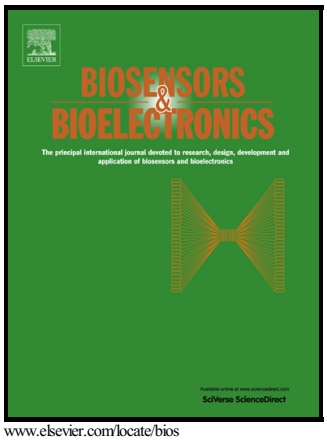

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Carbon nanotubes functionalized electrospun nanofibers formed 3D electrode enables highly strong ECL of peroxydisulfate and its application in immunoassay

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Abstract

A new biosensing platform based on electrospun crabon nanotubes nanofibers (CNTs@PNFs) composite, which enabled strong electrochemiluminescent emission of peroxydisulfate, was firstly developed for immunoassay with favorable analytical performances, and then was utilized to evaluate the interaction between antibody and antigen in vitro. Moreover, the obvious ECL image of peroxydisulfate on the prepared sensing platform was firstly recorded in this report. In order to expand the application of peroxydisulfate ECL, the specific recognization biomolecules, α-fetoprotein (AFP) antibody was bound to the functionalized film via electrostatic interaction for fabricating label-free ECL immunosensor to detect α-AFP. Based on the ECL change resulting from the specific immunoreaction between antigen and antibody, the quantitative analysis for AFP with wide dynamic response in the range from 0.1 pg mL^{-1} to 160 ng mL^{-1} was realized. And the limit of detection was estimated to be 0.09 pg mL^{-1}. Therefore, the flexible sensing platform not only acted as the sensitized sensing element, but also offered a suitable carrier for immobilization of biological recognition elements with low-toxicity and eco-friendliness, which opened a promising approach to developing further electrospun nanofiber based amplified ECL biosensor with favorable analytical performances.

Keywords: electrospun nanofibers; peroxydisulfate; immunoassay;

1. Introduction

Electrochemiluminescent (ECL) technique is concerned with interplay between

optical information and electrochemistry, which has attracted tremendous attention in developing ultrasensitive immunosensors for determining trace biomarkers owing to its excellent properties such as high sensitivity, simplicity, versatility, stability, rapidity, easy controllability and low background (Yuan et al., 2012; Wei and Wang, 2011). To improve the analytical properties of ECL immunosensor, co-reactants were usually applied in the detection. In the past, peroxydisulfate usually played an important role as the ECL co-reactant in quantum dot based ECL systems (Yang et al., 2010; Tian et al., 2010; Dai et al., 2010), which attribute to the strong oxidation of its electrochemical products and peroxydisulfate could cause the classical "Reductive-oxidative" type ECL emission. Through the negative potential scanning, the electron-hole recombination could lead to obviously enhancement of the ECL responses in quantum dots based system. Nevertheless, the potential interference would be induced by the existences of multi components in ECL assay system. Furthermore, the classic ECL luminescent reagents suffer from quite high analytical cost. Accordingly, on the basis of economic criteria and the need in the ultrasensitive assay, exploration for new, cheap and strong ECL reagents without co-reactant is inevitable. However, till now, the investigations involved the ECL emission from peroxydisulfate were still limited (Koval'chuk, 1999; Yao, 2008; Niu and Yuan, 2011; Yao, et al., 2008). The pursuit of sensitized ECL platform to trigger strong ECL emission from peroxydisulfate for practical application is an issue still expected to be solved. Nanometer size materials act as the sensitized element for constructing sensing interface for peroxydisulfate. Nowadays, various nanometer material

functional nanofibers have caused considerable attention and made them highly attractive to different applications in filtration, drug delivery platforms, tissue engineering and so on. Electrospinning, as a simple technique to obtain one-dimensional nanomateials (Gao, et al., 2012; Moon, et al., 2013; Chronakis, et al., 2009;), which is a process carried out at room temperature that allows the production of polymer fibres. The key feature of electrospinning is the elongation of a polymer solution by an electrostatic field, which easily allows the fabrication of continuous polymeric and hybrid fibers with diameters down to a few tens of nanometers.(Santos, et al., 2014) It is the most versatile and widely used technique for the fabrication of polymer nanofibers. It opens new economically viable possibilities to produce nanofiber (PNFs) with high-quality and low cost. To improve the conductivity of the polymer nanofibers, carbon nanotubes (CNTs) are an ideal candidate to act as a novel adulterant, due to their superior structure, chemical, electrical and thermal performance. (Gao, et al., 2012; Moon, et al., 2013) Herein, for the first time, we reported the highly strong and stable ECL emission of peroxydisulfate observed on CNTs@PNFs without any co-reactant, and the ECL image was recorded. Afterwards, the ECL mechanism of peroxydisulfate was investigated and its preliminary application in fabricating a label-free ECL biosensor for AFP with wide dynamic response, ultrasensitivity and satisfactory reproducibility based on the change caused by specific recognization process was also demonstrated, suggesting peroxydisulfate can be promising and cost-effective candidate for the ECL biologic analysis. Furthermore, the binding constant of AFP and its antibody in vitro was calculated.

2. Experimental Section

2.1. Reagents and instruments

AFP, α-fetoprotein antibody (anti-AFP, monoclone), AFP ELISA kits and bovine serum albumin (BSA, 96–99%) were purchased from Biss Inc. (Beijing, China). Chitosan (CS), Poly(methyl methacrylate) (PMMA) and luminol were obtained from Sigma (St. Louis, MO, USA) and used without further purification. Other reagents were of analytical regent grade. All solutions were prepared with deionized water by a Milli-Q water purification system (Millipore, Milford, MA, USA). ECL intensity versus potential consisted of a BPCL Ultra-Weak Chemiluminescence Analyzer controlled by a personal computer with BPCL program (Institute of Biophysics, Chinese Academy of Sciences) in conjunction with a CHI 760 electrochemical analyzer (Shanghai Chenghua Instrument Co., China). The detail description of this system has been given in previous report (Dai, 2011).

2.2. Construction of ECL immunosensor

First, a disk glassy carbon electrode (GCE) with 3 mm diameter as working electrode was polished to a mirror-like surface with define alumina powder, then it was sonicated thoroughly in ethanol and deionized water for 5 min, respectively. The CNTs@PNFs /CS solution was prepared by dispersing 600μL of 2mg/ml CNTs@PNFs solution and 300μL of 1% CS solution in 100μL water in strong ultrasonic wave field. Then the GCE was immersed into this solution by applying the potential between 0.0V and -1.8V for several cycles. This modified electrode was rinsed with water and dried in the air, then CNTs@PNFs /GCE was obtained. As a

comparison, the PMMA nanofibers and CS modified electrode (PNFs-CS/GCE) and CS modified electrode (CS/GC) were similarly prepared.

AFP antibodies were immobilized on the sensing interface by immersing the modified electrode in anti-AFP solution (100μg/ml) for 80 min at room temperature, and then incubated into BSA solution (0.1wt%) for about 30 min at room temperature to block possible active sites. Then the resultant biosensor was incubated in different concentration of AFP solution for 60 min, as it was showed in Scheme 1. The modified electrode could be stored in refrigerator at 4 ℃ when not in use.

3. Results and Discussion

Scheme 1

3.1. The scheme of the electrochemiluminescent sensor

The electrochemiluminescent sensing strategy for detection of AFP was shown in Scheme 1. CNTs were incorporated in the PMMA to form nanofibers by electrospinning technique. Through electrodeposition, the CS and CNTs@PNFs formed three dimensional network structure for sensing. Benefited from the unique properties of this netlike interface, strong ECL emission of peroxydisulfate was generated. Afterwards, the antibody of AFP, was chosen as a model to conjugate to the hybrid film. When the reaction between antibody and AFP occurred, the ECL response of peroxydisulfate dramatically decreased owing to the non-conductive properties of immunocomplex. Therefore, a specific quantitative ECL immunosensor was developed, which enabled a high loading of the active biochemical receptor and

met the requirement of rapid and specific quantitative detection in disease diagnosis. From SEM image in Fig.1, the thin nanofibers about 700nm in average diameter were initially produced together without large beads at 8 wt% PMMA solution containing 0.5 wt% CNTs, which indicated that the appropriate surface tension of this complex for electrospinning. CNTs did not appear to exist on the surface of the nanofibers in the SEM image. Nevertheless, the TEM image indicated that the end of CNTs actually ranged in sequence at the surface of the nanofibers due to the effect of electric field. It's well known that the ends of CNTs owned highly electrochemical catalysis. Hence, the unique construction of this electrospun material possibly possessed some advantages in fabricating CNTs assisted biosensing platform.

Fig. 2

3.2. Highly sensitive performance of electrochemiluminescent sensor

As it demonstrated in Figure 2A, compared with the electrochemical performance of GCE for $K_3Fe(CN)_6$, the electrodeposited casting film of CS obviously inhibited the electrochemical reaction occurred on the sensing platform due to the compact structure of CS film. However, when the PMMA nanofibers were introduced into CS film, the corresponding electrochemical behavior of $Fe(CN)_6^{3-/4-}$ redox probe at this PMMA nanofibers and CS modified electrode was evidently restrained, which may be attributed to a much higher electron transfer resistance of PMMA nanofibers. Since CNTs were highly conductive and were often utilized as the catalysts own to the unique and charming nanostructure and electronic properties, the peak redox current at the CNTs@PNFs nanostructured electrode was about 3 folds

higher than that at the PMMA nanofibers and CS modified electrode, indicating the bigger active surface and the inner catalysis of this nanostructure nanofibers. Furthermore, this result also demonstrated the highly conductive and facilitated electron transfer kinetics of the CNTs functionalized PMMA nanofibers, illuminating the potential application of this nanomaterial in electroanalysis. A cyclic voltammogram of peroxydisulfate was shown in insert of Figure 2B. At the negative potential side, an irreversible wave corresponding to the reduction of peroxydisulfate was observed at around -1.5V. Meanwhile, an ECL emission could be triggered responding to the reductive process of peroxydisulfate. Fig. 2B illustrated at the bare GCE, only a very weak ECL emission could be observed. Nevertheless, the greatly enhanced ECL response from peroxydisulfate could be generated by adopting the PMMA nanofibers and CS as the modified component to construct the sensing platform. By comparing the ECL curve a, b, c in Figure 2B, the electrospun PMMA nanofibers were found could evidently promote the emission of peroxydisulfate which was put down to the high porosity and the three dimensional construction. The utilization of electrospun nanofibers as the incorporation of CNTs within the fibrous structure resulted in prodigious amplification for peroxydisulfate ECL, ascribing to the anisotropic properties such as electrical conductivity of this electrospun material. By employing CCD camera, the first ECL image of peroxydisulfate at the CNTs@PNFs modified electrode was recorded. As it shown in Figure 2C, dark orange ECL image could be observed at the prepared sensor. Compared with the ECL image of Internal, a bright ECL image could be observed at circle. To understand the

detail ECL mechanism of peroxydisulfate, a series of experiments were executed in order to gain a further understanding of the peroxydisulfate ECL (Fig. S2). A clear ECL emission peak could be observed at the as-prepared modified electrode in peroxydisulfate air-saturated PBS solution, while nearly no ECL emission was observed in N_2 saturated peroxydisulfate air-saturated PBS solution. This proved the dissolved oxygen played a crucial role in the peroxydisulfate ECL. In general, several reactive oxygen species (ROS), such as $O^{2\bullet-}$, H_2O_2 and •OH, may be produced by the electro-reduction of oxygen. To test which kind of ROS might be essential for ECL, H_2O_2 was first added to the N_2 saturated peroxydisulfate solution, and the ECL emission of peroxydisulfate did not change with the addition of H_2O_2, which strongly demonstrated that $O^{2\bullet-}$ or $HO_2^{\bullet}$ rather than other ROS was involved in the ECL process. An additional experimental result showed that ECL was pH-dependent, i.e., the lower the pH, the stronger the ECL intensity in 2×10^{-3} M peroxydisulfate solution. Consequently, it led to a conclusion that $HO_2^{\bullet}$ rather other $O_2^{\bullet-}$ acts as the highly reducing species in this ECL process. According to the previous discussion, the ECL mechanism of peroxydisulfate was that the dissolved oxygen was the first reduced to $HO_2^{\bullet}$ at the modified electrode, $S_2O_8^{2-}$ was reduced to the strong oxidant $SO_4^{\cdot-}$ at the same time, then $HO_2^{\bullet}$ could react with $SO_4^{\cdot-}$ produced light-emitting species $^1(O_2)^{2*}$, because $^1(O_2)^{2*}$ was not stable, which jumped to ground state became triplet state 2^3O_2 and released energy. (Yao et al., 2008; Niu and Bard, 1982) Thereby the correesponding ECL process of peroxydisulfate could be described as it shown in S2. (Jie et al., 2008).

To extend the application range of this kind electrospun nanofibers modified electrode in peroxydisulfate ECL, the antibody of α-fetoprotein was chosen to immobilize onto the electrospun nanofibers based sensing platform. Upon the optimized conditions, as Fig S3 and Fig S4 shown, an ECL immunosensor for AFP was developed with sensitive detection and wide dynamic response range. As Fig S4D displayed, results showed the ECL intensity of this biosensor in the absence and presence of different concentrations of AFP. With the increment of AFP, the ECL intensity decreased gradually with the increased concentration of AFP, owing to the formation of non-conductive immune-complex. As has already been shown in the ECL profiles(Figure 3), the dynamic response range for APF by the proposed method was from 0.1 pg mL^{-1} to 160 ng mL^{-1}, the linear response range was wider than those of others strategies for the quantitative determination of AFP with the detection limit of 0.09 pg mL^{-1}, which was much lower than that reported previously (Table S1). Moreover, as Fig 4S showed, the prepared sensor possessed good repeatability and stability and showed potential for practical applications. To assess the specificity of this ECL immunosensor, the influences of the same concentration of other proteins such as CA125, CA19-9, CEA, PSA were examined under the same experimental conditions. None of these chose proteins could cause obvious alteration of the ECL signal. The result elucidated that the as-proposed immunosensor exhibited good selectivity for AFP assay.

Fig. 3

3.3. The interaction process of antibody and antigen

The coincident ECL emission of this immunosensor for the same concentration of AFP was also tested, which illuminated the potential practical application in clinical diagnosis. Assuming the interaction process of antibody and antigen could meet the requirements of the Langmuir isotherm (Langmuir, 1916; Norio, 2003; Sun et al., 2010). Hence, the binding constant (equilibrium constant, Kb) could be expressed as followed:

$$\text{Antibody} + \text{Antigen} = \text{Antibody} * \text{Antigen} \qquad (1—1)$$

$$K_b = \frac{[\text{Antibody} * \text{Antigen}]}{[\text{Antibody}][\text{Antigen}]} \qquad (1—2)$$

where [Antibody], [Antigen], and [Antibody*Antigen] represent the surface concentration of antibody on modified electrode, the solution concentration of antigen, and the surface concentration of conjugated antibody-antigen on modified electrode, respectively. Thus, according to Langmuir equation, the binding constant (K_b) can be described as follows:

$$|\Delta I_{ECL}| = |\Delta I_{ECL}^{max}| \frac{[Antibody] K_b}{1 + K_b [Antigen]} \qquad (1—3)$$

Then, the equation 3 could be rearranged as equation 4:

$$\frac{[\text{Antigen}]}{|\Delta I_{ECL}|} = \frac{1}{|\Delta I_{ECL}^{max}| * K_b} + \frac{[\text{Antigen}]}{|\Delta I_{ECL}^{max}|} \qquad (1—4)$$

Therefore, as it described in equation 4, $[Antigen]/\Delta I_{ECL}$ versus $[Antigen]/\Delta I_{ECL}^{max}$ exhibited linear relationship (Figure 3). Figure 3b depicted a plot, which resulted in an intercept of 5.02×10^{-4} and a slope of 2.22×10^{-4} ($R^2 = 0.999$). Accordingly, ΔI_{ECL}^{max} =4484 (a.u.) and K_b = 0.44 mL/ng (or 3.15×10^{10} M^{-1}) were obtained. The large K_b value suggested that under present experimental conditions the binding strength

between Antigen and the surface-confined Antibody was significantly strong. Other studies have shown that the binding constants were highly target dependent and ranged from the 10^{12} M^{-1} to 10^{6} M^{-1} for various targets (Nimjee, et al., 2005, Cho, et al., 2009, Rusconi, et al., 2002). This result indicated the firm bonding between AFP and its antibody (Nirio, 2003; Sun et al., 2010).

4. Conclusions

In summary, a new strategy for triggering highly active ECL of peroxydisulfate by utilizing three dimensional nanocomposite modified sensing platform was proposed. This 3D nanocomposite modified electrode aroused strong ECL emission of peroxydisulfate, which was nearly 35 folds higher than that case on GCE. Moreover, the obvious ECL image of peroxydisulfate on the prepared sensing platform was firstly recorded in this report. Then the specific recognization biomolecules, α-fetoprotein (AFP) antibody was bound to the functionalized film via physisorption electrostatic interaction for fabricating label-free ECL immunosensor to detect α-AFP with wide dynamic response, ultra sensitivity and satisfactory reproducibility. Furthermore, the binding constant of AFP and its antibody was firstly calculated in vitro. This flexible sensing platform not only can acted as the sensitized sensing element for peroxydisulfate ECL, but also offered a suitable carrier for immobilization of biological recognition elements with low-toxicity and eco-friendliness, which opens a promising approach to develop further electrospun nanofibers based amplified ECL biosensors with favorable analytical performances.

Acknowledgment

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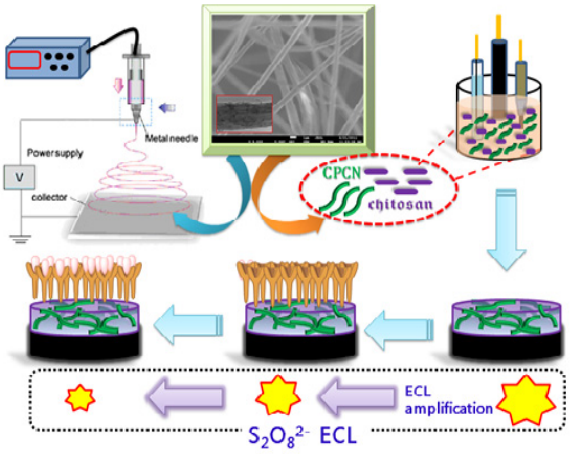
Figure captions

Figure 1. Schematic routine of the label-free ECL sensing platform for APF.

Figure 2 (A) Cyclic Voltammetrys of different modified electrodes: in 0.1mol/L KCL solution contained 5mmol/L $[Fe(CN)_6]^{3-}$. (B) ECL intensity and potential curve of different modified electrodes in 0.1mol/L pH 7.0 PBS solution containing 0.1mol/L peroxydisulfate. The inset was electrochemical behaviours of the corresponding electrode. (a) GCE, (b) CS/GCE, (c)PMMA PNFs/CS/GCE (d)CNTs@PNFs/CS/GCE, scan rate: 0.1V/s. (C) The ECL image of peroxydisulfate at the CNTs@PNFs modified electrode at the same condition as B.

Figure 3 Langmuir isotherms obtained from the immunosensor after interactions with various concentrations of AFP. (A) Non-linear regression between the change of ECL emission at immunosensor and the concentration of AFP, (B) linear regression between [AFP]/ΔIECL and the concentration of AFP.

Figure 1



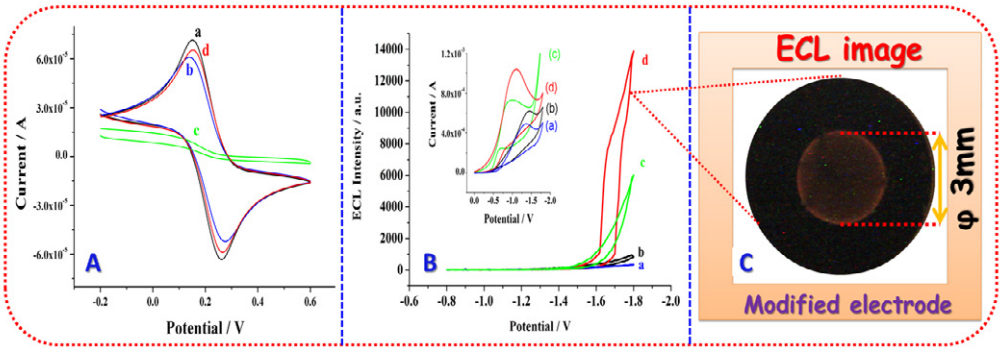


Figure 2

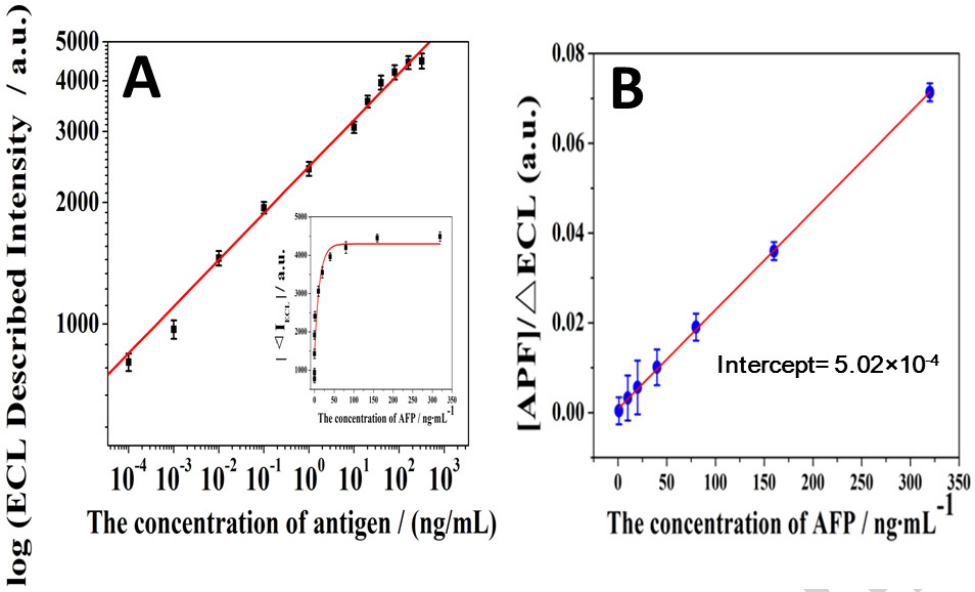


Figure 3

Highligts

- Peroxydisulfate was used as a new cheap, strong ECL reagent for immunoassay.
- Using electrospun composite nanofibers to form 3D electrode for ECL immunoassay.
- An ECL image of peroxydisulfate on the sensing platform was firstly recorded.
- Offerring a method to caculate the interaction of biological recognition system.