14] M.D. Shults, K.A. Janes, D.A. Lauffenburger, B. Imperiali, *Nat. Methods* 2 (2005) 277–283.
- [15] E. Pazos, O. Va'zquez, J.L. Mascaren, E.M. Va'zquez, *Chem. Soc. Rev.* 38 (2009) 3348–3359.
- [16] R.P. Johnson, A.F. El-Yazbi, M.F. Hughes, D.C. Schriemer, E.J. Walsh, M.P. Walsh, W.C. Cole, *J. Biol. Chem.* 284 (2009) 16562–16574.
- [17] J.H. Moon, P. Maclean, W. McDaniel, L.F. Hancock, *Chem. Commun.* 46 (2007) 4910–4912.
- [18] J.H. Kim, S. Lee, K. Kim, H. Jeon, R.W. Park, I.S. Kim, K. Choi, I.C. Kwon, *Chem. Commun.* 13 (2007) 1346–1348.
- [19] Y.P. Kim, E. Oh, Y.H. Oh, D.W. Moon, T.G. Lee, H.S. Kim, *Angew. Chem. Int. Ed.* 46 (2007) 6816–6819.
- [20] M.G. Shapiro, J.O. Zablowski, R. Langer, A. Jasanoff, *J. Am. Chem. Soc.* 131 (2009) 2484–2486.
- [21] Y. Choi, N.H. Ho, C.H. Tung, *Angew. Chem. Int. Ed.* 46 (2007) 707–709.
- [22] J. Oishi, Y. Asami, T. Mori, J.H. Kang, T. Niidome, Y. Katayama, *Biomacromolecules* 9 (2008) 2301–2308.
- [23] J. Oishi, Y. Asami, T. Mori, J.H. Kang, M. Tanabe, T. Niidome, Y. Katayama, *ChemBioChem* 8 (2007) 875–879.
- [24] X. Xu, Z. Nie, J. Chen, Y. Fu, W. Li, Q. Shen, S. Yao, *Chem. Commun.* 45 (2009) 6946–6948.
- [25] K. Kerman, M. Chikae, S. Yamamura, E. Tamiya, *Anal. Chim. Acta* 588 (2007) 26–33.
- [26] K. Kerman, H.B. Kraatz, *Chem. Commun.* 47 (2007) 5019–5021.
- [27] K. Kerman, H.B. Kraatz, *Biosens. Bioelectron.* 24 (2009) 1484–1489.
- [28] J. Ji, H. Yang, Y. Liu, H. Chen, J. Kong, B. Liu, *Chem. Commun.* 12 (2009) 1508–1510.
- [29] T. Li, L. Guo, Z. Wang, *Biosens. Bioelectron.* 23 (2008) 1125–1129.
- [30] T. Li, D. Liu, Z. Wang, *Biosens. Bioelectron.* 24 (2009) 3335–3339.
- [31] M. Steinitz, *Anal. Biochem.* 282 (2000) 232–238.
- [32] J. Wang, W. Meng, X. Zheng, S. Liu, G. Li, *Biosens. Bioelectron.* 24 (2009) 1598–1602.
- [33] K.C. Grabar, R.G. Freeman, M.B. Hommer, M.J. Natan, *Anal. Chem.* 67 (1995) 735–743.
- [34] J. Li, S.P. Song, X.F. Liu, L.H. Wang, D. Pan, Q. Huang, Y. Zhao, C.H. Fan, *Adv. Mater.* 20 (2008) 497–499.
- [35] J. Wang, Y. Cao, Y. Xu, G. Li, *Biosens. Bioelectron.* 25 (2009) 532–536.
- [36] G. Cozza, P. Bonvini, E. Zorzi, G. Poletto, M.A. Pagano, S. Sarno, A. Donella-Deana, G. Zagotto, A. Rosolen, L.A. Pinna, F. Meggio, S. Moro, *J. Med. Chem.* 49 (2006) 2363–2366.
- [37] B. Zhang, B. Yan, *Anal. Bioanal. Chem.* 396 (2010) 973–982.