Simple and free-labeled voltammetry determination of glioma cell

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Abstract
Glioma is one of the most frequent brain tumors with substantial mortality and morbidity, thus designing a simple sensor for achieving highly efficient determination of glioma cell is of great importance. In this work, by preparing 3,4,9,10-perylene tetraacarboxylic acid (PTCA) non-covalently functionalized carbon black (CB) nanohybrids (CB-PTCA) as matrix and using angiopep-2 peptide (Ang-2) as receptor to recognize selectively glioma cell, a simple and free-labeled voltammetry sensor was developed for the first time to detect glioma cell by using Ang-2 and CB-PTCA modified glassy carbon electrode (Ang-2/CB/GCE): via introducing typical [Fe(CN)6]4−/3− as the signal probe, its electrochemical signal would be suppressed when glioma cells were recognized by Ang-2, and the values of peak current difference varied along with the concentrations of glioma cells. After optimizing the related testing conditions (the amounts of CB-PTCA, concentration of Ang-2 and recognition time of Ang-2 towards glioma cells), a wide linearity from $10^2$ to $10^6$ cells mL$^{-1}$ and a low analytic limitation of 30 cells mL$^{-1}$ were achieved for glioma cell. Furthermore, the application of the proposed immunosensor in human serum was also studied, revealing that the results are reliable and the designed proposal offers a significant clinical application for glioma detection.

Keywords Glioma · Glioma cell · Brain tumor · Electrochemical sensing · Angiopep-2 peptide · Carbon black

Introduction
As one of the most frequent brain tumors, glioma is diagnosed in ~100,000 persons associated with high mortality/morbidity according to the Global Cancer Observatory 2018 report [1, 2]. Therefore, developing a rapid, simple and effective method to monitor glioma cell is of great significance for treating brain tumors. Various analytical techniques including Raman spectroscopy, PCR, telomerase promoter-based assay, and surface plasmon resonance have been reported for detecting glioma cell [3–5]. Nevertheless, these means are generally time-consuming, high cost or rely on skilled technicians. Since the electrochemical method is simpler, more sensitive, easy to operate, time-saving and low-cost compared to the other methods, several electrochemical immuno-sensing platforms have been developed for glioma cell based on electrochemical impedance spectroscopy (EIS) [6, 7]. In general, compared to the EIS method, the voltammetry technology exhibits higher sensitivity and better resolution. However, there is no voltammetry immuno-sensing reported for glioma cell.

As we all know, designing appropriate materials as sensing platform to facilitate electron transfer and increase loading amount of the antibodies is one of the key aspects for constructing effective electrochemical immuno-sensor [8]. In the past decades, many carbon-based nanomaterials (e.g., graphene, carbon nanotube) drawn wide attentions in electroanalysis since they can offer large surface area, high conductivity and chemical stability [9–12]. However, the production procedures for most of them are relative tough. Interestingly, carbon black (CB) consisted of amorphous quasi-graphitic primary nanoparticles, has received more and more attentions recently since it be obtained from abundant sources via simple and cost-effective procedures [13–16]. In addition, CB can also boast many interesting chemical/physical superiorities similar to the other carbon nanomaterials [17–20], enabling it with significant applications in electroanalysis [21–23]. However, there is no related method reported for constructing electrochemical immuno-sensor of glioma cell based on CB. Furthermore, the
dispersibility of CB is poor and there are no effective functional groups on the surface of CB for loading antibodies. Perylene tetracarboxylic acid (PTCA) is a bifunctional organic molecule consisting of a pyrenyl group and four \(-\text{COOH}\), which cannot only as a functional unit to improve the dispersibility of CB but also offer the capability to immobilize antibodies: the pyrenyl group of PTCA can attach to CB surface via non-covalent mean and the carboxyl could link to antibodies \([24, 25]\). Therefore, the fabrication of CB-PTCA nano-composite as the matrix is an effective mean to construct electrochemical immunosensor for sensitively and selectively detecting glioma cells. Angiopep-2 peptide (Ang-2), with length around 19 amino acids, can attach and recognize selectively to LRP1 (low-density lipoprotein receptor protein 1). Since the LRP1 receptors are expressed abundantly only in the glioma cell, Ang-2 can distinguish and detect selectively the glioma cells to other cells \([26, 27]\). In consequence, the specific affinity of Ang-2 towards LRP1 receptor could serve as probe in brain tumor determination. In this work, by preparing CB-PTCA as matrix to link Ang-2 (Ang-2/CB) and choosing \([\text{Fe(CN)}_6]^{3-/4-}\) as probe, a simple and free-labeled voltammetry immunosensor was developed for the first time for the detection of glioma cells. After optimizing various experimental conditions, the designed immunosensor exhibited low detection limit (LOD), wide linearity and good selectivity, which showing important potential applications for the detection of glioma cell (Scheme 1).

**Experimental section**

**Reagents and apparatus**

N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were commercially available from Sigma. CB was purchased from Shenzhen Nanotech Port Ltd. PTCA was obtained according to previous work \([24]\). Ang-2 was obtained from NavoPro. All the other reagents are of analytical grade. Super-pure water (\(\geq 18 \text{ M}\Omega\)) was introduced to prepare all the solutions. The electroanalysis measurements were performed on CHI 660E Electrochemical Workstation.

**Preparation of CB-PTCA and Ang-2/CB**

Firstly, CB-PTCA was prepared via a simple stirring method: 50.0 mg CB powder was added in to 100.0 mL PTCA solution (0.1 mg mL\(^{-1}\)) and stirred for 1 h continuously, and the CB-PTCA product can be obtained after filtration, washing and drying.

Next, Ang-2/CB was prepared through the covalent method: the as-obtained CB-PTCA nanohybrids were mixed with the solution of EDC/NHS and then Ang-2 (10 \(\mu\text{g mL}^{-1}\)) was added further for occurring the chemical reaction between the \(-\text{COOH}\) group from PTCA and cysteine terminal from Ang-2; next, the residual EDC/NHS was removed through dialysis, and the prepared Ang-2/CB nanocomposite was stored for further use at 4 \(^\circ\)C.

**Construction of glioma cell immunosensor**

Before the modification of electrode, glassy carbon electrode (GCE) was polished with different alumina powders and suffered a sonication in water. Next, the GCE surface was covered with Ang-2/CB solution of 8.0 \(\mu\text{L} (1.0 \text{ mg mL}^{-1})\) to form Ang-2/CB/GCE. For detecting glioma cell, the glioma cell solution with various concentrations were dropped onto Ang-2/CB/GCE and undergo incubation for 15 min. The unbound glioma cells need to be removed from the surface of the electrode via gently washing with phosphate buffer solution (pH 7.4). Then, the electrochemical tests were studied in the \([\text{Fe(CN)}_6]^{3-/4-}\) solution of 5.0 mM containing 0.1 M KCl.

**Results and discussion**

**Characterization of CB-PTCA**

Scanning-electron microscopy (SEM) and transmission-electron microscopy (TEM) were firstly used to study CB. As shown in Fig. 1, it’s noted that CB presents unique and uniform nanoballs with \(\sim 28 \text{ nm}\) diameter. Then, Fourier transform infrared spectroscopy (FT-IR) was introduced to characterize CB-PTCA (Fig. 1C). It’s observed CB-PTCA exhibits C–C band at 1128 cm\(^{-1}\) and C=C conjugation at 1588 cm\(^{-1}\); meanwhile it displays stretching bands at \(\sim 1755 \text{ cm}^{-1}\) for \(\text{C}=\text{O}, \sim 1030\) and 3160 cm\(^{-1}\) for O–H resulted from the \(-\text{COOH}\) group of PTCA, indicating the successful preparation of CB-PTCA via non-covalent mean. The CB-PTCA dispersibility in water was also investigated (Fig. 1D), a homogeneous and dark dispersion without precipitate was presented for CB-PTCA; while the pure CB
shows poor dispersion ability in water. The excellent dispersibility of CB-PTCA is very important for efficiently enhancing the utilization of nanohybrids and improving the reproducibility and sensitivity of the sensor. In addition, the prepared CB-PTCA was evaluated via the thermogravimetric analysis (TGA) that was carried out at N₂ atmosphere in the temperature range of 0–800 °C and the obtained results were displayed in Fig. 2. It can be noted that the pristine CB is stable and nearly no weight loss. However, for the prepared CB-PTCA, there is a remarkable weight loss observed at ~410 °C resulted from the PTCA decomposition, and the loss value is ~7.0 wt%.

**Electrochemical sensing of glioma cell**

The build process of the immunosensor was firstly studied by introducing electrochemical impedance spectroscopy (EIS) that was carried out at the following conditions: initial potential, 0.25 V; low frequency, 1 Hz; high frequency, 1,000,000 Hz. Figure 3 displayed the EIS plots of pure GCE (a), CB-PTCA/GCE (b), Ang-2/CB/GCE before (c) and after (d) incubation with glioma cell. The results revealed CB-PTCA/GCE only possesses a very weak resistance ($R_{CT}$) compared to GCE, suggesting the CB-PTCA nanohybrid has excellent conductivity. When Ang-2 was immobilized
cells, the peak current ($I'_p$) is further reduced comparing to that at Ang-2/CB/GCE. These results are consistent with those obtained by EIS analysis, indicating the designed proposal for detecting glioma cell is feasible. Since the principle for the designed sensor is on the basis of the restrain towards the current of probe, the absolute value of peak current difference ($|\Delta I| = |I'_p - I''_p|$) was used as the recorded signal in the following sections.

For achieving the best analytical performances, the influences of several experimental parameters including the amounts of CB-PTCA, concentration of Ang-2 and recognition time of Ang-2 towards glioma cells on the sensing performance of Ang-2/CB/GCE were optimized. Figure 5A shows the influence of the amount of CB-PTCA, it can be found that the $|\Delta I|$ value of the sensor increases when the CB-PTCA amount increases from 2 to 8 µL and then decreases upon the increase in loading amount to 8 µL. Therefore, 8 µL CB-PTCA is used for the further experiments. Figure 5B exhibits the influence from the concentration of Ang-2, and the results showed that the $|\Delta I|$ values increase along with the increase of Ang-2 concentrations, while when the loading of Ang-2 is higher than 10 µg mL$^{-1}$, there is no obvious changes observed, indicating that Ang-2 of 10 µg mL$^{-1}$ is the best concentration for constructing immunosensor of glioma cell. In addition, the effect from the recognition time of Ang-2 towards glioma cells was displayed in Fig. 5C, it is clear that the $|\Delta I|$ value increases upon increasing the time, while there is no obvious increase when the time exceed 20 min, indicating that the time of 20 min is the optimal recognition time and is then introduced for the further experiments of glioma cell determination.

Under the above optimized experimental conditions, the detection performance of the proposed simple immunosensor was investigated based on the increase in $|\Delta I|$ value to the Ang-2/CB/GCE sensor when gliomas cells were bio-conjugated with Ang-2 on the Ang-2/CB/GCE surface. Through plotting the related calibration trace which consists of the $|\Delta I|$ value of probe (y-axis) upon the increase of glioma cell concentrations (x-axis). It can be found from the curve that along with the rise of glioma cell concentration ranging from $10^2$ to $10^6$ cells mL$^{-1}$, the $|\Delta I|$ value linearly increases (Fig. 6) and the corresponding linear equation is $|\Delta I|$ (µA) = $-0.234 + 2.95$LogC (cells mL$^{-1}$) ($R^2 = 0.996$).

Based on S/N = 3, the LOD value was calculated to be 30 cells mL$^{-1}$. Table 1 displayed the comparisons between the proposed immunosensor and previous studies about the analytical performance, the results revealed that the obtained performances in present work for detecting glioma cell exhibits lower LOD and wider linearity than those showed in the almost of previous literatures.

The reproducibility, specificity and real application are three key parameters for the sensing properties of the
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In order to investigate the reproducibility of the proposed immunosensor, six parallel-constructed Ang-2/CB/GCE sensors were utilized to measure the DPV current of 1000 cells mL⁻¹ glioma cells. The relative standard deviation (RSD) value of the \(|\Delta I|\) value was obtained to be 4.66\% (Fig. 7A), demonstrating that the immunosensor has desirable reproducibility. The specificity of the immunosensor for glioma cell was evaluated also (Fig. 7B). By comparing the \(|\Delta I|\) values in the presence of interfering cells. The results displayed there is almost no interference obtained for the cells including MCF-10, HeLa, b.End 3 and MCF-7 (10.0 times of glioma cell concentration), demonstrating the specificity of the designed immunosensor is satisfactory for glioma cell analysis. Furthermore, for studying the feasibility of the immunosensor in the practical application, the determination of the human serum was performed through introducing standard addition method. Through adding various concentrations of glioma cells to the samples, the current peak of sensor was obtained after the incubation of the immunosensor in samples and the recovery was adopted to study the feasibility in practical samples. As displayed in Table 2, the formed recovery was found in the range between 92.6 and 97.5\%, and the related RSD values are < 5.0\%, indicating that constructed immunosensor can monitor successfully glioma cell in the real samples.

**Conclusion**

By preparing CB-PTCA as sensing platform to link Ang-2 that can recognize selectively glioma cell, a simple and free-labeled voltammetry immunosensor of glioma cell was designed for the first time in this work. By introducing \([\text{Fe(CN)}_6]^{4-/3-}\) as probe, its peak current based on Ang-2/CB/GCE decreases linearly with the increase of glioma cell concentration owing to the specific recognition between Ang-2 and glioma cell. After optimizing several related conditions, the constructed immunosensor of glioma cell displays excellent detection performances with wide
Furthermore, the designed immunosensor exhibited desirable reproducibility and selectivity as well as practicability, it’s thus confirmed that the developed method for gliomas cell analysis will have important potential for the early and clinical diagnosis of glioma.

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