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Short Communication

Nitrogen-doped Graphene Modified Glassy Carbon Electrode for Electrochemical Determination of Breast Cancer Marker Carbohydrate Antigen 15-3

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In this study, an electrochemical immunosensor based on nitrogen-doped graphene sheets (N-GS) for label-free and sensitive determination of CA15-3 was prepared. The linear range of this new immunosensor was as broad as 0.1–20 U/mL, and the limit of detection (LOD) was as low as 0.017 U/mL. Traditional immunosensors are similar to complex labelling systems, and the separation requires plenty of time. However, our proposed immunosensor is facile and requires no labelling. Our proposed immunosensor has potential uses in clinical analysis and diagnosis applications.

Keywords: Breast cancer; Biomarker; Electrochemical immunoassay; CA15-3; Graphene

1. INTRODUCTION

Breast cancer commonly occurs as a malignant tumour in females, of which the most vital biological marker is cancer antigen 15-3 (CA15-3). The normal concentration of CA15-3 in human serum is below 25 U/mL. A significant increase in CA15-3 serum concentration is observed in 30–50% of breast cancer patients. In addition, patients with CA15-3 concentrations of more than 100 U/mL can be diagnosed with breast cancer. CA15-3 serum concentration is not only useful in monitoring the state of patients after operation but is also predictive of the recurrence and metastasis of breast cancer [1-3].

Enzyme-linked immunosorbent assays, chemiluminescence immunoassays, enzyme immunoassays, radio-immunoassays and many other techniques have been used to determine the

concentration of CA15-3 in human serum [4]. However, in these techniques, the label processing is usually complex, and the separations often require plenty of time. In addition, they are not able to meet the increasing clinical demands of fast CA15-3 determination. Additionally, these methods are not sensitive enough to function with the extremely low biogenic concentrations seen in the clinical setting. Therefore, it is vital for a sensitive and fast CA15-3 determination method to be developed. The improvement in the fabrication of immunosensors, especially electrochemical immunosensors, has the potential to support breakthroughs in the diagnosis of cancer [5, 6]. Fast determination of tumour markers can be achieved by these immune-sensing approaches, where the specific interaction between antigen and antibody yields electrochemical signals which are easily transduced, thus a direct readout can be realized. A significant contribution to this field is an electrochemical measurement that works by the discrimination of probe signals based on redox potential [7]. Hansen and co-workers reported the synthesis of dual label probes of PbS and CdS semiconductor nanocrystals to perform protein detection, where Pb^{2+} and Cd^{2+} are dissolute and accumulated, and then the recognition and scaling of the lysozyme and thrombin levels are conducted using square wave voltammetry (SWV) tripping [8]. In previous studies, we proposed the combination of nanoparticles or liposomes and redox probes labelling to carry out dual, or even triple, analyte detection [9, 10]. This multi-label detection usually adopted the covalent label as a common antibodies-probes conjugation [11]. It has been well established that an antibody comprises a constant region and a variable region. The signal labelling is usually achieved using the amino acid sequence on the constant region. Meanwhile, the specificity to an antigen is determined by the changeable amino acid sequence on the variable region. The recognition antibody may face the risk of being deactivated due to a negative effect induced by the non-specific covalent label on the variable region. In this case, it is common for low current responses to be observed.

As biotechnology and biocompatible nanomaterials have developed in recent years, third generation biosensors have shown better performance [12, 13]. Nanomaterials are characteristic of immobilized enzymes [14-21]. As a two-dimensional crystal, graphene sheets (GS) have received substantial attention as a material to be used for biosensor preparations since they are highly conductive, desirably biocompatible, and possess favourable electronic features [22, 23]. Nevertheless, due to the lack of a bandgap, complete control over the electrical conductivity of graphene has not been achieved. The carbon atoms can be replaced by foreign atoms via the doping of graphene, suggesting that the modulation of conductive types and the opening of the bandgap can be achieved. The dopant atoms can potentially be extensively used through the tailoring of the physicochemical features and electronic band structure of graphene [24, 25]. In recent years, N-GS have received substantial attention and have been used in fields such as electrochemical biosensors, batteries, electrocatalysis, and supercapacitors since this material is highly electrically conductive and possesses large numbers of edge sites and high surface area [26-29].

This study presents a new label-free electrochemical immunosensor capable of the sensitive detection of CA15-3. Label-free immunosensing and detection were achieved by enhanced sensitivity, ascribed to the high conductivity of N-GS caused by greatly improved electron transfer. Therefore, the proposed immunosensors have the potential to be used for clinical diagnostic application.

2. EXPERIMENTS

2.1. Chemicals

Commercial graphene slices and graphite powder were purchased from Shanghai Carbon Co., Ltd. (China). *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide (EDC) were procured from Sinopharm Chemical Reagent Co., Ltd. (China). Carbohydrate antigen 15-3 (i.e., CA15-3) and Anti-CA15-3 antibody were acquired from Yixin Biochemical Reagents (China). Dimethylformamide (DMF), potassium ferricyanide and bovine serum albumin (BSA) were purchased from Beijing Chemical Factory (China) and Sigma (USA), respectively.

2.2. Synthesis of N-GS

Graphite oxides (GO) were prepared from graphite powder by a modified Hummers' method. N-GS were synthesized from GO through thermal annealing of GO in ammonia [26]. Initially, 140 mg of the as-prepared GO was dispersed in water. Subsequently, $NH_3 \cdot H_2O$ (30%) was added to reach a pH of 10 for the aforementioned solution. Then, the mixed solution was stirred for 10 min, after which 2 mL of hydrazine was added. The solution was allowed to incubate for more than 12 h at 80 °C to allow the hydrazine–induced reduction of the graphite oxides evidenced by black precipitate. After filtration and washing with doubly distilled water, the pH of the precipitate solution was adjusted to neutral. Eventually N-GS was obtained after drying the solids produced in the last step.

2.3. Instrument

A D8 ADVANCE X-ray diffractometer (Bruker AXS, Germany) was used for X-ray diffraction (XRD) measurements. Monochromatic Al Kα radiation was applied to an ESCALAB 250 (ThermoFisher Scientific, England) to carry out X-ray photoemission spectroscopy (XPS) measurements. A CHI 660D electrochemistry workstation with a triple-electrode configuration was employed for all electrochemical measurements, where the working, auxiliary and reference electrode were, respectively, a modified Au electrode (GE, $\phi = 4$ mm), a platinum wire and a saturated calomel electrode (SCE). A pH meter and differential pulse voltammetry (DPV) were used to perform the pH assays and EC measurements, respectively. The parameters for the EC measurements were potential (−0.6 to 0.4 V), potential step (5 mV), amplitude (50 mV), the first pulse width (0.2 s), the second pulse width (0.06 s) and a pulse period (0.5 s). Prior to signal secondary antibody loading, the immunosensor construction was controlled by electrochemical impedance spectroscopy (EIS). The stepwise detection was performed in 5.0 mM [$Fe(CN)_6^{4-/3-}$] (pH 7.0), where the high frequency, low frequency, and amplitude of 10000 Hz, 0.05 Hz and 5 mV, respectively.

2.4. Modification of electrodes

Typically, the working electrode was modified by attaching the target antibody (i.e., Anti-CA15-3 antibody) to the surface of the graphene, where the amidation reaction occurred between the

amino group of the antibody and the carboxylic group remaining on *N-GS* [30]. Graphene sheets (2 mg), EDC (100 mM) and NHS (100 mM) were added to PBS (1 mL) and kept for 4 h (denoted as N-GS-modified GCE). Subsequently, Anti-CA15-3 antibody (100 μg/mL, 1 mL; PBS) was added to the system for a further 12 h (denoted as Anti-CA15-3/N-GS-modified GCE). The resulting graphene–Anti-CA15-3 suspension was centrifuged and re-dispersed in 1 mL of PBS at 4 °C for further use.

2.5. Immunosensor fabrication

Initially, the glassy-carbon electrode (GCE) was polished using 1, 0.3, and 0.05 μm alumina powders. This was followed by incubation with BSA solution (1 wt%) to block non-specific binding sites between the antigen and electrode (denoted as BSA/Anti-CA15-3/N-GS-modified GCE). Afterwards, various concentrations of CA15-3 were added to the as-prepared electrode, and left to react for 3 h. After washing and cleaning, the final electrodes were stored at 4 °C before use. Additionally, the $Fe(CN)_6^{3-/4-}$-electrode electron transfer during the immunological reaction of CA15-3 was monitored by the redox probe, potassium ferricyanide. The variation in the current response to the immunological reaction with CA15-3 at varying concentrations was also investigated.

3. RESULTS AND DISCUSSION

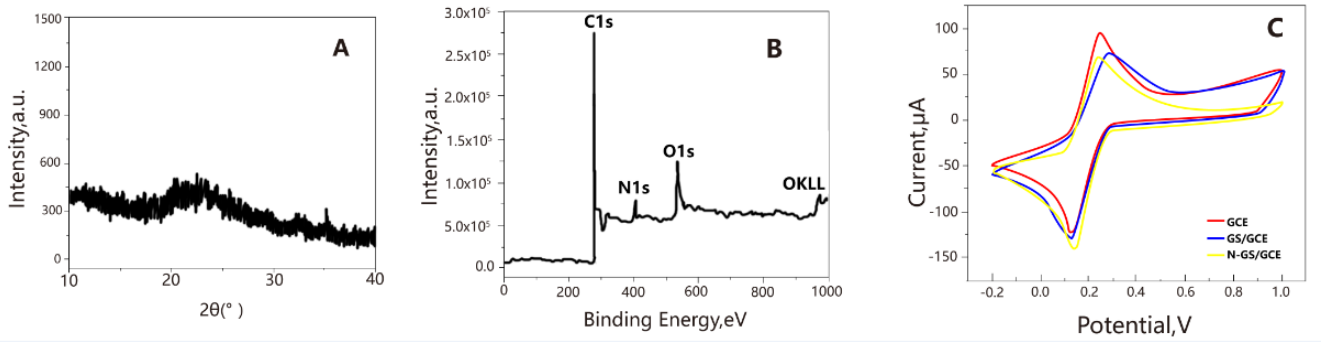

Figure 1. (A) XRD profiles of N-GS. (B) XPS spectra of N-GS. (C) CVs of GCE, GS-modified GCE and N-GS-modified GCE in $K_3[Fe(CN)_6]$ (5.0 mM).

Fig. 1A shows the XRD profiles of N-GS. It can be seen that a broad peak is centred *ca.* 24°, corresponding to the recovery of graphitic crystal structure. We also performed an XPS analysis to confirm that graphene was successfully doped with N. The primary C 1s, O 1s, and N 1s core levels were observed in the scan spectrum from XPS (Fig. 1B) without any traces of impurities. The bare GCE, GS-modified GCE, N-GS-modified GCE and CS-modified GCE were characterized via CVs in Fig. 1C. The GS-modified GCE and N-GS-modified GCE displayed significantly higher redox peaks in comparison to those of the bare GCE, due to the distinct electrical features of GS and N-GS. It can

also be observed that there was a clear redox peak current between GS-modified GCE and N-GS-modified GCE, firmly suggesting easier electron transfer with N-GS than with graphene.

The dependence of the N-GS concentration on the peak current was observed. Initially, an increase was detected for the variation in the peak currents, which was then decreased. The concentration of N-GS was optimized at approximately 2 mg/mL, which was then set for the development and control of our proposed immunosensor.

Fig. 2 shows the electrochemical impedance spectra for the performance of the preparation and assembly of our proposed immunosensor. The semicircle section was observed at higher frequencies, associated with the electrochemical processes involving electron transfer, with the diameter representing the electron-transferring resistance [31]. For comparison, we recorded the stepwise coating and assembly process during the preparation of our proposed immunosensor. A linear relationship was confirmed for both the electrodes after N-GS modification and the bare GCE, as indicated in the Nyquist plots. The semicircle section was found to be enhanced after the electrodes were coated using CA15-3, BSA, and anti-CA15-3. This phenomenon suggests that the electron transfer was decreased after the electrode surfaces were modified, which corresponds to the decreased channels caused by the coating using the aforementioned biomolecules. It can be clearly seen that these biomolecules were successfully assembled on the electrode surface. It was confirmed that the electron transfer was enhanced in the sensing system after the addition of N-GS.

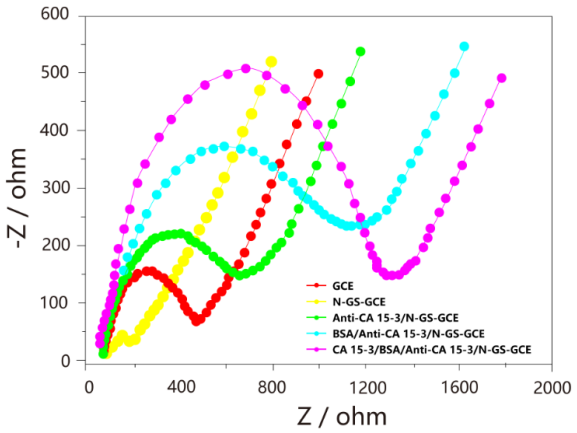

Figure 2. Electrochemical impedance spectra (EIS) of the response of the immunosensor to CA15-3 antigen (4 U/mL) at bare GCE, N-GS-modified GCE, Anti-CA15-3/N-GS-modified GCE, BSA/Anti-CA15-3/N-GS-modified GCE, along with CA15-3/BSA/Anti-CA15-3/N-GS-modified GCE in pH 7.4 PBS that contained 1 mM $K_3Fe(CN)_6$ (0.1 to 10^5 Hz).

The detection of CA15-3 (0.1-18 U/mL) was investigated using our developed immunosensors, as indicated in Fig. 3. As the concentration of CA15-3 increased, a decrease in the amperometric signal was observed. A broad linear range of 0.1-20 U/mL was obtained for this immunosensor. Compared with the LOD obtained with traditional labelling detection of 0.025 U/mL, this immunosensor had a lower LOD of 0.017 U/mL, and the correlation coefficient was 0.998 [32]. The sensitivity of the proposed sensor was compared to that of other reported CA15-3 sensors and the results are presented

in Table 1. With a large linear detection range and a low LOD, our proposed immunosensor is highly competitive against other sensors for clinical analysis and diagnosis applications.

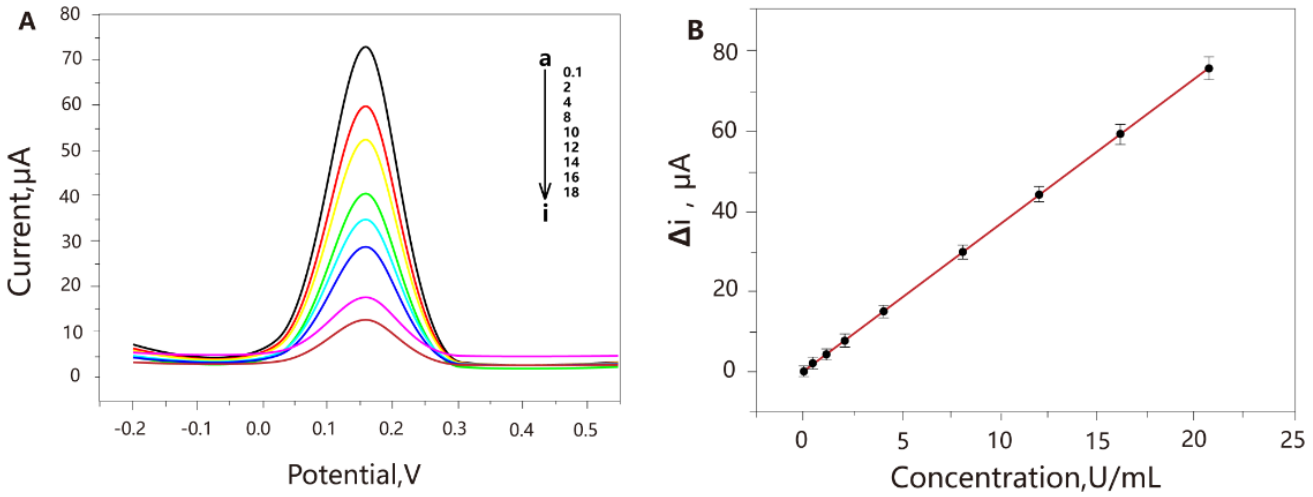

Figure 3. (A) Differential pulse voltammograms (DPVs) measurement of CA15-3 at the immunosensor in pH 7.4 PBS that contained 5 mM potassium ferricyanide at an amplitude of 50 mV and a pulse period of 0.2 s (B) Calibration curve with error bar.

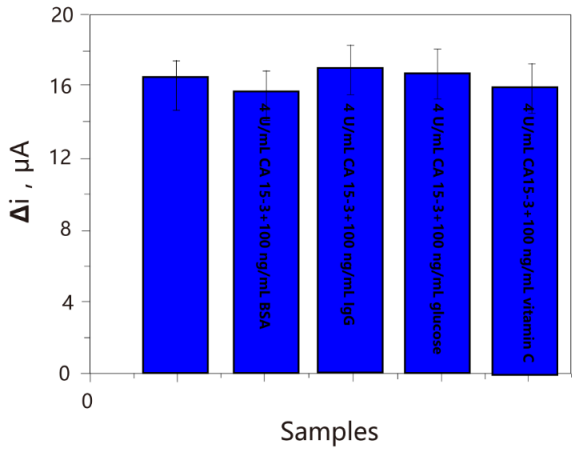

Figure 4. Effect of interference on the CA 15-3 determination in pH 7.4PBS that contained 1 mM potassium ferricyanide with interfering agents.

Table 1. Comparison of the present electrochemical sensor with other CA 15-3 determination methods.

Method	Linear detection range	Detection limit	Reference
N-doped graphene	2–30 U/mL	0.72 U/mL	[33]
Gold nanoparticle doped CA 15-3 antibody	1–10 U/mL	0.25 U/mL	[34]
Thionine-nanoporous gold-graphene	0.5–20 U/mL	0.2 U/mL	[35]
CA 15-3/BSA/Anti-CA 15-3/N-GS modified GCE	0.1-20 U/mL	0.017 U/mL	This work

We also tested the reliability of our proposed immunosensor by measuring 4 U/mL CA15-3 solutions (pH 7.4) that contained interference agents (i.e., vitamin C, glucose, human IgG, BSA, etc.). As indicated in Fig. 4, these interference agents exhibited no obvious effects on CA15-3 determination. In comparison with the control groups, the interference agents showed a current variation of below 5%. Therefore, our proposed immunosensor showed desirable selectivity for further use. Afterwards, the reproducibility was investigated using six working electrodes fabricated under similar conditions, with a standard deviation obtained of 3.9%. Thus, the reproducibility was acceptable in this experiment. The reactivity of our proposed immunosensor to CA15-3 did not vary significantly after 1 month of storage, suggesting a desirable stability.

The CA15-3 was also determined in real specimens in serum (Table 2). The CA15-3 was added into CA15-3-free human blood serum to obtain a test specimen. CA15-3-free human blood serum was used for the dilution of serum specimens, where the CA15-3 level exceeded the calibration curve range. The relative standard deviations (RSD) for 2 U/mL, 5 U/mL and 10 U/mL were obtained as 2.44%, 1.71% and 4.22%, respectively. The corresponding CA15-3 recoveries were calculated as 99.4%, 100.34% and 101.3%, respectively.

Table 2. Analytical data of DPV assays for CA 15-3 in serum samples.

Sample	Addition	Found	ELISA	Recovery	RSD
1	2 U/mL	1.988 U/mL	1.907U/mL	99.4 %	2.44 %
2	5 U/mL	5.017 U/mL	5.020U/mL	100.34 %	1.71 %
3	10U/mL	10.108 U/mL	9.983 U/mL	101.08%	4.22 %

4. CONCLUSIONS

In this work, CA15-3 was successfully detected with an N-GS-modified GCE using a facile electrochemical technique. The proposed immunosensor greatly enhanced electron transfer and showed a desirable sensitivity in the detection of CA15-3 due to the significant conductivity of the graphene-modified electrode. The linear range was as broad as 0.1–20 U/mL, and the LOD was 0.017 U/mL.

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