Glypican-3 electrochemical aptasensor based on CuO-rGO nanocomposite

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Abstract. Herein, a novel electrochemical immunosensor was fabricated based on copper oxide-reduction graphene oxide (CuO-rGO) nanocomposites for Glypican-3 (GPC3) detection. Firstly, CuO-rGO nanocomposite as nanoenzyme with good peroxidase-like properties is synthesized by one-step hydrothermal. Then CuO-rGO nanoenzyme captures GPC3 aptamer to form a signal probe, finally GPC3 antibody is used as the recognition probe to form an "antibody-antigen-aptamer" sandwich structure. Due to the peroxidase-like properties of the probe, this sandwich structure can effectively catalyze the reaction between hydrogen peroxide (H$_2$O$_2$) and silver nitrate (AgNO$_3$) and AgNPs is deposited on the surface of the electrode to amplify the final measured current. The Differential Pulse Voltammetry (DPV) method was employed to record the response signal of the immunosensor. The current response presented a linear relationship to the GPC3 concentration in the range of 1 to 40 μg/mL with the detection limit of 0.45 μg/mL.

1. Introduction
Hepatocellular carcinoma (HCC) has been the third cause of cancer death and the leading cause of mortality among cirrhotic patients. GPC3 is a polyphase sulfate glycoprotein anchored on the cell membrane by glycosylphosphatidylinositol. GPC3 can play a role in the growth, differentiation and migration of cells, and has a close relationship with HCC[1]. GPC3 is not expressed or expressed at a low level in normal adult tissues, but specifically expressed at a high level in HCC[2]. Thus, GPC3 has been a prospective tumor biomark for HCC in recent years. To date, various methods have been developed for the detection of GPC3, such as fluorescence immunoassay, enzyme-linked immunosorbent assay, electrochemiluminescence, electrochemical immunosensor [3]. Among them, electrochemical immunosensor has aroused great interests and been applied in the detection of tumor markers owing to high sensitivity, facile operation, low cost and ease miniaturization[4].

Recent years, nano-enzyme provide a multifunctional platform for immunosensing interface due to their unique properties such as the excellent electrical conductivity, large specific surface area and so on[5]. As a p-type semiconductor with a narrow band gap of 1.2 eV, copper oxide (CuO) has been widely exploited and excellent electrocatalytic activity at lower over-potentials[6]. Jagadeesan et al. have produced an enzyme-free glucose sensor using ellipsoidal copper oxide nanoparticles as nanoenzymes. The linear dynamic range, from 2.0 μM to 2.5 mM, and sensitivity of 627.3 μA mM$^{-1}$ cm$^{-2}$[7]. Significantly, composite nanoenzyme has versatile physical and biological properties which have a wide range of applications in catalysis and biosensing compared to single-component nanoenzymes.

Herein, copper oxide-reduction graphene oxide (CuO-rGO) nanocomposite is combined as novel nanoenzyme with good peroxidase-like properties. Then, we constructed an electrochemical immunosensor for the detection of GPC3 based on the CuO-rGO nanoenzyme. The sensor uses CuO-
rGO nanomaterials to capture GPC3 aptamer and form a signal probe. The GPC3 antibody is used as the recognition probe to form an "antibody-antigen-aptamer" sandwich structure. Due to the peroxidase-like properties of the probe, this sandwich structure can effectively catalyze the reaction of hydrogen peroxide ($H_2O_2$) and silver nitrate ($AgNO_3$), and AgNPs is deposited on the surface of the electrode to amplify the final measured current. The differential pulse voltammetry (DPV) method was employed to record the response signal of the immunosensor.

2. Experimental

2.1. Reagents and apparatus

GO was purchased from Xianfeng NANO Materials Tech Co., Ltd. (Nanjing, China). All analytical reagents used in the experiment were obtained from Aladdin Reagents Ltd (Shanghai, China) and Sinopharm chemical Reagent Co., Ltd (Beijing, China), and used without further purification. All the solutions were prepared with ultrapure water of 18 Ω·cm purified from a Milli-Q purification system (Milli-Pore, Bedford, MA, USA). The human blood serum was acquired from 924st Hospital of Chinese People's Liberation Army (Guilin, China). Electrochemical analysis was carried out on ch1660e electrochemical workstation (Shanghai Chenhua Instrument Co., Ltd.)

2.2. Synthesis of the CuO-rGO and CuO-rGO-GPC3Apt target probe

Firstly, 35 mL 0.1 mg/mL of GO was vigorous stirring with ultrasonic for 2 h to form a uniform suspension. Secondly, 70 mg AA was slowly added and stirred for 4 h to attain rGO. Then, CuO was prepared by mixing 0.5625g of Cu(NO$_3$)$_2$ and 0.24g of NaOH in a hydrothermal kettle at 150°C for 4 hours. Finally, CuO-rGO nanomaterials were prepared by mixing 10mL 1.0mg/mL rGO solution and 10mL 5mg/mL CuO solution, adding 10mg NHS and 40mg EDC, stirring for 12 hours, centrifugal washing and drying.

Mix 125 μL of 5 uM GPC3 aptamer and 250 μL of 0.5 mg/ml CuO-rGO solution, stir for 10 minutes, and incubate overnight in 4 °C refrigerator. In order to explore whether NH$_2$-GPC3$_{Apt}$ is well combined with CuO-rGO, 200 μL of CuO-rGO solution with concentration of 0.5 mg/mL, the supernatants of NH$_2$-GPC3$_{Apt}$ solution with concentration of 5 μmol/L, CuO-rGO-GPC3$_{Apt}$ solution with concentration of 0.5 mg/mL and CuO-rGO-GPC3$_{Apt}$ solution after centrifugation with concentration of 0.5 mg/mL. The binding rate of GPC3$_{Apt}$ is 82.9%.

2.3. TeConstruction of GPC3 electrochemical aptasensor

Firstly, the SPE was immersed in 5 mL of H$_2$SO$_4$ solution (0.5 M) and activated by electrochemical cyclic scanning method with scanning speed of 100 mV s$^{-1}$ and potential range from 0.2 to 1.0 V for 20 cycles. Secondly, Au NPs were deposited on surface of the activated SPE through electrodeposition in 5 mL 0.01% HAuCl$_4$ solution under the potential of −0.5 V for 120 s while the magnetic stirring. Thirdly, GPC3$_{Ab}$ (2 μL, 10 μg/mL) was added dropwise to the surface of Au NPs-SPE and incubated at 25 °C for 1 h. Fourthly, 2 μL BSA solution (1.0%) was added to the surface of GPC3$_{Ab}$-Au NPs-SPE to block nonspecific site.

2.4. Electrochemical detection of GPC3 with the electrochemical aptasensor

Firstly, a total of 3 μL of GPC3 standard solution (different concentration) was added to the surface of GPC3$_{Ab}$-Au NPs-SPE and incubated at 25 °C for 30 min. Secondly, 2 μL of CuO-rGO-GPC3$_{Apt}$ solution (0.83 mg/mL) was added dropwise and incubated at 25 °C for another 60 min. Then, $H_2O_2$ (2 μL, 100 mM) and AgNO$_3$ solution (1 μL, 50 mM) were added dropwise to the surface of CuO-rGO-GPC3$_{Apt}$-GPC3-GPC3$_{Ab}$-Au NPs-SPE. After incubation for 30 min in the dark at 25 °C, the electrode was washed twice with glycine-NaOH buffer solution. Finally, the electrode was inserted into a 4.0 mL glycine-NaOH buffer solution (0.05 M, pH 8.5) containing 0.1 M HNO$_3$ and 0.6 M KNO$_3$ and recorded the electrochemical responses with differential pulse voltammetry (DPV) method with scanning range from
− 0.2 to 0.2 V and scanning rate of 100 mV s⁻¹. Each sample was detected for three times, and the results were calculated as mean ± RSD

3. Results and discussion

3.1. Characterization of the CuO-rGO nanocomposites
The appearance of the CuO-rGO nanocomposite was characterized by SEM in Figure 1A. It showed monoclinic phase structure of CuO and fold-like structure of rGO. UV characterization of CuO-rGO was shown in Figure 1B, the characteristic peaks of RGO and CuO are at 260 nm and 370 nm respectively. XRD characterization of CuO-rGO is shown in Figure 1C, the structure of CuO crystal corresponds to the standard card No. 45-0937. CuO-rGO has diffraction peaks at 2θ angles of 35.66, 38.81 and 48.95 which can be associated to the (111), (200) and (202) crystallographic planes of monoclinic CuO. Figure 1D is the Raman characterization of CuO-rGO. The peak of CuO at 630 cm⁻¹ is close to other reported results. CuO-rGO has a strong D peak at 1338 cm⁻¹ and a strong G peak at 1597 cm⁻¹, and I(D)/I(G)=0.97, indicating that CuO and rGO are well combined.

![Figure 1](image)

Figure 1 (A) SEM characterization of CuO-rGO nanoenzyme; (B) UV characterization of CuO-rGO nanoenzyme; (C) XRD characterization of CuO-rGO nanoenzyme; (D) Raman characterization of CuO-rGO nanoenzyme.

3.2. Principle of GPC3 electrochemical aptasensor based on CuO-rGO nanoenzyme
The principle of GPC3 electrochemical aptasensor based on CuO-rGO nanoenzyme was schematically shown in Figure 2. Firstly, the electrode was activated in dilute sulfuric acid, and gold nanoparticles (Au NPs) were electrodeposited on the activated screen printed electrode. Secondly, GPC3 antibody (GPC3 Ab) was dripped on the electrode surface, and the antibody was adsorbed and fixed on the electrode surface. Then, bovine serum albumin (BSA) was used to block the unbound sites. After that, GPC3 protein and CuO-rGO-GPC3 Apt were dripped. After incubation for a period of time, the sandwich product of "antibody-antigen-aptamer" was formed. Finally, AgNO₃ and H₂O₂ were dripped on the surface of the electrode. Under the action of the probe, silver nanoparticles were deposited on the surface of the electrode. The amount of deposited Ag, which was derived from the amount of GPC3, was
quantified by differential pulse voltammetry (DPV) method. Therefore, GPC3 can be detected with high sensitivity by measuring DPV current.

Figure 2 The principle of GPC3 electrochemical aptasensor based on CuO-rGO nanoenzyme.

3.3. The feasibility analysis of GPC3 electrochemical aptasensor
For the feasibility study of GPC3 electrochemical aptasensor, Figure 3A explores the source of the signal, where curve a is GPC3/ GPC3Ab/ Au NPs/ SPE, curve b is CuO-rGO-GPC3Apt/GPC3/ GPC3Ab/ Au NPs/ SPE, and curve c is Ag/ CuO-rGO-GPC3Apt/ GPC3/ GPC3Ab/ Au NPs/ SPE. From Figure 3A, it can be seen that only curve c has an absorption peak, indicating that the determination of GPC3 is achieved by depositing Ag. Figure 3B explores the relationship between the sensor and protein concentration, in which curve a is GPC3 solution with concentration of 40 μg/mL, curve b is GPC3 solution with concentration of 10 μg/mL, and curve c is no GPC3 solution. It can be seen from Figure 3B that the current value corresponding to curve c is the smallest, and the higher the protein concentration is, the greater the response current has, which indicating that the GPC3 electrochemical aptasensor can be used to detect GPC3 protein.

Figure 3 (A) DPV scan chart of the process of sensor construction. (B) Protein concentration affects DPV scanning.

3.4. Characterization of GPC3 electrochemical aptasensor
Cyclic voltammetry (CV) was used to observe the electrochemical behavior of the electrodes in different stages of the biosensor construction. Figure 4A is the CV characterization of each stage of the sensor with 5 mM K4Fe(CN)6/K3Fe(CN)6 and 0.1 M KCl solution as the scanning medium, the scanning voltage is -0.8 ~ 0.8V, and the scanning rate is 0.1V/s. The CV potential of bare electrode can be obtained by
curve a; It can be seen from the curve b that the conductivity of the electrode is improved after the deposition of Au NPs, so the CV potential increases; It can be seen from the curve c that after dropping GPC3 on the electrode surface, the electron transfer is hindered and the CV potential decreases; It can be seen from the curve d that after dropping GPC3 protein on the electrode surface, the overall impedance is further increased and the CV potential decreases again; It can be seen from the curve e that after dropping CuO-rGO-GPC3 Apt probe on the electrode surface, the CV potential increases because of the good conductivity of RGO. It can be seen from the curve f that the deposition of silver on the electrode surface greatly promotes the electron transfer and increases the CV potential.

EIS is another method to further characterize the electron transfer properties of the different modified electrodes. Figure 4B is the characterization of EIS in each stage of the sensor with 5 mM K₄Fe(CN)₆/K₃Fe(CN)₆ and 0.1 M KCl solution as the scanning medium, with scanning voltage of 0.24 V and scanning range of 0.00001-100 KHz. From curve a, it can be seen that the impedance of the bare electrode is about 8792 Ω; It can be seen from curve b that the impedance value is greatly reduced after the gold nanoparticles are deposited, and the impedance value is about 4136 Ω; It can be seen from curve c that the electron transfer of the sensor is hindered by the addition of GPC3 antibody, and the impedance value is about 15020 Ω; It can be seen from curve d that after adding GPC3 protein, the impedance of the protein itself further hinders the electron transfer of the sensor and increases the overall impedance. The impedance value is about 14390 Ω; It can be seen from curve e that after adding CuO-rGO-GPC3 Apt probe on the electrode surface, because of its good conductivity, the overall impedance value is reduced, and the impedance value is about 18260 Ω; It can be seen from curve f that after silver monolith deposited on the electrode surface, the electron transfer is greatly promoted, and the total impedance value is reduced sharply, and the impedance value is about 4543 Ω.

Figure 4 (A) CV and (B) EIS characterization of the process of sensor construction in 5 mM K₄Fe(CN)₆/K₃Fe(CN)₆ and 0.1 M KCl solution.

3.5. Analytical performance of GPC3 electrochemical aptasensor
Under the optimized experimental conditions, the GPC3 detection was performed by the electrochemical aptasensor based on CuO-rGO nanoenzyme. Figure 5A was electrochemical signals of different concentrations of GPC3, As the concentration of GPC3 increasing, a larger amount of GPC3 was specifically recognized by Ab and CuO-rGO-GPC3 Apt to form large sandwich complex, leading to the gradually increasing Ag ions deposition of metallic Ag and increasing the electrochemical signal response. The linear relationship between electrochemical signals and the concentration of GPC3 within the range of 1-40 µg/mL was shown in Figure 5B, the regression equation was Y=0.74086X+30.07645 with a correlation coefficient of 0.99293. The limit of detection (LOD) was calculated to be 0.45 µg/mL at a signal-to-noise ratio of 3. Figure 5C was an analysis of the specificity of CuO-rGO electrochemical biosensor, the responses caused by AFP, IgG, IgE and HSA could be negligible, which indicated that the GPC3 aptasensor based on CuO-rGO nanoenzyme has a good selectivity and high specificity and can be used for the detection of GPC3 without any effect caused by possible interferents.
3.6. Detection of real serum by GPC3 electrochemical aptasensor

In order to verify the constructed sensor in practical applicability in the serum, recovery experiments were performed under the best experimental conditions by adding different concentrations of GPC3 to real human serum samples. The serum samples are from the Key Laboratory of metabolic diseases research in Guangxi, hospital 924 of the people’s Liberation Army (Guilin, China), and comply with the ethical committee requirements of Guangxi Key Laboratory of metabolic diseases research of the 924 Hospital of the people’s Liberation Army. The three kinds of different concentrations of standard GPC3 solution (5 μg/mL, 10 μg/mL, 20. μg/mL) added to the actual human serum, and tested by DPV method. The results shown in Table 1 show that the recoveries of known samples are between 105% and 121%, with RSD values of 0.46-4.74%. The results show that the GPC3 electrochemical aptasensor can detect the actual serum and has a good application prospect in medical diagnosis.

| Human serum samples | Spiked concentration(μg/mL) | Detection concentration(μg/mL) | Recovery (%) | RSD(%) |
|---------------------|-----------------------------|--------------------------------|--------------|--------|
| a Sample1           | 5.0                         | 5.8                            | 116          | 1.23   |
|                     | 10.0                        | 10.6                           | 106          | 0.83   |
|                     | 20.0                        | 21.0                           | 105          | 1.16   |
|                     | 5.0                         | 5.3                            | 106          | 0.16   |
| b Sample2           | 10.0                        | 11.1                           | 111          | 1.52   |
|                     | 20.0                        | 24.2                           | 121          | 4.74   |
|                     | 5.0                         | 5.5                            | 110          | 0.77   |
| c Sample3           | 10.0                        | 10.9                           | 109          | 1.25   |
|                     | 20.0                        | 23.5                           | 118          | 3.97   |

a: Sample 1 :AFP concentration of 5.14ng/mL.
b: Sample 2 :AFP concentration of 88.27ng/mL.
c: Sample 3 :AFP concentration of 223.88ng/mL.

4. Conclusion

In conclusion, a highly sensitive GPC3 electrochemical aptasensor was constructed based on the peroxidase like properties of CuO@gO nanoenzyme. In the range of 1 μg/mL~40 μg/mL, the response current of the sensor is linear with the concentration of GPC3, the linear equation is Y=0.74086X+30.07645, and the correlation coefficient is 0.99293. The electrochemical aptasensor has
good recovery and low standard deviation in the detection of human serum samples. Thus, the GPC3 electrochemical aptasensor has great potential in clinical diagnosis.

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