Gold microstructures/polyaniline/reduced graphene oxide/prussian blue composite as stable redox matrix for label-free electrochemical immunoassay of α-fetoprotein

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Abstract

Sensitivity amplification strategies in label-free electrochemical immunosensors are mainly limited by redox molecules leaking and degradation of electrical conductivity caused by layers of decoration. Herein, a relatively stable and sensitive label-free electrochemical immunosensor based on hierarchically flower-like gold microstructures/polyaniline/reduced graphene oxide/prussian blue (HFG/PANI/rGO/PB) composite modified electrode was stepwise fabricated for determination of α-fetoprotein (AFP). In this process, the effect of PANI and rGO on the proposed immunosensor was studied. In detail, PANI/rGO due to the unique electrochemical properties can effectively prevent PB leakage and form a stable sensing platform, which cause sensitive responsiveness and thus the more satisfied detection limit. Meanwhile, the HFG with good biological compatibility can effectively immobilize plenty of antibodies. Under optimal conditions, the HFG/PANI/rGO/PB modified immunosensor exhibited an excellent linearity (0.01-30 ng/mL), a low detection limit (0.003 ng/mL) (S/N=3), suitable specificity, stability and reproducibility towards AFP. The present work offered a promising platform for clinical hepatocellular carcinoma diagnostics.

Keywords: α-Fetoprotein; Immunosensor; Prussian blue; Reduced graphene oxide; Polyaniline;
Introduction

α-Fetoprotein (AFP) is an acid glycoprotein that may accumulate to high concentration levels in human plasma as a result of pathological changes of liver \(^1,^2\). It is present in the adult plasma of 60-70% of patients with hepatocellular carcinoma (HCC) \(^3-^5\). As a consequence, it is necessary for medical and biological research to establish an accurate and sensitive analytical technique for the determination of AFP. Recently, electrochemical immunosensors are attracting special attention on account of their fast analysis time, ease of use and cost effectiveness \(^6-^10\). Furthermore, fascinating advantages could be achieved by label-free electrochemical immunosensors, such as directly monitoring the binding process of antibody-antigen reaction and also avoiding interferences from conjugated markers \(^1,^11,^12\).

For the label-free electrochemical immunosensors, a crucial issue of detection is to develop various electrode material with high electroconductivity and good biological compatibility to efficiently and effectively immobilize antibodies \(^1,^11\). Prussian blue (PB), a redox-active species, can be utilized in electrochemical immunosensors as an electron transfer mediator. However, the PB usually exhibits low conductivity and poor stability \(^12-^14\). To overcome these limitations, further surface modification of the working electrode is often required. It is worth to mention that carbon nanomaterials have been used in PB based electrochemical system, which thereby significantly promotes the electron transfer efficiency hence amplifying its electrochemical signals \(^5,^15\). Yang et al. synthesized AuNPs-PEDOT/PB-rGO and exploited its application in bioanalysis \(^4\). Jiang et al.
constructed an amperometric immunosensor for the analysis of AFP based upon GNP/PB/MWCNT, showing well-defined current response towards the successive addition of AFP. In recent years, graphene oxide (GO) is an excellent electrode modification material owing to the characteristics of large specific surface area and remarkable electrochemical conductivity. Moreover, it is demonstrated that polyaniline (PANI)-GO nanocomposite possessed a synergistic interaction of PANI and GO. GO with various oxygen functional groups (such as hydroxyl, epoxy, and carbonyl groups) can act as anchor sites and attach the subsequent in situ polymerization of PANI to the surface and edge of the GO sheets. Therefore, the PANI-GO nanocomposite can significantly improve the conductivity and sensitivity of the immunosensor. In comparison with GO, reduced graphene oxide (rGO) converts the sp$^3$ hybridized carbon to sp$^2$, resulting in the restoration of conjugation and hence a higher electrical conductivity can be realized. As is well known, PANI has attracted a growing interest in biosensor applications due to its favorable and controllable electrochemical properties, its unique environmental electrochemical stability, and its interesting electronic and electrooptical properties. It has been shown that PANI can improve the stability of PB and can easily form nanostructured films on the electrode surface using electrophoretic deposition or electropolymerization.

Herein, in order to prevent PB leakage and improve its conductivity, a PANI/rGO/PB composite film was fabricated by step-by-step modification on glassy carbon electrode (GCE) for the first time. Then, hierarchically flower-like gold...
microstructures (HFG) were loaded on PANI/rGO/PB composite film by a convenient electrodeposition technique, which could immobilize plenty of antibodies. The HFG/PANI/rGO/PB composite with good redox and biological properties was acted as both electron transfer mediators and electrode materials. Moreover, the fabricated immunosensor exhibited good analytical performance, such as excellent linear response, high sensitivity, suitable specificity, and good accuracy to detect AFP in real sample.

**Experimental**

*Chemicals*

AFP, anti-AFP and carcinoembryonic antigen (CEA) were purchased from Biocell Biotech Co., Ltd. Potassium ferricyanide (K$_3$Fe(CN)$_6$), aniline, anhydrous ferric chloride (FeCl$_3$), ascorbic acid (AA), arginine (Arg), potassium nitrate (KNO$_3$), bovine serum albumin (BSA) and chloroauric acid (HAuCl$_4$$\cdot$4H$_2$O) were obtained from the Sinopharm Chemical Reagent Co., Ltd. rGO was obtained from Institute of Coal Chemistry, Chinese academy of sciences. Phosphate buffer solution (PBS) of different pH values were prepared by dissolving NaH$_2$PO$_4$, Na$_2$HPO$_4$ and KCl in ultrapure water. All reagents used were of analytical grade.

*Apparatus*

A standard three-electrode system included a glassy carbon working electrode (4 mm in diameter), a saturated calomel reference electrode, and a Pt counter electrode. All electrochemical measurements, including cyclic voltammetry (CV),
electrochemical impedance spectroscopy (EIS) and differential pulse voltammetry (DPV) were conducted on a CHI 660E electrochemical analyzer (Shanghai CH Instruments Co., China). A JSM-7100F scanning electron microscopy (SEM, Japan Electron, Japan) was used for electrode surface characterization.

Fabrication of the immunosensor

Before every use of GCE, they were burnished smoothly with Al₂O₃ power of 1.0, 0.3 and 0.05 μm. The polished GCE was covered by 6.0 μL of PBS (10 mM FeCl₃ and K₃[Fe(CN)₆], pH 1.5) and protected from light. Next, 6.0 μL of rGO suspension (0.5 M) was dropped on the electrode. Subsequently, the GCE was immersed in 0.5 M H₂SO₄ solution containing 0.05 M aniline and electrochemically deposited 60 s at 0.75 V. After washed with ultrapure water, the PANI/rGO/PB/GCE was soaked in 0.1 M KNO₃ solution containing 12 mM HAuCl₄, then a constant potential (-200 mV) for 600 s was used to obtain HFG film. The label-free electrochemical immunoassay determines the concentration of the analyte by directly measuring the antigen-antibody specific recognition of the change in the electrochemical signal that is generated after binding. Therefore, 6 μL of anti-AFP (Ab) solution (200 μg/mL) was modified the GCE and incubated overnight at 4 °C. HFG can firmly adsorb antibody due to their large specific surface area, good biocompatibility and high surface free energy. Moreover, a lot of the functional groups such as amino group, mercapto group on the antibody have a high affinity for Au. The method also can avoid the complex cross-linking process 26. After washing, the GCE was incubated with BSA (1.0 wt%) for 1 h to block the remain active sites.
Ultimately, the obtained immunosensor was washed with PBS (pH 6.5) to remove the loosely absorbed species. The construction process of the fabricated immunosensor was displayed in Scheme 1.

**Determination of AFP**

Based on antigen-antibody specific reactions, the well-prepared immunosensor was incubated with 6 µL of AFP (Ag) solution of different concentrations at 37 °C for 1 h. After washed with PBS (pH 6.5), the obtained electrodes were studied by DPV in PBS (pH 6.5). The measurement parameters are listed: potential range, -0.4 to 0.6 V; potential amplitude, 50 mV.

**Scheme 1.**

**Results and Discussions**

**Characterization of PB, rGO/PB, PANI/rGO/PB and HFG/PANI/rGO/PB**

The morphologies of PB, rGO/PB, PANI/rGO/PB and HFG/PANI/rGO/PB were confirmed by SEM, respectively. As shown in Fig. 1A, the PB nanoparticles have formed uniform spherical morphology, with an average diameter of 20 nm. The SEM images also reveal that rGO exhibited a single layer and fold structure, which could provide more active nucleation sites for PANI (Fig. 1B)\(^{21}\). A dendritic structure was observed after the electropolymerization of aniline on rGO/PB/GCE, suggesting the formation of PANI film on the surface of rGO/PB/GCE (Fig. 1C). After HFG were electrodeposited onto the PANI/rGO/PB/GCE, the superstructures of flower-like...
HFG with average diameter of about 300 nm were uniformly dispersed on the PANI surface without aggregation (Fig. 1D). This flower structure could form a rough surface so that increases a large surface area that connecting more biomolecules to improve the analytical properties of the proposed immunosensor 27.

**Figure 1.**

*Electrochemical characterization on electrode surface*

The electronic transfer properties of the stepwise manufacturing process were evaluated by CV and EIS. As shown in Fig. 2A, when rGO/PB was coated on the surface of bare GCE (curve b), the redox peak current obviously increased comparing with the redox peak current of bare GCE (curve a), because the rGO with excellent conductivity can effectively accelerate electron transfer. The modification of the rGO also provided the conducting bridges for the electron-transfer of $\text{[Fe(CN)}_6^{3-/4-}$]. After the electrodeposition of PANI (curve c) and HFG (curve d), the peak current increased gradually, this might be caused by the PANI and HFG can accelerate the electron transfer. However, the redox peak current decreased orderly when the anti-AFP (curve d), BSA (curve e), and AFP (curve f) were coated onto the surface of electrode in accordance with the sequence of immunosensor assembly, because the bioactive substances greatly inhibited the efficiency of electron transfer.

Fig. 2B illustrated the Nyquist plots of the well-known standard redox probe $\text{[Fe(CN)}_6^{3-/4-}$ at different stages. $R_{et}$ is the electron transfer resistance which reflects
the surface property of surface of the electrode. Observed from the Fig. 2B, the unmodified GCE showed a relatively large interfacial resistance (curve a, the electron transfer resistance: $R_{et} = 120 \Omega$). After the GCE modified with rGO/PB (curve b, $R_{et} = 15 \Omega$), PANI (curve c, $R_{et} = 12 \Omega$) and HFG (curve d, $R_{et} = 8 \Omega$), respectively, the interfacial resistance gradually decreased. The reason is that the HFG/PANI/rGO/PB can accelerate the electron transfer. However, the interfacial resistance increased gradually when the GCE was coated with anti-AFP (curve e, $R_{et} = 20 \Omega$), BSA (curve f, $R_{et} = 70 \Omega$) and AFP (curve g, $R_{et} = 80 \Omega$) in due succession, indicating that these proteins perturbed the interfacial and retarded the efficiency of electron transfer. All those observations illustrated that the proposed immunosensor is successfully developed.

**Figure 2.**

*Optimal parameters*

The analytical conditions including pH of PBS, incubation time of antigen and the ratio of PANI and rGO were systematically optimized to achieve the best DPV response for AFP determination. The influence of pH value of PBS was investigated with 5 ng/mL AFP between pH 5.5 and pH 8.0. As shown in Fig. 3A, increasing the pH from 5.5 to 6.5 resulted in a decreased DPV response, further increases in pH resulted in the DPV response increasing. The reason is that highly alkaline or acidic environment would determine the connected biomolecules activity and stability.
Thereby, PBS pH at 6.5 was chosen for all subsequent experiments. Furthermore, incubation time of antigen also directly influences the activity of the biomolecules. By the same token, the influence of antigen incubation time was investigated in the time periods from 20 to 70 min. The DPV response decreased with increasing incubation time and tended to reach a steady value after 60 min (Fig. 3B). Thus, 60 min was used throughout this work.

The ratio of PANI and rGO is a key factor that affects the morphologies of HFG and the electrochemical response of the developed immunosensor. Hence, we have optimized PANI electrodeposition times on the surface of rGO (the SEM image characterization of the HFG/PANI rGO/PB/GCE at different deposition times of PANI in Fig. S1). The conductive PANI film as an extension of the modified electrode increases the area for effective electrodeposition of HFG. HFG are evenly dotted on the nanostructured PANI film by electrodeposition time of 60 s, which may be attributed to the fact that PANI film with suitable thickness could be formed by electrodeposition of 60 s. Meanwhile, the amine groups of PANI ensures the electrodeposition of firmly deposited HFG by forming Au-N bonds on the PANI film, which can enhance the performance of the as-prepared immunosensor. Therefore, a deposition time of 60 minutes was used in the subsequent work.

Figure 3.
Assay performance

Under the optimized conditions, the HFG/PANI/rGO/PB modified immunosensor was incubated with AFP solutions with various concentrations. As shown in Fig. 4A and 4B, a well-defined DPV signal related to the electrochemical signal of PB was achieved. When AFP as target was introduced, the DPV response decreased with the increase of AFP levels from 0.01 to 30 ng/mL, implying a good linear relationship between the DPV response and logarithm of AFP level. The linear equation was $y = -6.5608x + 24.5159$, where $R^2$ was 0.9289. The detection limit was evaluated to be 0.003 ng/mL according to the formula $\text{LOD} = 3S_b/m$, where $S_b$ and $m$ were the standard deviation of blank samples and the slope of calibration plot, respectively. However, there appeared other multi-peaks as the concentration of AFP increases, which might be attributed to the redox reaction of OH$^-$ with the residual functional groups in the rGO matrix, suggesting the incomplete reduction of rGO$^{28}$.

In addition, the calibration plots of the HFG/PANI/PB modified immunosensor and HFG/rGO/PB modified immunosensor were also studied, as shown in Fig. 4B. Table 1 summarizes the assay performance of each modified immunosensor. As is listed, the detection range of HFG/rGO/PB modified immunosensor is much narrow. PB usually exhibits poor stability under alkaline or neutral environments$^{12-14}$.$^{12-14}$ The presence of the PANI not only prevented the leakage of PB, but also enhanced the analytical performances of the immunosensor$^{21, 24}$.$^{21, 24}$ With the aid of PANI, the DPV response was significantly strengthened in comparison with HFG/rGO/PB. Compared with HFG/PANI/PB modified immunosensor, the HFG/PANI/rGO/PB
modified immunosensor achieves a larger linear slope, that is to say, the HFG/PANI/rGO/PB modified immunosensor has higher sensitivity\textsuperscript{11}. This might be caused by the unique conductivity of rGO, which accelerated fast electron transfer process. Compared to published literature (Table 2),\textsuperscript{5,11,17,29-33} the immunosensor based on HFG/PANI/rGO/PB composite shows good analytical properties. Some possible explanations may be as follows: First, the PANI/rGO/PB composite film with remarkable electroconductibility and stability can observably accelerate the electron transfer. Second, HFG can firmly adsorb antibodies because of its good biological compatibility. Meanwhile, the amino or mercapto groups of the antibodies have a high affinity for Au\textsuperscript{12}.

Figure 4.

Table 1.

Table 2.

Specificity, stability and reproducibility

To research the reliability of the HFG/PANI/rGO/PB modified immunosensor, the specificity, stability and reproducibility of this system were systematically analyzed. AA, Arg, BSA and CEA were used as potential interferents to analyze the specificity of the HFG/PANI/rGO/PB modified immunosensor. 5 ng/mL AFP
solution comprising 50 ng/mL different interferents were evaluated. As shown in Fig. 5A, the DPV response was almost the same as that with pure AFP (5 ng/mL), confirming excellent specificity.

To evaluate the stability, the DPV response to AFP (5 ng/mL) was recorded per five days. As shown in Fig. 5B, the DPV response maintained 93.8 %, 91.2 % and 89.6 % of its initial value after 5, 10 and 15 days respectively, which declared satisfactory stability of the immunosensor for AFP detection.

Furthermore, the reproducibility of the immunosensor was inspected. Five equally prepared electrodes were utilized to assay AFP (5 ng/mL). Five immunosensors showed similar DPV response and the relative standard deviation (RSD) was 4.4 % (Fig. 5C). These preliminary results indicated that propitious reproducibility of the well-prepared immunosensor.

Figure 5.

Practical applications

To analyze the analytical feasibility of the immunosensor to real sample, 10 human serum samples containing AFP were measured in comparison with ELISA. As summarized in Table 3, t-test was employed to evaluate the significant difference between two methods. The $t_{\text{exp}}$ values in all samples were less than $t_{\text{crit}}$ ($t_{\text{crit}}=2.571$), indicating the present method could be a bright tool in the clinical detection of AFP levels in real samples.
**Table 3.**

**Conclusions**

Herein, an electrochemical immunosensor was developed based upon HFG/PANI/rGO/PB composite. The HFG/PANI/rGO/PB composite showed large surface area, efficient redox, and excellent conductivity, allowing them be acted as both electron mediators and electrode materials, enhancing the number of connected biomolecules and efficiently avoiding the leakage of redox molecules. Moreover, the designed immunosensor also could accurate detection of AFP in real samples. Thus, the convenient operation and sensitivity of this method offers a stable promising platform for determining biomarkers in clinical applications.

**Conflicts of interest**

There are no conflicts to declare.

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Table

**Table 1.** A list of assay performance of each modified immunosensor.

**Table 2.** Comparison with various immunosensors for AFP detection.

**Table 3.** Comparsion with ELISA for AFP determination.
Table 1. A list of assay performance of each modified immunosensor.

| Methods            | Linear Equation | Correlation Coefficient | Linear range (ng/mL) | Detection limit (ng/mL) |
|--------------------|-----------------|--------------------------|----------------------|-------------------------|
| HFG/PANI/rGO/PB    | \( y = -6.56x + 24.52 \) | 0.9289                   | 0.01 to 30           | 0.003                   |
| HFG/PANI/PB        | \( y = -2.53x + 13.96 \) | 0.8217                   | 0.01 to 10           | 0.004                   |
| HFG/rGO/PB         | \( y = -6.60x + 7.30 \) | -                        | 0.01 to 1            | 0.003                   |
| Methods                       | Linear range (ng/mL) | Detection limit (ng/mL) | Reference |
|-------------------------------|----------------------|-------------------------|-----------|
| GNRs                          | 0.1-200              | 0.04                    | 11        |
| GNP/PB/MWCNT                  | 0.01-300             | 0.003                   | 5         |
| GNWs/GO/CuS-GO               | 0.001-10             | 0.0005                  | 17        |
| Graphene/Au-Pd                | 0.05-30              | 0.005                   | 29        |
| AuNPs/Fc-IL-CHO/AuNPs-PAM     | 0.05-30              | 0.02                    | 30        |
| GS-TH/Fe₃O₄-MSN-HRP           | 0.01-25              | 0.004                   | 31        |
| AuNPs/HRP                     | 5.0-80               | 3.7                     | 32        |
| PdNPs-rGO                    | 0.01-12              | 0.005                   | 33        |
| HFG/PANI/rGO/PB              | 0.01-30              | 0.003                   | Present Work |
Table 3. Comparison with ELISA for AFP determination

| Serum sample No. | This method (ng/mL) | ELISA<sup>a</sup> (ng/L) | t<sub>exp</sub> |
|------------------|---------------------|--------------------------|--------------|
| 1                | 0.05±0.38           | 0.05±0.52                | 0.000        |
| 2                | 0.15±0.32           | 0.14±0.29                | 0.052        |
| 3                | 0.52±0.14           | 0.55±0.21                | -0.266       |
| 4                | 1.01±0.26           | 1.06±0.31                | -0.276       |
| 5                | 10.50±0.42          | 10.40±1.23               | 0.172        |
| 6                | 12.32±1.23          | 12.21±0.43               | 0.189        |
| 7                | 15.46±1.34          | 16.16±0.63               | -1.057       |
| 8                | 17.27±0.78          | 16.93±1.13               | 0.554        |
| 9                | 20.38±1.14          | 21.17±0.89               | -1.221       |
| 10               | 25.02±0.79          | 24.32±0.52               | 1.655        |

<sup>a</sup> Mean value ± SD of five measurements.
Figure Captions

**Scheme 1.** Construction of the Ag/BSA/Ab/HFG/PANI/rGO/PB/GCE.

**Figure 1.** SEM images of (A) PB, (B) rGO/PB, (C) PANI/rGO/PB, and (D) HFG/PANI/rGO/PB.

**Figure 2.** CVs (A) and EIS (B) of bare GCE (a), rGO/PB/GCE (b), PANI/rGO/PB/GCE (c), HFG/PANI/rGO/PB/GCE (d), anti-AFP/HFG/PANI/rGO/PB/GCE (e), BSA/anti-AFP/HFG/PANI/rGO/PB/GCE (f), AFP/BSA/anti-AFP/HFG/PANI/rGO/PB/GCE (g).

**Figure 3.** Effects of (A) pH of PBS, (B) incubation time of antigen on the DPV response of the immunosensor to 5 ng/mL AFP. Error bar = RSD (n = 5).

**Figure 4.** (A) DPV signals of the immunosensor in the presence of 0, 0.01, 0.1, 1, 5, 10 and 30 ng/mL AFP (a~g) in PBS (pH 6.5). (B) Calibration curve.

**Figure 5.** The (A) Specificity, (B) stability and (C) reproducibility of the AFP immunosensor.
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