

## One Novel Nano Magnetic Fe<sub>3</sub>O<sub>4</sub>/ZrO<sub>2</sub>/nano Au Composite Membrane Modified Amperometric Immunosensor for $\alpha$ -Fetoprotein in Human Serum

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One novel amperometric immunosensor for  $\alpha$ -fetoprotein(AFP) was fabricated based on AFP antibody(anti AFP) modified glassy carbon electrode(GCE) which was immobilized in nano Fe<sub>3</sub>O<sub>4</sub>(core)/ZrO<sub>2</sub>(shell)-nano Au -poly Lysine(Fe<sub>3</sub>O<sub>4</sub>-ZrO<sub>2</sub>-nano Au-pLL) composite membrane. Firstly, the deoxyribonucleic acid-phenoxyacetic acid isoniazid schiff base Co(II) complex (DNA-CoRb) mixture film was cast on surface of multicarbon nanotubes(MWCNTs) modified glassy carbon electrode (GCE/CNTs/DNA-CoRb). Then Fe<sub>3</sub>O<sub>4</sub>-ZrO<sub>2</sub>-Au-pLL was employed to immobilize the antibody of  $\alpha$ -fetoprotein(anti AFP) to produce the magnetic probes for AFP. Finally the probes were modified on GCE/ CNTs/DNA-CoRb through the specific absorption ability between DNA and ZrO<sub>2</sub> to form a novel membrane immunosensor for AFP(GCE/ CNTs/ DNA-CoRb/Fe<sub>3</sub>O<sub>4</sub>-ZrO<sub>2</sub>-Au-pLL-anti AFP). The function of immunosensor was investigated by scanning electron microscopy (SEM), cyclic voltammetry (CV), and electrochemical impedance spectroscopy (EIS). The results indicated that the immunosensor combined the advantages of the MWNTs-DNA material and Fe<sub>3</sub>O<sub>4</sub>-ZrO<sub>2</sub>-Au-pLL-anti AFP nano probes with excellent sensitivity and selectivity to AFP. The modified electrode was sensitive to AFP with a linear relationship between 0.05 and 10 ng · mL<sup>-1</sup> and a correlation coefficient of 0.9905. The detection limit at a signal to noise ratio of 3 was 0.01 ng·mL<sup>-1</sup> under the optimal conditions. The described preparation of the immunosensor and immunoassay methods offer promise for simple and cost-effective analysis of AFP in serum samples.

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**Keywords:** cyclic voltammetry (CV), Fe<sub>3</sub>O<sub>4</sub>(core)/ZrO<sub>2</sub>(shell) nanoparticles, nano gold colloid, poly Lysine (pLL), deoxyribonucleic acid (DNA),  $\alpha$ -fetoprotein (AFP)

### 1. INTRODUCTION

It is well known that  $\alpha$ -fetoprotein (AFP), an oncofetalgly coprotein with a molecular weight of approximately 70,000 Da, is an important tumor marker. It has been reported that hepatic cancer can

be suspected if the total AFP level is more than  $20 \text{ ng}\cdot\text{mL}^{-1}$  in adult serum [1–2]. Conventional immunoassay methods which are employed to detect AFP in serum include radio immunoassays, single radial immunodiffusion, immuno-turbidimetry and enzyme-linked immuneosorbent assays [3–4]. Compared with the above conventional techniques, recently the electrochemical immunosensor, which possesses the promising properties of specific and simple detection, low cost, short assay time, and suitable miniaturization, has recently attracted increasing interest.

Although a low detection limit can be achieved via electrochemical immunosensor, most antibodies and antigens are intrinsically unable to act as redox partners. Thus, electron mediators should be attached to the electrode surface or dissolved in the detection solution. The electroactive product of the electron mediators can be used to produce the electrochemical signal [5–7]. In recent years, our research group reported many immunosensor based on Cu complex as electron mediator which have excellent electrocatalytic behavior towards  $\text{H}_2\text{O}_2$  to amplify the current signals' change in the immunoreaction with high sensitivity and accuracy [8]. In this work, one novel phenoxy acetic acid isoniazid schiff base Co(II) complex (referred to as CoRb) was employed as an electron transfer mediator. DNA can not only provide a biocompatible microenvironment for bimolecular, but also greatly increase the coverage of bimolecular on the electrode surface. Moreover, CoRb exhibited excellent electrocatalytic behavior towards carbamide peroxide (CP) which can amplify the current signals' change by the immunoreaction of the immunosensor .

Nano gold colloid (GNP) can provide a natural environment for bimolecular immobilization and facilitate the electron-transfer of biosensor because of their high surface area, interesting electrochemical properties, and good biocompatibility. The long-branch chains of poly-Lysine(pLL) can decrease the repulsive force between antibody and the surface, and maintain the native functional state of a protein[9]. So Au-pLL can improve the antibody immobilization on the modifie delectrode. Researchers have demonstrated that  $\text{ZrO}_2$  had strong affinity for the phosphoric group and proved to be a fixed ideal material of biological molecules [10–11].  $\text{ZrO}_2$  which can attach with the phosphate group of DNA was used for the antibody loading, which can not only prevent the leakage of antibody, but also accelerat the electrons between the protein molecule and electrode [12]. Therefore nano  $\text{Fe}_3\text{O}_4$ - $\text{ZrO}_2$ -Au-pLL composite particles can be easily incorporated DNA which can be employed as electron transfer and membrane matrix to incorporate AFP antibody (anti AFP) as probes for AFP. Furthermore, the probes can be easily separated from the solution through external magnetism.

In this paper, firstly, CoRb-DNA mixture film was cast on surface of multi carbon nanotubes(MWCNTs) modified glassy carbon electrode(GCE/CNTs/DNA-CoRb) which have excellent electro conductive for MWCNTs' unique electronic and mechanical properties, and CNTs have been widely used in the fields of electroanalysis including electrochemical sensor or electrocatalysis..Then the composite nanoparticles ( $\text{Fe}_3\text{O}_4$ - $\text{ZrO}_2$ -Au-pLL) was employed to immobilize the antibody of AFP to produce the probes( $\text{Fe}_3\text{O}_4$ - $\text{ZrO}_2$ -Au-pLL-anti AFP) for AFP. Finally the probes was modified on GCE/CNTs/ DNA-CoRb to form a novel nanocomposite membrane immunosensor for AFP(GCE/CNTs/ DNA-CoRb/  $\text{Fe}_3\text{O}_4$ - $\text{ZrO}_2$ -Au-pLL-antiAFP). And the detection functions of immunosensor for AFP were investigated by electrochemical and spectrum methods in details.

## 2. EXPERIMENTAL PART

### 2.1. Chemicals and materials

Carbon nanotubes (CNTs), hydrochloroauric acid, poly-Lysine (pLL) were purchased from Shenzhen Nanoport and Sinopharm Chemical Reagent Co. Ltd in China. Double-stranded calf thymus (DNA) and AFP enzyme-linked immunosorbent assay (ELISA) kits were purchased from Autobio Diagnostics Co.Ltd in USA.  $0.1 \text{ mol}\cdot\text{L}^{-1}$  phosphate buffer solutions (PBS) with different pH values were prepared by mixing the stock standard solutions of  $\text{KH}_2\text{PO}_4$  and NaOH. All solutions were made with double distilled water. Other reagents were of analytical reagent grade.

### 2.2. Instrumentations and Apparatus

Scanning electron microscopy (SEM) images of the modified electrode surface were obtained using a S-3400N scanning electron microscope (Hitachi, Japan). Absorption spectra were acquired using a UV-vis spectrophotometer (Beijing ,China). Cyclic voltammeteries (CV) and Electrochemical impedance spectroscopy (EIS) measurements were performed on electrochemical Analyzer (Chenhua Instrumental Co., China) CHI660A controlled by a personal computer. A three-electrode system was employed, including a modified glassy carbon electrode as the working electrode, a platinum wire as the auxiliary electrode, and saturated calomel electrode electrode as the reference electrode.

### 2.3. Preparation of soluble CNTs

CNTs were chemically shortened by ultrasonic agitation in a mixture of sulfuric acid and nitric acid (3:1) for 4 h. The resulting CNTs were separated and washed repeatedly with distilled water by centrifugation until the pH was 7.0. 1.0 mg CNTs were dissolved in 1.0 mL dimethylformamide (DMF) solutions using ultrasonic liquid processors for 15 min to give a homogeneous black solution.

### 2.4. Preparation of DNA-CoRb

Phenoxyacetic acid isoniazid Schiff base (HL) were prepared by refluxing isoniazid (10 mmol) with oxygen acid aldehyde (10 mmol) in isopropanol solution (80 mL) at  $80 \text{ }^\circ\text{C}$  for 3 h. The ligands wer filtered, dried. Phenoxyacetic acid isoniazid Schiff base Co (CoRb) were prepared by refluxing 0.5 g HL (10 mmol) with 0.4832 g Co (II) perchlorate in isopropanol solution (80 mL) at  $80 \text{ }^\circ\text{C}$  for 48 h. The product was filtered, dried. 0.1 mg DNA were solubilized in 10 mL 10 mmol/L Tris-HCl

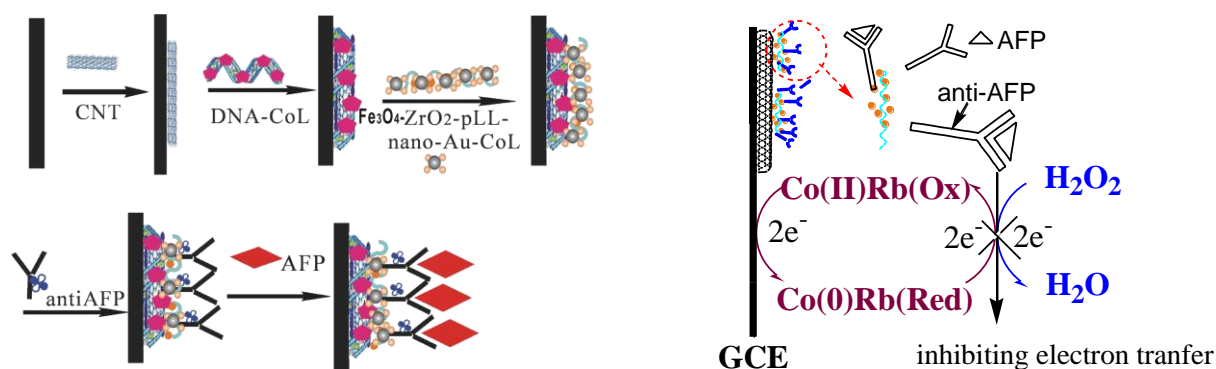
(pH=8.0), 0.2 mmol/L EDTA buffer. Then 0.5 mL of DNA solution was mixed with the equivalent volume of CoRb saturated solution.

### 2.5. Preparation of $Fe_3O_4$ - $ZrO_2$ -Au-pLL particles

Nano  $ZrO_2$  colloid was synthesized according to literature [25] and Au-pLL colloid according to literature [22]. Colloidal Au was prepared by dissolving 8.8 mg chloroauric acid in 15 mL of deionized water, 2 mL of ethanol, and 3 mL of 0.1% pLL-solution. The mixture was then maintained at 80 °C for 3 hours. The resulted colloidal Au was stored in a brown glass bottle at 4 °C. Then 10 mL of  $ZrO_2$  suspension was mixed with the equivalent volume of Au-pLL colloid solutions. The obtained magnetic particles ( $Fe_3O_4$ - $ZrO_2$ -Au-pLL) were separated by magnetic decantation and washed with PBS for three times.

### 2.6. Preparation of the immunosensor

The pretreated glassy carbon electrode (GCE) (diam=3 mm) was dropped 5  $\mu$ L 1000  $mg \cdot L^{-1}$  CNTs solution. The modified electrode (GCE/CNTs) was washed with double distilled water.



**Figure 1.** Preparation process of GCE/CNTs/DNA-CoRb/ $Fe_3O_4$ - $ZrO_2$ -Au-pLL-anti-AFP immunosensor

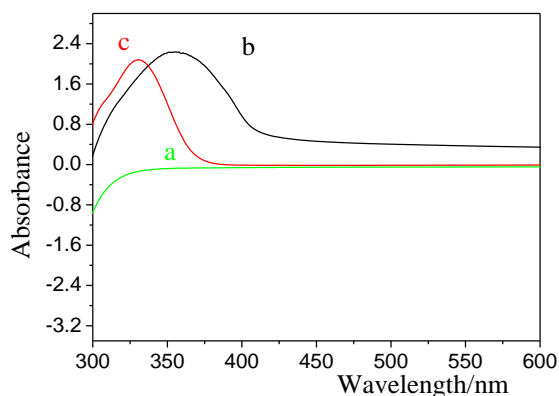
Then 20  $\mu$ L of freshly prepared 400  $mmol \cdot L^{-1}$  EDC and 100  $mmol \cdot L^{-1}$  NHS in water were placed onto the CNTs forest electrodes, and washed off after 10 min. A volume of 5  $\mu$ L DNA-CoRb composite solution was piped onto the surface of the GCE/CNTs. Then the modified electrode (GCE/CNTs /DNA-CoRb) was washed with double distilled water, and 5  $\mu$ L  $Fe_3O_4$ - $ZrO_2$ -Au-pLL colloid solution was dropped on it to dry at 4 °C overnight. Then the modified electrode was incubated in AFP antibody solution by 3 hours. The procedures used for construction of the immunosensor were showed in Figure 1. The GCE/CNTs/DNA-CoRb/ $Fe_3O_4$ - $ZrO_2$ -Au-pLL-anti-AFP was incubated in horseradish peroxidase (HRP) solution (w/w, 0.25%) about 1 h at 35 °C in order to block possible

remaining active sites and avoid the non-specific adsorption. The finished immunosensor was stored at 4 °C before use.

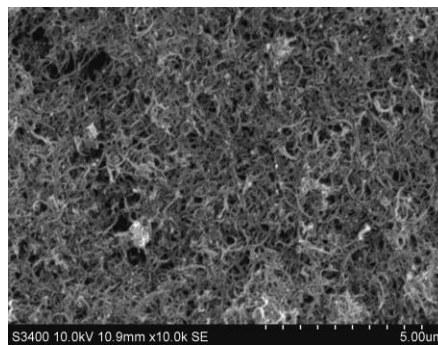
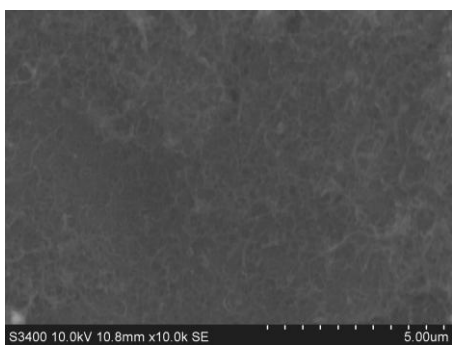
### 3. RESULTS AND DISCUSSION

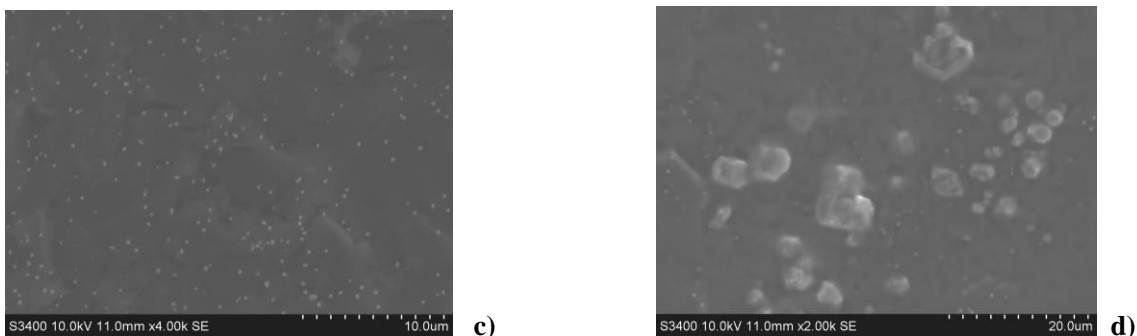
#### 3.1. Characterization

The interaction of CoRb with DNA was investigated using ultra violet(UV) and visible(vs) absorption spectra from 300 nm to 600 nm in Figure 2. The spectrum of DNA showed a smooth curve (Figure 2a). The adsorption peak at 355 nm was the characteristic peak of free CoRb in Figure 2b. However, a blue shift of the previous 355 nm peak to 330 nm when CoRb was added into DNA solution in Figure 2c which proved the intercalative binding of CoRb molecules with DNA.



**Figure 2.** UV-vis absorption spectra of solution of DNA (a), CoRb (b), DNA-CoRb composites (c).



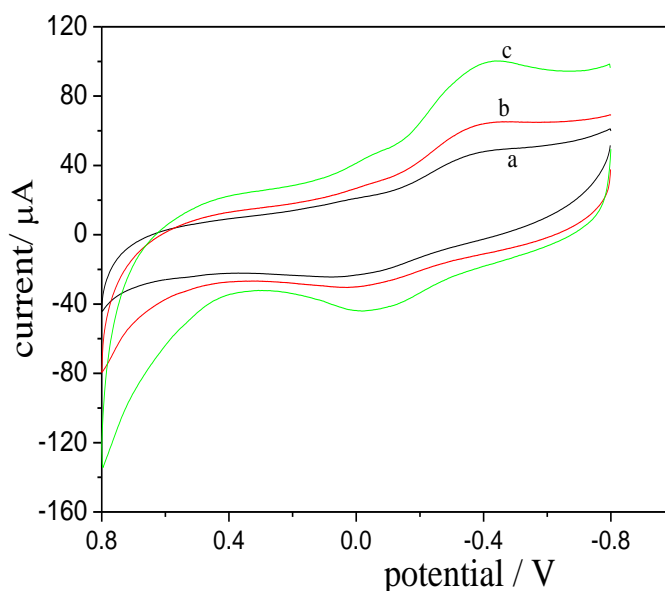


**Figure 3.** The SEM images of GCE/CNTs (a), GCE/CNTs/DNA-CoRb (b), GCE/CNTs /DNA-CoRb /ZrO<sub>2</sub>-pLL-Au (c), GCE/CNTs/DNA/ZrO<sub>2</sub>-pLL-Au-anti-AFP (d).

The SEMs were used to observe the surface morphology of GCE/CNTs (a), GCE/CNTs/DNA-CoRb (b), GCE/CNTs/DNA-CoRb/ZrO<sub>2</sub>-pLL-Au (c), GCE/CNTs /DNA-CoRb /ZrO<sub>2</sub>-pLL-Au-anti-AFP (d) in Figure 3. According to Figure 3a, the homogeneous dispersion CNTs were in the form of small single tubes. When DNA-CoRb was coated on the CNTs film, DNA-CoRb could be clearly seen on CNTs surfaces and the surfaces became dense membrane (Figure 3b). After ZrO<sub>2</sub>-pLL-Au coated on the DNA film (Figure 3c), it can be seen that Au were dispersed uniformly on the ZrO<sub>2</sub> film and the average diameter of the nano-Au was about 20 nm. When the anti-AFP molecules were immobilized on the CNTs/DNA/ZrO<sub>2</sub>-pLL-Au composite film (Figure 3d), the nano-Au was almost covered and the surface became rougher.

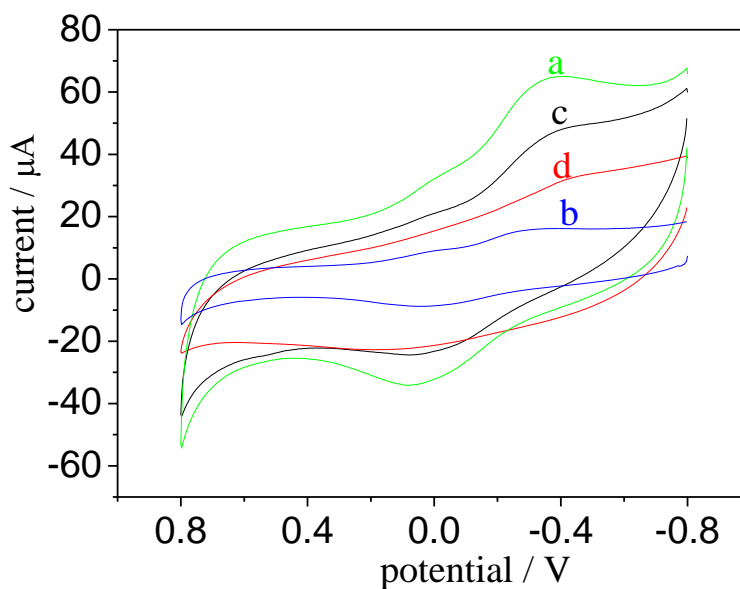
### 3.2. Cyclic voltammetric characterization of the immunosensor

Cyclic voltammetry (CV) is a valuable and convenient tool for providing electro activity information regarding the modified immunosensors, as well as the activity and stability of immobilised reagents.



**Figure 4.** Cyclic voltammetry (CV) of GCE/CoRb (a), GCE/CNTs/CoRb (b), GCE/CNTs/ DNA-CoRb (c) in pH 7.0 PBS adding 5 mmol·L<sup>-1</sup> CP (scan rates: at 100 mV·s<sup>-1</sup>)

Figure 4 showed the cyclic voltammogram of different electrodes in  $0.1 \text{ mol}\cdot\text{L}^{-1}$  pH 7.0 oxygen-free PBS adding  $5 \text{ mmol}\cdot\text{L}^{-1}$  CP at  $100 \text{ mV}\cdot\text{s}^{-1}$ . When CoRb was casted directly on the electrode surface, the GCE/CoRb, GCE/CNTs/CoRb also showed the response of CoRb (Figure 4a and b), but the response was smaller than that of DNA-CoRb. This can be ascribed to the negatively charged phosphate groups of DNA accelerating the access of CoRb to the GCE/DNA-CNTs electrode via the negative-positive charge interaction[26].

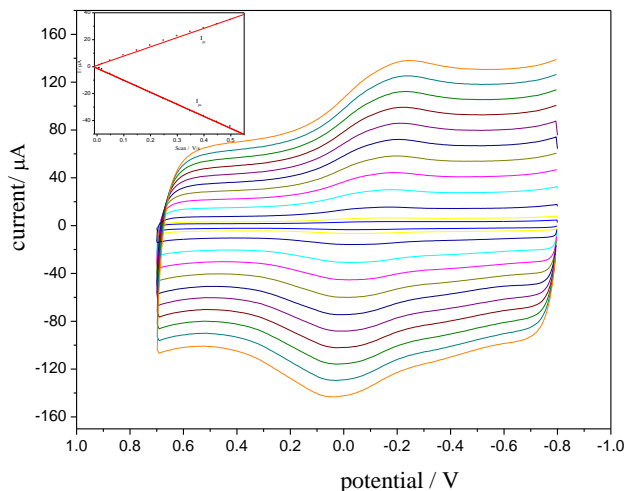


**Figure 5.** Cyclic voltammetry (CV) of GCE/CNTs/DNA-CoRb (a), GCE/CNTs/DNA-CoRb/ZrO<sub>2</sub>-Au-pLL-anti-AFP (b), GCE/CNTs/DNA-CoRb/ZrO<sub>2</sub>-Au-pLL-anti-AFP blocked by HRP (c), GCE/CNTs/DNA-CoRb/ Fe<sub>3</sub>O<sub>4</sub>-ZrO<sub>2</sub>-Au-pLL-anti-AFP-HRP/AFP (d) in pH 7.0 PBS adding  $5 \text{ mmol}\cdot\text{L}^{-1}$  CP (scan rates: at  $100 \text{ mV}\cdot\text{s}^{-1}$ )

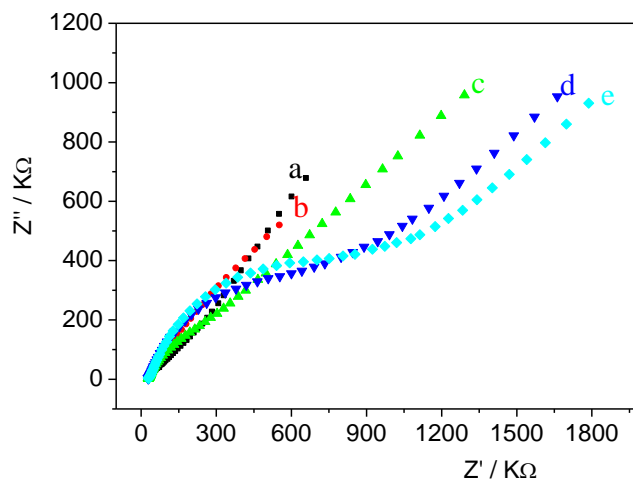
Figure 5a showed the CV of the GCE/CNTs/DNA-CoRb electrode in pH 7.0 PBS adding  $5 \text{ mmol}\cdot\text{L}^{-1}$  CP (scan rates: at  $100 \text{ mV}\cdot\text{s}^{-1}$ ) which was induced by CP the catalyzed by CoRb. Figure 5b showed there was an obvious reduction in current response at  $-0.400 \text{ V}$  when the electrode of (a) was modified with ZrO<sub>2</sub>-Au-pLL-anti-AFP, composite membrane. Obviously, the presence of non-conductive composite membrane decreased the reductive current response of electrode (a). The reductive current of CP increased obviously when the immunosensor blocked with HRP as showed in Figure 5c because HRP can catalyze the reaction between CoRb and CP. However the current response of the immunosensor decreased when it was incubated with AFP because the produced AFP-anti-AFP immunocomplexes increased the resistance of the immunosensor(Figure 5d). Figure 6 showed the CVs of the immunosensor in pH 7.0 PBS at scan rates ranging from 10 to  $500 \text{ mV}\cdot\text{s}^{-1}$ . The inset of Figure 4 showed the linear dependence of the peak current on scan rate, indicating that the electrochemical process of CoRb at GCE/DNA-CNTs electrode was, as expected, a surface-confined one.

### 3.3. Electrochemical impedance characterization of the modified electrode

Electric impedance spectroscopy (EIS) is a valuable and convenient tool to monitor the barrier of the modified electrode, and it is an effective method to prove that the interface properties of the surface-modified electrodes could be successfully applied in an immunoassay [27–29]. The complex impedance plots of different layer-modified electrodes were shown in Figure 7.



**Figure 6.** CVs of immunosensor in pH 7.0 PBS at 10, 20, 50, 100, 150, 200, 250, 300, 400, 450, and 500  $\text{mV}\cdot\text{s}^{-1}$  (from lowest to highest peak currents). Inset: plots of peak currents vs scan rate.



**Figure 7.** The Nyquist plots of bare and different modified electrodes. GCE electrode (a) and GCE/CNTs (b), GCE/CNTs/DNA-CoRb (c), and GCE/CNTs/DNA-CoRb/ $\text{Fe}_3\text{O}_4$ - $\text{ZrO}_2$ -Au-pLL-anti-AFP (d), GCE/CNTs /DNA-CoRb/ $\text{Fe}_3\text{O}_4$ - $\text{ZrO}_2$ -Au-pLL-anti-AFP/HRP (e).

The bare and modified electrodes were compared by EIS in hexacyanoferrate solutions at a frequency range from  $5 \times 10^{-2}$  to  $1 \times 10^5$  Hz in a given open circuit voltage with an amplitude of 10 mV. The EIS of the bare electrode is shown in Figure 7b. As expected, a small semicircle was obtained. After modifying the electrode surface with CNTs (Figure 7a), were observed a slightly smaller semicircle,



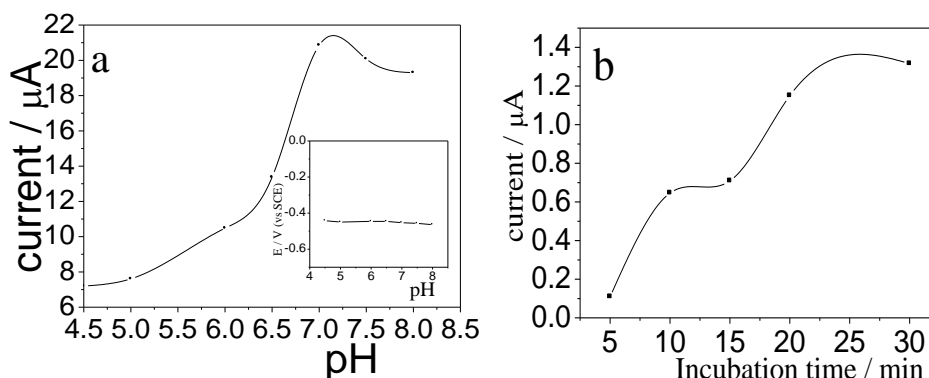
indicating a lower electron transfer resistance at the modified electrode due to the good electron transfer ability of CNTs. After immobilisation of DNA-CoRb onto the electrode surface, an increased interfacial Ret ( $R_{et} = 400 \Omega$ ) was observed (Figure 7c). After immobilization of anti-AFP onto the electrode surface, an increased interfacial Ret ( $R_{et} = 600 \Omega$ ) was observed (Figure 7d), and the Ret increased to  $900 \Omega$  after the electrode was blocked with HRP (Figure 7e). This increase was attributed to the fact that the outer protein membrane insulated the conductive support and counteracted the interfacial electron transfer.

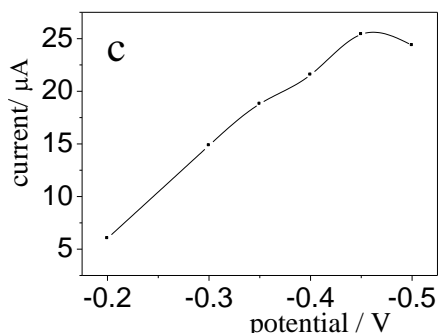
### 3.4 Optimization of experimental parameters

In order to provide an immunosensor with a good performance, the pH of working buffer, the incubation time and the applied potential for the antibody immobilization were investigated.

The effect of pH on the immunosensor behavior was induced mainly by two aspects: one is the activity of CoRb, the other is the impact of peak potential. As shown in Figure 8a, the peak current of the sensor increased with increasing pH value from 4.5 to 7.0 and decreased after further increasing pH value. The maximum current response occurred at pH 7.0. So PBS of pH 7.0 was chosen throughout this study. Besides, the change of peak potential with pH values showed that the electrode reaction process was not involved in proton (the inset of Figure 8a).

The effect of incubation time was investigated being showed in Figure 8b. The results showed that the response current of immunosensor was rapidly up with the duration of incubation time from 5 to 25 min. The longer incubation time did not result in further change of the current response, which indicated the saturated formation of immunocomplex in the matrix. Therefore, 25 min was used for incubation time of all the subsequent assays. The effect of the applied potential on the response current of the GCE/CNTs/DNA-CoRb/ $Fe_3O_4$ - $ZrO_2$ -Au-pLL- anti-AFP electrode was illustrated in Figure 8c. When the applied potential was changed from  $-0.2$  V to  $-0.5$  V, the maximum response current was observed at  $-0.45$  V. So a potential of  $-0.45$  V was selected as the optimum applied potential.

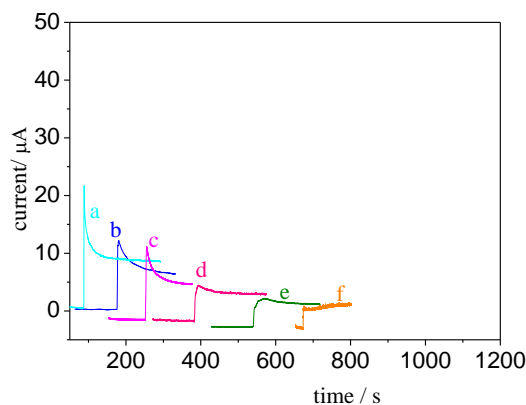




**Figure 8.** Effects of pH of substrate solution (a), incubation time (b), potential (c) of  $100 \text{ mV s}^{-1}$  in pH 7.0 PBS.

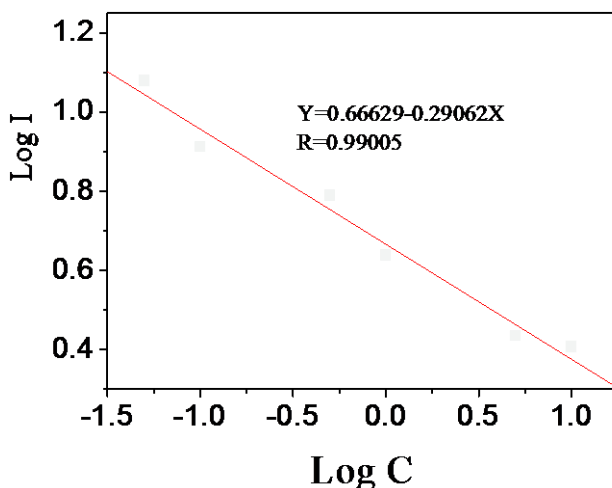
### 3.5. Electrochemical detection of the immunosensor to AFP concentration

Under the optimization of experimental condition, the immunosensor was used to react with AFP of different concentration. It was found that the decrease of current results from the immobilization of antibodies and the binding of AFP to the electrode could increase the electro-transfer resistance, which led to a decrease of the catalytic efficiency of CP in AFP concentration ranges from  $0.05 \sim 10 \text{ ng}\cdot\text{mL}^{-1}$ .



**Figure 9.** Amperometric response for immunosensors incubated with AFP in  $10 \mu\text{L}$  for 25 min, (a)  $0.05 \text{ ng}\cdot\text{mL}^{-1}$  (b)  $0.1 \text{ ng}\cdot\text{mL}^{-1}$  (c)  $0.5 \text{ ng}\cdot\text{mL}^{-1}$  (d)  $1.0 \text{ ng}\cdot\text{mL}^{-1}$  (e)  $5 \text{ ng}\cdot\text{mL}^{-1}$  (f)  $10 \text{ ng}\cdot\text{mL}^{-1}$  (g) AFP at  $-0.45 \text{ V}$  and  $3000 \text{ rpm}$  after placing electrodes in buffer injecting CP to  $5 \text{ mM}$  to develop the signal.

The regression equation was  $Y = 0.66629 - 0.29062X$ , with a detection limit of  $0.01 \text{ ng}\cdot\text{mL}^{-1}$  based on a signal to noise ratio of 3. The linear correlation coefficient (0.99005) showed high matching between both current and concentration when it was drawn on a logarithmic scale in Figure 9. The analytical performance of the proposed immunoassay had been compared with those of other AFP immunoassays reported (Table 1). The comparative data suggested superiority of the present sensor over some earlier reported methods, especially the detection limit.



**Figure 10.** Calibration curve of AFP ranging from 0.05 ng·mL<sup>-1</sup> until 10 ng·mL<sup>-1</sup>.

**Table 1.** Comparison of different electrochemical immunosensors.

Detection methods	Linear range (ng·mL <sup>-1</sup> )	Detection limit (ng·mL <sup>-1</sup> )	References
Differential pulse voltammetry	0.5-80.0	0.28	[30]
Amperometric flow/stop-Flow injection	5.0-150.0	2.0	[31]
Cyclic voltammetry	1.0-55.0	0.6	[32]
Cyclic voltammetry	0.01–10.0,10.0–200.0	0.04	[33]
Cyclic voltammetry	0.25-250.0	0.08	[34]
Amperometric response	0.5-160.0	0.1	[35]
Amperometric response	0.05-10	0.01	This work

### 3.6. Selectivity of the immunosensor

The selectivity of the immunosensor plays an important role in analyzing biological samples in situ without separation. The effect of possible interferences that might interfere with the determination of target analytes was investigated. The immunosensor was incubated in 5.0 ng·mL<sup>-1</sup> AFP containing carcinoembryonic antigen (CEA, 100 ng/mL), human IgG ( HIgG, 1µg/mL), carbohydrate antigen 19-9 (CA19-9, 20 ng·mL<sup>-1</sup>), human chorionic gonadotropin antigen (HCG, 20 ng·mL<sup>-1</sup>), BSA (2 µg·mL<sup>-1</sup>), ascorbic acid (AA, 2 µg·mL<sup>-1</sup>), dopamine (DA, 2 µg·mL<sup>-1</sup>), L-lysine (2 µg·mL<sup>-1</sup>), uric acid (UA, 2.5 µg·mL<sup>-1</sup>), glucose (glu, 2.5 µg·mL<sup>-1</sup>), Ascorbic Acid (AA, 5 µg·mL<sup>-1</sup>), bovine serum albumin (BSA, 5 µg·mL<sup>-1</sup>) and Na<sup>+</sup> (5 µg·mL<sup>-1</sup>). The results showed that the coexistents did not interfere with the determination of 5.0 ng·mL<sup>-1</sup> AFP (signal changed below 5%). The results suggested that the immunosensor displayed good selectivity for the determination of AFP.

### 3.7. Reproducibility and stability of the AFP immunosensor

The successive stability of the immunosensor was evaluated. A relative standard derivation (R.S.D.) of 2.5% was obtained after 30 CV measurements were performed in working buffer with the proposed immunosensor. The stability of the immunosensor was investigated on a 10-days period. When the immunosensor was stored at 4 °C and measured intermittently (every 3~5 days), no apparent change in the working buffer was found over 30 days, it yielded a 3.0% R.S.D.. The good stability may be due to the fact that the Fe<sub>3</sub>O<sub>4</sub>-ZrO<sub>2</sub>-Au-pLL membrane was consistent and protein molecules were attached firmly onto the surface of GCE.

### 3.8. Application

The feasibility and the accuracy of AFP determination was examined by comparing the results obtained from five human serum samples with the proposed method and ELISA, which was a useful and powerful method for serum samples analysis. The average concentration of AFP obtained by this method were 4.56 ng·mL<sup>-1</sup>(n=3). Compared with the results obtained by ELISA which were 4.89 ng·mL<sup>-1</sup>, the results were satisfactory. It obviously indicated that there was no significant difference between the results and ELISA method. Thus, the developed immunosensor could be satisfactorily applied to the clinical determination of AFP levels in human serum.

## 4. CONCLUSIONS

A stable, sensitive and separation-free amperometric immunosensor for rapid determination of AFP has been successfully fabricated based on CNTs, DNA and Fe<sub>3</sub>O<sub>4</sub>-ZrO<sub>2</sub>-Au-pLL sol-gel film. This method was used to construct a new immunosensor for the determination of AFP with good sensitivity, selectivity, stability and long-term maintenance of bioactivity. The usage of MWNTs-DNA-CoRb nano composite film can enlarge the electrochemical signal of the immunosensor and the increase of the sensitivity for AFP detection has not been reported previously. At the same time the Fe<sub>3</sub>O<sub>4</sub>-ZrO<sub>2</sub>-Au-pLL nanomaterials can easily immobilized on DNA membrane for the specific connection between the phosphate base of DNA and ZrO<sub>2</sub> which can also form a porous structure to provide an enhanced effective surface area and active sites for electron transfer. Although we illustrated our strategy with only a single antibody/antigen pair (anti-AFP and AFP), we expect that the application of our general method to other antibodies shall yield immunosensor for the detection of other antigens or compounds of biological importance in the "window period".

## ACKNOWLEDGMENTS

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