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Label-Free Electrochemical Sensing Platform for the Detection of Protease

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Proteases have been used by proponents of alternative therapies, or identified in materials of traditional or folk medicine. In this work, we reported a label-free strategy for the design of electrochemical biosensors for protease detection based on the change of interfacial features of the electrode. To demonstrate the feasibility and sensitivity, active prostate specific antigen (PSA, a serine protease produced by both normal prostate glandular cells and prostate cancer cells) was tested as model analyte. Specifically, peptide with a sequence of CHSSKLQK was immobilized onto gold electrode. The positively charged lysine residues facilitate the access of the negatively charged $[Fe(CN)_6]^{3-/4-}$ probes to the electrode surface. The cleavage of peptide by PSA will induce loss of the positively charged lysine residues, leading to neutral SAMs that form a barrier for the electron transfer between redox probe and electrode. The decrease in the current of $[Fe(CN)_6]^{3-/4-}$ is proportional to the increase of PSA concentration. The present sensor obviates the use of labeled probe and could reduce the operation complexity and assay cost.

Keywords: protease; electrochemical biosensors; label-free; prostate specific antigen

1. INTRODUCTION

Enzymes, serving a wide variety of functions inside living organisms, are indispensable for signal transduction and cell regulation. Enzymes analytical detection is important for the screening of noxious toxins and pathologies associated with their presence, and for the development of effective and selective therapeutics [1,2]. Protease is one of the most important physiological enzymes because proteolytic processing is the final step in the expression of the activity of a great variety of proteins [3]. The current assays for proteases including those based on radioisotopes or fluorogenic substrates are usually time-consuming, lack sensitivity and/or require complicated instruments [4]. Therefore, there remains significant room for the development of a theoretically and technically simple approach for protease detection.

In recent years, electrochemical biosensors have been shown to be a promising alternative to mass- and fluorescence-based sensors for the specific detection of protein, DNA and small biomolecules in view of its high sensitivity, simplicity, rapid response, and compatibility with miniaturization [5-10]. Among kinds of electrochemical biosensors, redox tags (most commonly methylene blue or MB and ferrocene or Fc) labeled probe site-specifically attached to an interrogating electrode is one of the most attractive approaches [11-18]. Change in the configuration or decrease in the amount of redox tags resulting from the cleavage of probe by enzymes leads to a readily detectable change in Faradaic current upon voltammetric interrogation. Based on this principle, Fc peptide-immobilized electrodes have been successfully used for an electrochemical analysis of proteases [19-24]. For example, Lin and co-workers have applied Fc-conjugated peptides to develop electrochemical biosensors for the detection of matrix metalloproteinases (MMPs) and prostate specific antigen (PSA) [20,22]. Xiao et al. evaluated the caspase activity processes involved in apoptosis using Fc peptide modified electrode [21]. The same approach was extended by Takenaka for the assay of plasmin activity [19]. Recently, Demaille and co-workers have optimized the gold electrode attached Fc-peptide systems for kinetic measurements of protease action [23,24]. Labeling of peptide or DNA probe with Fc offers better sensitivity. However, Fc-labeled sensor exhibits significant signal loss after multiple regeneration/assay cycles and long-term storage due to the degeneration of Fc tags [25,26]. Moreover, the labeling process would make the experiments complex and affect the bioaffinity between the probes and their targets. Therefore, there has been interest in developing label-free and low cost biosensors in recent years.

It is well known that the terminal groups of self-assembled monolayers (SAMs) have a great impact on the redox response and electron-transfer resistance of redox probes in aqueous solutions due to the electrostatic interaction between the terminal groups and ionic redox species. For example, the voltammetric response of $[Fe(CN)_6]^{3-/4-}$ at the SAM-modified electrode is decreased in the order of the terminal group NH_2 > OH > COOH, while the response of $[Ru(NH_3)_6]^{3+/2+}$ is increased in the order of NH_2 < OH < COOH [27]. Based on the change of interfacial features of the electrode, a series of electrochemical biosensors have been fabricated for the detection of proteins, DNA and small molecules [28-36]. Recently, our group investigated the carbodiimide-mediated amine coupling reaction based on measurements of the electrochemical response of SAMs in the presence of $[Fe(CN)_6]^{3-/4-}$ as a redox probe [37]. The results indicated that the magnitude of the increase/decrease in electron-transfer resistance and redox current is related to the change in the surface charge. In the present work, we reported a label-free electrochemical strategy for the detection of proteases based on the change of interfacial features of the electrode.

2. EXPERIMENTAL

2.1 Chemicals and materials

The acetylated peptide (Ac-CHSSKLQK) was synthesized and purified by ChinaPeptides Co., Ltd (Shanghai, China). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and 6-mercapto-1-hexanol (MCH) were obtained from Sigma-Aldrich. Prostate specific antigen (PSA) was obtained from

Linc-Bio Science Co. Ltd. (Shanghai, China). All aqueous solutions were prepared with a Millipore system (Simplicity Plus, Millipore Corp.).

2.2 Preparation of peptide-modified electrode

The gold disk electrode with a diameter of 2 mm was polished with diamond pastes down to 3 μm and alumina pastes down to 0.3 μm, and then sonicated in ethanol and water. The peptide-modified electrode was prepared by immersing the cleaned gold electrodes in a solution of 10 μM peptide containing 50 μM TCEP in the darkness for 12 hours. The amount of peptide immobilized on gold electrode was chosen according to the well-established protocol [20]. This step was followed by washing the electrode thoroughly with water and soaking the electrode in a 1 mM MCH for 1 hour to block the unreacted gold surface. Again, the electrode was rinsed with ethanol/water to rid any non-specifically adsorbed substance.

2.3. Electrochemical measurements

Cyclic voltammograms (CVs) and differential pulse voltammograms (DPVs) were collected on a uECS-PRO electrochemical workstation (Changchun Institute of Applied Chemistry Chinese Academy of Science, China) in a homemade plastic three-electrode cell. The three-electrode system consists of a gold disk electrode, a platinum wire auxiliary electrode, and an Ag/AgCl reference electrode. Potential scanned from 0.7 to −0.3 V with a scan rate of 100 mV/s. Electrochemical impedance spectroscopy measurements were performed on a CHI 660D electrochemical workstation (CH Instruments), and the redox mediator used was 1 mM $[Fe(CN)_6]^{3-/4-}$ (1:1) solution containing 0.1 M KCl. For the assay of PSA, the peptide/MCH-covered electrodes were incubated with PBS solutions containing a given concentration of PSA. Unless otherwise noted, the incubation time was 30 min, and the reaction was conducted at room temperature. The change in the current was used to evaluate the performances of the sensor.

3. RESULTS AND DISCUSSION

3.1 Principle of the method

To prove the feasibility of our strategy, PSA, a 30-kDa serine protease produced by both normal prostate glandular cells and prostate cancer cells [20,38], was tested as model analyte. The analysis principle of this method was shown in Fig. 1. It is based on measurement of the electrochemical response of peptide/MCH-covered gold electrode in the presence of the negatively charged $[Fe(CN)_6]^{3-/4-}$ as a redox probe. The label-free peptide with a sequence of Ac-CHSSKLQK was immobilized on a gold electrode surface by self-assembling. The positively charged peptide/MCH SAMs facilitate the access of the redox probe to the electrode surface. The cleavage of peptide by PSA will induce loss of the positively charged lysine residues, leading to neutral SAMs that form a barrier for the electron transfer between redox probe and electrode. Thus, the concentration of PSA can be determined.

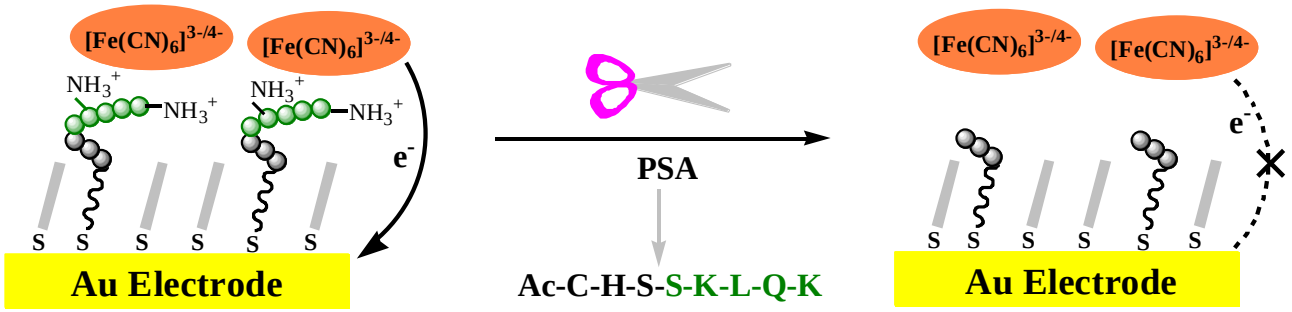

Figure 1. Scheme representation showing the PSA-catalyzed cleavage reaction at peptide modified electrode and the change of interfacial features of the electrode.

3.2 CVs of peptide-modified electrode

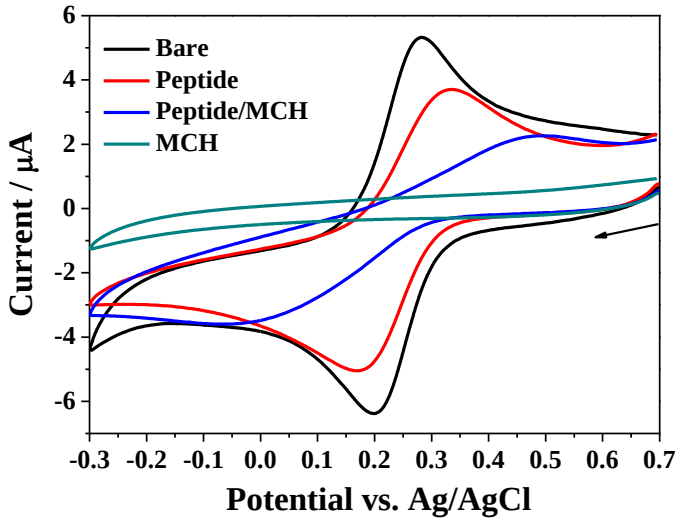

Figure 2. CVs of $Fe(CN)_6^{3-/4-}$ at bare and peptide-, peptide/MCH- and MCH-covered electrodes. The solid arrow indicates the scan direction.

When electrode surface has been modified by some materials, the electron transfer kinetics of $[Fe(CN)_6]^{3-/4-}$ is perturbed. Fig. 2 shows the cyclic voltammograms (CVs) of $[Fe(CN)_6]^{3-/4-}$ at a bare gold electrode (black curve), peptide-covered electrode (red curve), peptide/MCH-covered electrode (blue curve) and MCH-covered electrode (dark cyan curve). Decrease in the amperometric response accompanying the increase in the peak potential separation (ΔEp) between the cathodic and anodic waves is indicative of the success of the peptide modification (cf. black curve and red curve). Further, MCH was employed to block the unreacted sites on gold surface. As shown in the blue curve, the current of $[Fe(CN)_6]^{3-/4-}$ at peptide/MCH-covered electrode is markedly lower than that at peptide-covered electrode. This is understandable since the assembly of MCH layer enhances the electron transfer barrier. Note that no redox wave was observed at MCH-covered electrode (dark cyan curve), indicating that the electrostatic interaction between positively charged lysine residues and the negatively charged $[Fe(CN)_6]^{3-/4-}$ probe facilitates the electron transfer. It has been reported that the electrode coated with positively charged 11-amino-n-undecylmercaptan ($HS(CH_2)_{11}NH_2$) showed

well-defined redox waves with ΔEp of 75 mV [27]. However, a couple of irreversible redox waves were observed at the peptide/MCH-covered electrode. A reasonable explanation is that the amount of amino groups on the peptide/MCH-covered electrode surface is less than that on the 11-amino-n-undecylmercaptan-covered electrode surface, which affects the distribution of the negatively charged redox species in the vicinity of the electrode surface and perturbs the interfacial electron transfer.

3.3 Feasibility for the PSA detection

As shown in Fig. 3A, after incubation of the peptide/MCH-covered electrode with the PSA solution, drastic decrease in the reduction and oxidation peak currents of $[Fe(CN)_6]^{3-/4-}$ and increase in the ΔEp (see black and blue curves) were observed, which is attributed to the decrease in the amount of positively charged lysine residues on the electrode surface resulting from the cleavage of the peptides by PSA. The control experiment was performed by incubating the electrode in PBS solution in the absence of PSA. As shown in the red curve, a negligible change in oxidation and reduction peak current was observed, demonstrating that the decrease in the peak current of blue curve is dependent on PSA. The results were further confirmed by electrochemical impedance analysis (Fig. 3B). The charge-transfer impedance at the peptide/MCH-covered electrode increased after the electrode was incubated with PSA solution, illustrating that the occurrence of cleavage reaction hinders electron transfer between the redox probe in the electrolyte solution and the electrode. No apparent change was observed in the absence of PSA. The results are consistent with those of CVs.

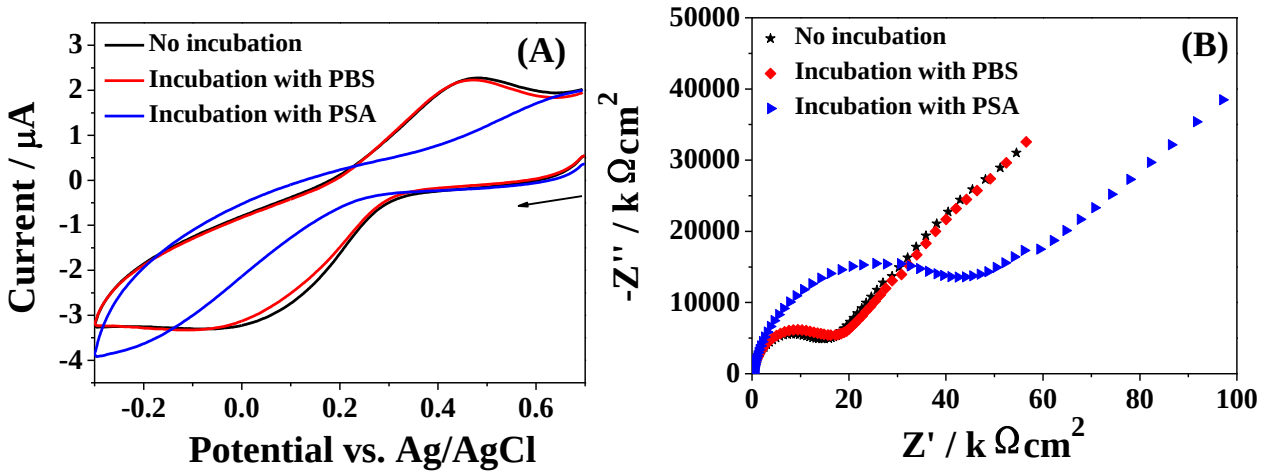

Figure 3. CVs (A) and electrochemical impendence spectra (B) of peptide/MCH-covered electrodes in $[Fe(CN)_6]^{3-/4-}$ before and after incubation with PSA. The concentration of PSA was 100 ng mL^{-1}. The incubation time for electrode in PSA solution was 30 min.

Differential pulse voltammetry can decrease the background charging currents and in turn increase the detection sensitivity. We investigated the effect of cleavage time on the current change with differential pulse voltammetry. Fig. 4A shows the representative differential pulse voltammograms (DPVs). The current decreases with the increase in the incubation time and begins to

level off beyond 30 min (Fig. 4B), indicating that the PSA-catalyzed cleavage reaction is completed within 30 min. The current did not finally drop to the background level, indicating that not all the peptides assembled onto the electrode surface were cut by PSA. The result was consistent with that reported previously [20].

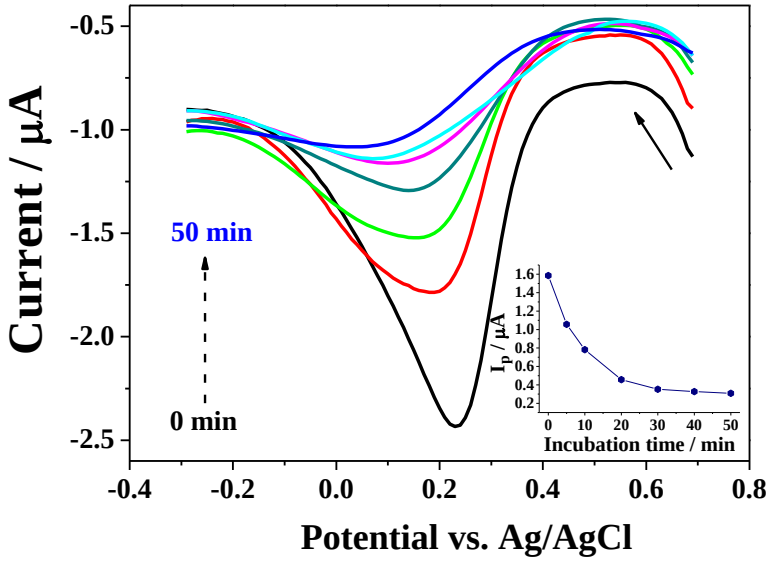

Figure 4. Effect of incubation time on PSA-catalyzed cleavage reaction. The experimental conditions are the same as those in Fig. 3.

3.4 Sensitivity to PSA

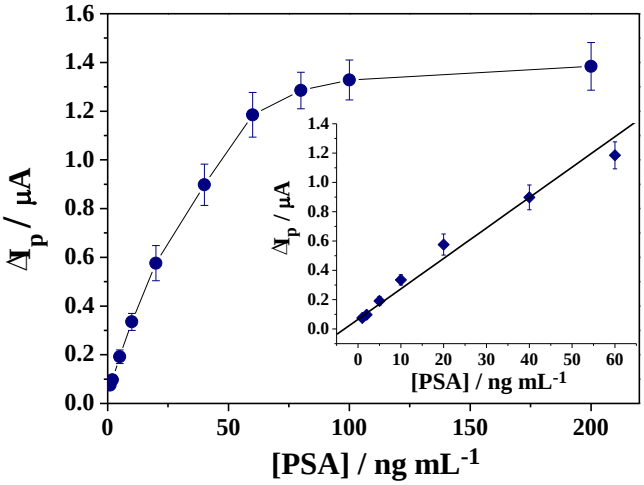

Figure 5. Dependence of the current variation (ΔI_p) on the PSA concentrations in the range of 1~200 ng mL^{-1}. Other experimental conditions are the same as those in Fig. 3. The absolute errors were deduced from at least three replicate measurements and are shown as the error bars.

The peptide/MCH-covered electrodes were further used to challenge different concentrations of active PSA. The variation of the current is given by ΔI_p ($\Delta I_p = I_p - I_p^0$, where I_p^0 and I_p represent the

current before and after the incubation with PSA, respectively). As shown in Fig. 5, ΔI_p linearly increased with the increase of the PSA concentration ranging from 1 to 60 ng mL^{-1}. The linear regression equation is expressed as $\Delta I_p = 0.65 + 0.21$ [PSA] (ng mL^{-1}) ($R^2 = 0.986$). The detection limit was estimated to be 0.5 ng mL^{-1}, which is comparable to that achievable using Fc-labeled peptide probe [20]. A point worth to mention is that Fc-labeled sensor exhibits a significant signal loss after multiple scans and long-term storage due to the degeneration of Fc tags [25, 26]. Therefore, label-free sensor presented here could improve the exactness and reduce the operation complexity and assay cost.

4. CONCLUSION

In conclusion, we reported a label-free electrochemical strategy for the detection of protease based on the change of the surface charge. The feasibility and sensitivity of this method was demonstrated with PSA as model analyte. The peptides with positively charge lysine residues facilitate the access of the negatively charge $[Fe(CN)_6]^{3-/4-}$ probe to the electrode surface. The cleavage of peptides by PSA induced the loss of the positively charged lysine residues, producing a barrier for the electron transfer between redox probe and electrode. The concentration of PSA was determined by monitoring the change in the redox current of $[Fe(CN)_6]^{3-/4-}$. We believe that the label-free approach could be valuable for the design of protease biosensors and likely lead to many detection applications in a biological matrix.

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References

1. J. T. Groves, *Nature*, 389 (1997) 329.
2. L. Hedstrom, *Chem. Rev.*, 102 (2002) 4501.
3. X. S. Puente, L. M. Sanchez, C. M. Overall and C. Lopez-Otin, *Nat. Rev. Genet.*, 4 (2003) 544.
4. C. Guarise, L. Pasquato, V. De Filippis and P. Scrimin, *Proc. Natl. Acad. Sci. USA*, 103 (2006) 3978.
5. L. Liu, J. Du, S. Li, B. Yuan, H. Han, M. Jing and N. Xia, *Biosens. Bioelectron.*, 41 (2013) 730.
6. N. Xia, D. Deng, L. Zhang, B. Yuan, M. Jing, J. Du and L. Liu, *Biosens. Bioelectron.*, 43 (2013) 155.
7. L. Krejcova, L. Krejcova, D. Hynek, V. Adam, J. Hubalek and R. Kizek, *Int. J. Electrochem. Sci.*, 7 (2012) 10779.
8. M. Pohanka, M. Hrabinova, J. Fusek, D. Hynek, V. Adam, J. Hubalek and R. Kizek, *Int. J. Electrochem. Sci.*, 7 (2012) 50.
9. R. M. Iost, J. M. Madurro, A. G. Brito-Madurro, I. L. Nantes, L. Caseli and F. N. Crespilho, *Int. J. Electrochem. Sci.*, 6 (2011) 2965.
10. L. Liu, G. Wang, Q. Feng, Y. Xing, H. Han and M. Jing, *Int. J. Electrochem. Sci.*, 8 (2013) 3814.
11. R. Y. Lai, K. W. Plaxco and A. J. Heeger, *Anal. Chem.*, 79 (2007) 229.
12. Y. Xiao, X. Qu, K. W. Plaxco and A. J. Heeger, *J. Am. Chem. Soc.*, 129 (2007) 11896.

13. D. M. Jenkins, B. Chami, M. Kreuzer, G. Presting, A. M. Alvarez and B. Y. Liaw, *Anal. Chem.*, 78 (2006) 2314.
14. Y. Lu, X. Li, L. Zhang, P. Yu, L. Su and L. Mao, *Anal. Chem.*, 80 (2008) 1883.
15. A.-E. Radi, J. L. A. Sánchez, E. Baldrich and C. K. O'Sullivan, *J. Am. Chem. Soc.*, 128 (2005) 117.
16. D. A. Di Giusto, W. A. Wlassoff, S. Giesebrecht, J. Justin Gooding and G. C. King, *J. Am. Chem. Soc.*, 126 (2004) 4120.
17. C. E. Immoos, S. J. Lee and M. W. Grinstaff, *J. Am. Chem. Soc.*, 126 (2004) 10814.
18. X. Zuo, S. Song, J. Zhang, D. Pan, L. Wang and C. Fan, *J. Am. Chem. Soc.*, 129 (2007) 1042.
19. K. Ohtsuka, I. Maekawa, M. Waki and S. Takenaka, *Anal. Biochem.*, 385 (2009) 293.
20. N. Zhao, Y. He, X. Mao, Y. Sun, X. Zhang, C.-Z. Li, Y. Lin and G. Liu, *Electrochem. Commun.*, 12 (2010) 471.
21. H. Xiao, L. Liu, F. Meng, J. Huang and G. Li, *Anal. Chem.*, 80 (2008) 5272.
22. G. Liu, J. Wang, D. S. Wunschel and Y. Lin, *J. Am. Chem. Soc.*, 128 (2006) 12382.
23. A. Anne, A. Chovin and C. Demaille, *Langmuir*, 28 (2012) 8804.
24. J. Adjémian, A. Anne, G. Cauet and C. Demaille, *Langmuir*, 26 (2010) 10347.
25. D. Ge and R. Levicky, *Chem. Commun.*, 46 (2010) 7190.
26. D. Kang, X. Zuo, R. Yang, F. Xia, K. W. Plaxco and R. J. White, *Anal. Chem.*, 81 (2009) 9109.
27. K. Takehara, H. Takemura and Y. Ide, *Electrochim. Acta*, 39 (1994) 817.
28. L.-P. Qiu, C.-C. Wang, P. Hu, Z.-S. Wu, G.-L. Shen and R.-Q. Yu, *Talanta*, 83 (2010) 42.
29. M. C. Rodriguez, A. N. Kawde and J. Wang, *Chem. Commun.*, (2005) 4267.
30. A. E. Radi, J. L. A. Sanchez, E. Baldrich and C. K. O'Sullivan, *Anal. Chem.*, 77 (2005) 6320.
31. D. K. Xu, D. W. Xu, X. B. Yu, Z. H. Liu, W. He and Z. Q. Ma, *Anal. Chem.*, 77 (2005) 5107.
32. S. D. Keighley, P. Li, P. Estrela and P. Migliorato, *Biosens. Bioelectron.*, 14 (2008) 1291.
33. D. L. Li, X. Q. Zou, Q. Shen and D. S. J., *Electrochem. Commun.*, 9 (2007) 191.
34. Q. Cao, H. Zhao, L. Zeng, J. Wang, R. Wang, X. Qiu and Y. He, *Talanta*, 80 (2009) 484.
35. F. Le Floch, H. A. Ho and M. Leclerc, *Anal. Chem.*, 78 (2006) 4727.
36. L. Shen, Z. Chen, Y. H. Li, P. Jing, S. B. Xie, S. L. He, P. L. He and Y. H. Shao, *Chem. Commun.*, (2007) 2169.
37. L. Liu, D. Deng, Y. Xing, S. Li, B. Yuan, J. Chen and N. Xia, *Electrochim. Acta*, 89 (2013) 616.
38. E. P. Diamandis, *Clin. Chem.*, 46 (2000) 896.