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Electrochemical Immunoassay for Breast Cancer Markers CA153 Determination Based on Carbon Nanotubes modified Electrode

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This work describes the fabrication of carbon nanotubes (CNTs) incorporated in a carbon paste electrode (CPE) modified by poly-glutamic acid (PGA) for the assessment of CA 153, a breast cancer biomarker. The developed immunosensor was highly sensitive to the level of CA 153 because of the considerably increased charge transfer, and the limit of detection (LOD) was calculated as 0.025 U/mL. The protocol designed for our proposed immunosensor has potential use for clinical study and practical diagnostic applications.

Keywords: Electrochemical Immunoassay; Breast Cancer Marker; CA153; Carbon nanotube; Poly-glutamic acid

1. INTRODUCTION

Breast cancer is a common malignant tumour in females. Cancer antigen 153 (CA153) is one of the most significant breast tumour markers, with a normal level of below 25 U/mL in human serum. A significant increase in CA153 concentration could be detected for 30–50% of breast cancer patients. Moreover, patients presenting a CA153 concentration of over 100 U/mL would be diagnosed with breast cancer [1-4]. The postoperative status of patients can be monitored directly by the concentration of CA153; the recurrence and metastasis of breast cancer can also be predicted by it [4-7]. A range of techniques, such as chemiluminescence immunoassays, enzyme immunoassays, radioimmunoassays, and enzyme-linked immunosorbent assays, have been developed for the determination of CA153 [8]. However, the above techniques suffer the disadvantages of time-consuming separations, a complex label processing, and difficulty with catering to the increasing clinical demands for the rapid determination of CA153. Furthermore, ultralow biogenic concentrations of tumour markers cannot be desirably detected using these strategies [9-15]. Therefore, it is necessary to develop novel strategies

for the sensitive and rapid determination of CA153. The development of immune sensors, especially electrochemical immune sensors, provides a promising route to settling the conundrums in the field of cancer diagnostics [16, 17]. These immune sensing strategies could rapidly detect tumour markers through a direct readout, since the electrochemical signals from the specific antibody–antigen interaction can be easily transduced.

In recent years, studies on the preparation, features, and mechanisms of conductive polymers have attracted increasing attention [18-20]. Because of their high permselectivity, reproducibility and stability, a large number of conductive polymers have gained application in the field of electroanalysis [21-23]. As one of 20 common amino acids, glutamic acid can form poly-glutamic acid (PGA) through its easy electropolymerization on an electrode surface. PGA exhibits excellent electrochemical features because of its glutamate repeat units and free protonated carboxylic groups (pK_a = 4.45) through the linking of the α-amino and δ-carboxylic acid functional groups [24]. Carbon nanotubes (CNTs) can enhance the charge transfer between the electrode surface and electroactive species, showing distinct features [25, 26], including excellent mechanical strength, desirable electronic features, high surface area, distinct chemical stability, etc. [27-30].

In the present work, a new electrochemical immunosensor was prepared for the label-free and sensitive determination of the level of CA153, a breast cancer biomarker. Since the significant charge transfer increase brought the desirable conductivity of PGA-CNT, this immunosensor is highly sensitive, thus supporting the development of a label-free immunosensing method. The present work aims to show that this new strategy could be used for the fabrication of the label-free immunosensors with potential use in clinical studies.

2. EXPERIMENTS

2.1. Chemicals

Carbohydrate antigen 15-3 (CA153) and an anti-CA153 antibody were purchased from Yixin Biochemical Reagents (China). Paraffin oil, graphite powder (spectral reagent), SDBS, glutamic acid, and CNTs were provided by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All reagents were of analytical grade. The supporting electrolyte used in this work was 0.1 M phosphate buffer solution (PBS).

2.2. Apparatus

All electrochemical measurements were carried out using a CHI 660C Electrochemical Workstation (Shanghai Chenhua Co., Ltd., China) with a traditional three-electrode geometry, where the working, reference, and counter electrode were PGA/CNTPE, a saturated calomel reference electrode (SCE) and a Pt foil, respectively. All potentials were applied according to that of the reference electrode. Fourier transform infrared (FTIR) spectra were performed using an AVATAR 370 Fourier transform infrared spectrometer (America).

2.3. Preparation of the PGA/CNTPE with Anti-CA 153

CNTPE was prepared by mixing CNTs, paraffin oil and carbon powder together at the proportion of 2:5:13 (w/w/w), respectively, in an agate mortar to full homogenization. A glass tube with an internal diameter of 3 mm was pre-ultrasonicated in a NaOH and HNO_3 solution and doubly distilled water in turns. Then, a portion of the as-prepared mixture was packed into one end of a glass tube with a copper wire inserted from the other end. The CNTPE was polished on a piece of weigh paper, and it was rinsed with doubly distilled water before use. Then, the as-prepared CNTPE was electro-polymerized in 0.1 M of pH 7.0 PBS that contained 0.05 M glutamic acid for 15 cycles (− 0.2 to 2.0 V at a scan rate of 100 mV/s) to yield the PGA/CNTPE. For comparison purposes, PGA/CPE was prepared without the CNTs. Prior to use, the as-prepared PGA/CNTPE was carefully washed using doubly distilled water. The working electrode was modified with the target antibody through an amidation reaction between the amino group of the antibody and the carboxylic group remaining on PGA/CNTPE. This was followed by immersing the as-prepared PGA/CNTPE in 100 mM EDC and 100 mM NHS containing 1 mL PBS, which was maintained for 4 h. Then, this system was mixed with 1 mL of the anti-CA 153 antibody (100 μg/mL; in PBS, indicated as Anti-CA 153/PGA/CNTPE) for an extra 12 h.

2.4. Fabrication of immunosensors

The non-specific binding sites between the electrode and antigen were blocked by incubating the as-prepared PGA/CNTPE/Anti-CA153 with 1% (wt) BSA solution (indicated as BSA/Anti-CA 153/PGA/CNTPE). Subsequently, different concentrations of CA 153 were added to the electrodes, and they were reacted for 180 min to obtain the final electrodes. After washing and cleaning, the resulting electrodes were stored at 4 °C before use. The charge transfer from $Fe(CN)_6^{3-/4-}$ to the electrode during the immunological reaction of CA 153 was recorded by the redox probe, potassium ferricyanide (indicated as CA 15-3/BSA/Anti-CA 153/PGA/CNTPE). The current change in the response to the immunological reaction vs. different concentrations of CA 153 was investigated as the analytical signal.

3. RESULTS AND DISCUSSION

The glutamic acid and PGA films were characterized via their Fourier transform infrared spectra (FTIR) in Fig. 1. The curve for glutamic acid had fine structures, which were not observed for PGA film. This was because glutamic acid was polymerized. The peaks of C—O stretching vibration at approximately 1261 cm^{-1}, C=O stretching vibration at 1642 cm^{-1}, and O—H, N—H, C—H stretching vibration at (2502 - 3502 cm^{-1}) constituted the FTIR absorption spectra of solid glutamic acid. The peaks observed at 3426 cm^{-1} were found to significantly broaden, as displayed in curve (b). This could be explained by the poly-reaction of glutamic acid. The peaks of C—O and C=O stretching vibration observed at 1115 cm^{-1} and 1634 cm^{-1}, respectively, corresponded to those observed at

1261 cm^{-1} and 1641 cm^{-1}, respectively, of glutamic acid, suggesting that the carboxylate group was present. Prior to the poly-reaction, one amino group and two carboxylate groups were present in every molecule, as indicated in the molecular formula of glutamic acid presented at the bottom of Fig. 1. Thus, condensation might take place between the amino group of the glutamic acid and one carboxylate group of another, one and the remaining carboxylate group may stay in the monomer of PGA. The molecular formula of glutamic acid [31]. There are two carboxylate groups and one amino group in every molecule before the poly-reaction [32].

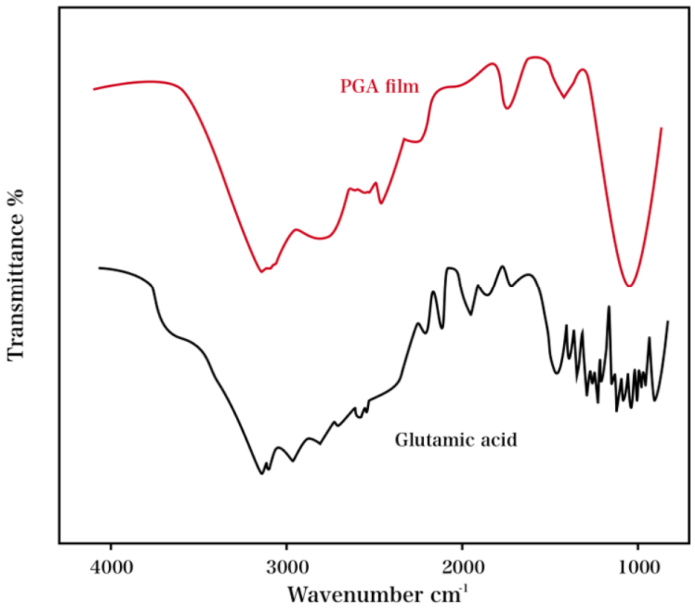

Figure 1. The FTIR of (A) the glutamic acid film and (B) the PGA film.

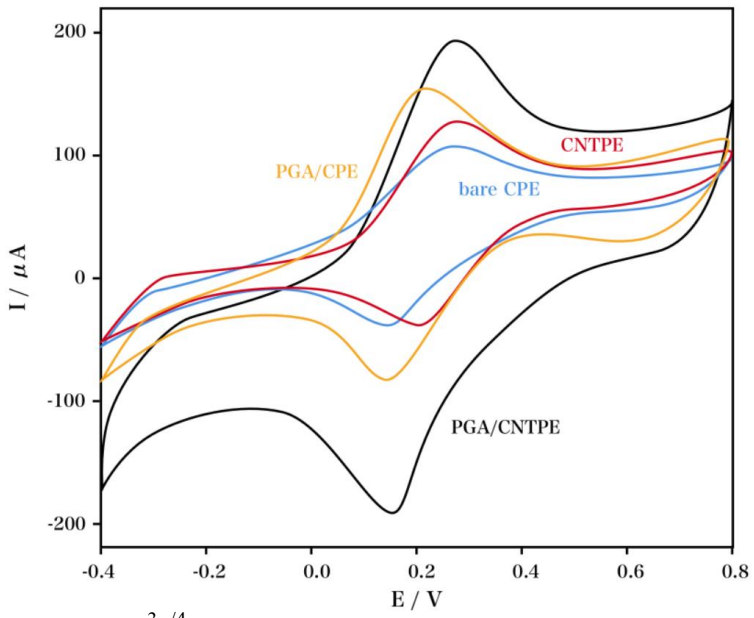

Figure 2. The CVs of $Fe(CN)_6^{3-/4-}$ (5 mM) that contained KCl solution (0.1 M) using bare CPE, CNTPE, PGA/CPE and PGA/CNTPE.

Cyclic voltammetry (CV) measurements were carried out in $Fe(CN)_6^{3-/4-}$ (5 mM) containing KCl (0.1 M) using the bare CPE, CNTPE, PGA/CPE and PGA/CNTPE to study the electrochemical characteristics of the electrodes, which is shown in Fig. 2. The bare CPE exhibited unclear and broad oxidation and reduction peaks, suggesting a slow charge transfer. However, the CNTPE exhibited a significantly enhanced response, with a relatively low peak - peak separation (ΔE_p) d), indicating that the PGA/CNTPE has a high electroactive surface area and good electrochemical properties. A pair of well-defined redox peaks were recorded after the modification with PGA, suggesting a significant increase in the current. When using PGA-modified CPE and PGA/CNTPE, the electrochemical reversibility of $Fe(CN)_6^{3-/4-}$ was more desirable than the ΔE_p obtained using bare CPE and CNTPE, which indicates that PGA/CPE and PGA/CNTPE provide the conducting bridges in order to promote the electron transfer of $Fe(CN)_6^{3-/4-}$ and accelerate the whole process of oxidation-reduction [33].

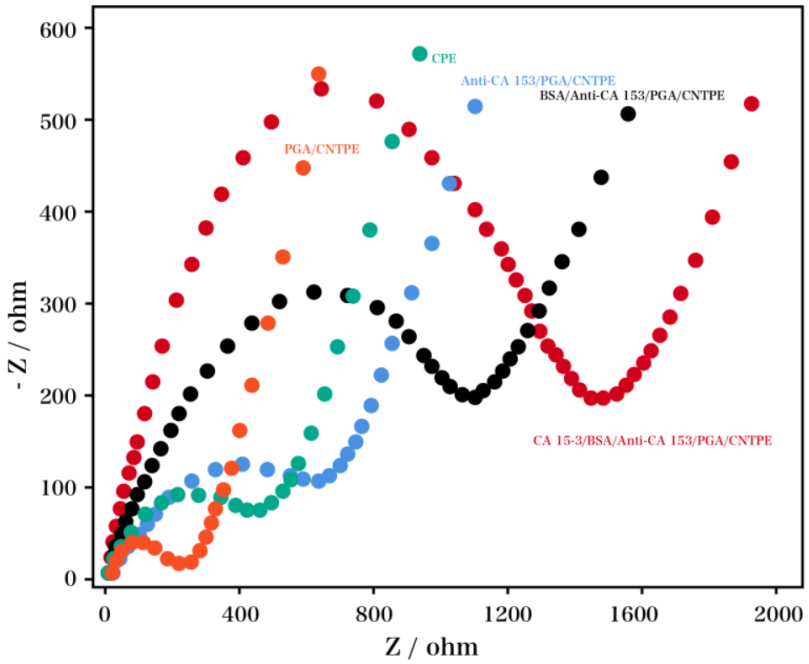

Figure 3. The EIS obtained from 0.1 to 10^5 Hz in PBS at pH 7.4 containing 1 mM $K_3Fe(CN)_6$ on the response of the immunosensor to the CA 153 antigen (4 U/mL) in the presence of bare CPE, PGA/CNTPE, Anti-CA 153/PGA/CNTPE, BSA/Anti-CA 153/PGA/CNTPE and CA 15-3/BSA/Anti-CA 153/PGA/CNTPE.

Fig. 3 shows the electrochemical impedance spectra (EIS) for the preparation and assembly performance of the immunosensor. It is generally known that the change in electrochemical impedances may reflect the chemical process occurring in the electrochemical sensors. A comparative study was conducted by studying the immunosensor fabrication in each assembly step while considering the stepwise assembly and coating. The linear portion at low frequencies is associated with the electrochemical behaviour limited by diffusion. The semicircle portion at high frequencies is, however, related to the electrochemical processes subject to electron transfer, where the diameter corresponds to the electron-transferring resistance [34]. Both CPE- and PGA-modified CNTPE were found to have almost linear Nyquist curves. The semicircle segments were enhanced after coating the

electrode with CA 153, BSA and anti-CA 153. This was because the modification over the electrode surfaces caused a decrease in the charge transfer, which corresponded to the biomolecule coating – induced access decrease. It was obvious that these biomolecules were successfully assembled onto the electrode surface. Notably, the charge transfer of the PGA/CNTPE-modified electrode was more desirable than that of the bare CPE. Thus, the PGA/CNTPE promoted the charge transfer under the sensing system.

The level of CA 153 (1 to 30 U/mL) was analysed and assessed using our developed immunosensor, as shown in Fig. 4. As the concentration of CA 153 increased, a decrease in the amperometric signal was observed. The results showed the desirable performance of our developed immunosensor with a low LOD of 0.025 U/mL, which is better than a recent report that resorted to conventional labelling detection (0.025 U/mL; [35]). The developed immunosensor clearly provides a prospective for clinical research and diagnostic applications. A broad linear range of 0.1–30 U/mL was obtained as well. The sensitivity of the proposed sensor was compared to that of other reported CA153 sensors, and the results are presented in Table 1.

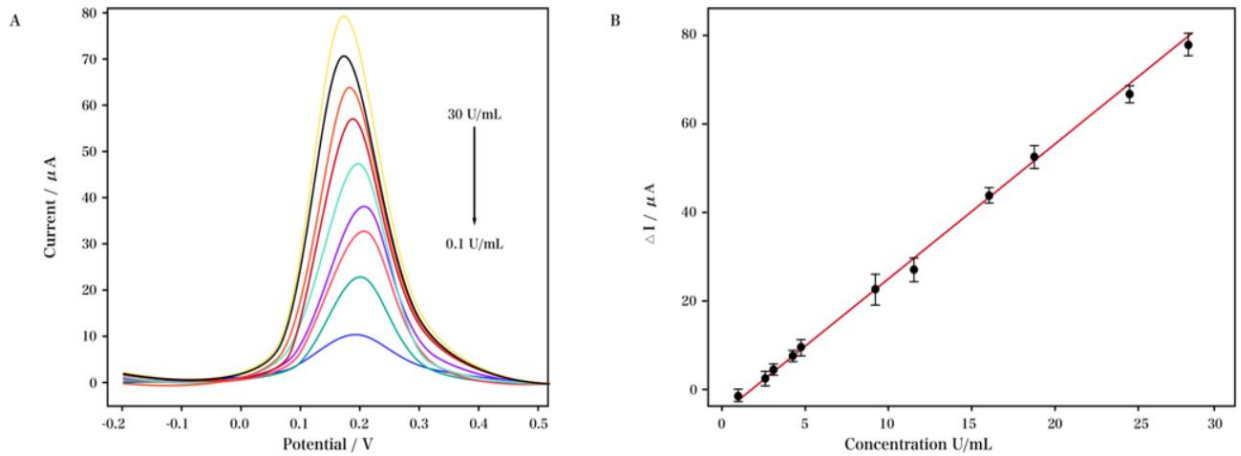

Figure 4. (A) The DPV profile of CA 153 in PBS at pH 7.4 that contained 5 mM potassium ferricyanide with the immunosensor. (B) The calibration curve with error bars for the RSD (*n*=5).

The effect of interference agents, such as 100 ng/mL glucose or vitamin C, 100 ng/mL human IgG, and 100 ng/mL BSA, on the detection of CA 153 was also studied to verify the reliability of our proposed immunosensor. As shown in Fig. 5, the determination of this target was not significantly affected by the above interference agents. Compared with the control group, the current change caused by the above interference agents was arguably less than 5%, suggesting a desirable selectivity for our proposed sensor. Afterwards, 5 working electrodes fabricated under similar conditions were used again to study the reproducibility of this sensor. After 5 experiments, the standard deviation was recorded as 4.2%, indicating that our proposed sensor was highly reproducible. The stability was also investigated by storing the immunosensor in PBS for a month. The results showed that the reactivity of this sensor to CA 153 did not obviously change.

Table 1. The 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	[34]
Gold nanoparticle doped CA 15-3 antibody	1–10 U/mL	0.25 U/mL	[35]
Thionine-nanoporous gold-graphene	0.5–20 U/mL	0.2 U/mL	[36]
CA 15-3/BSA/Anti-CA 153/PGA/CNTPE	0.1-30 U/mL	0.025 U/mL	This work

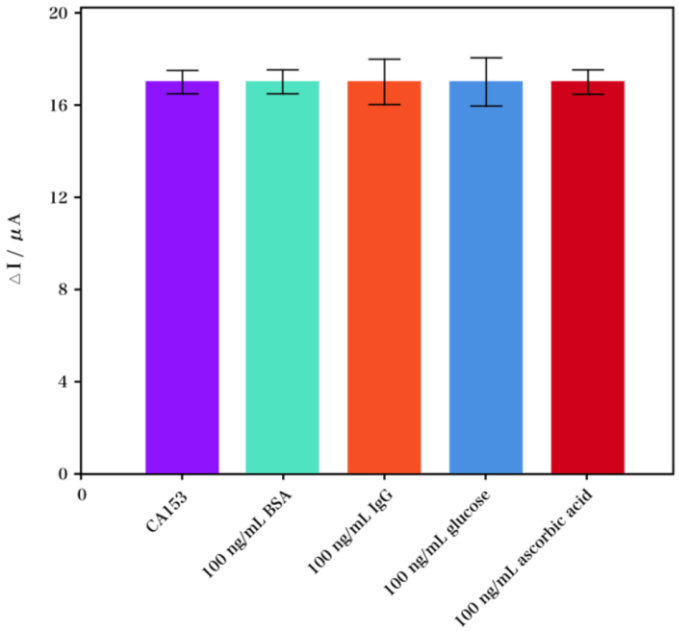

Figure 5. The effect of interference agents on the CA 153 determination in PBS at pH 7.4 that contained 1 mM potassium ferricyanide.

Table 2. The determination of serum specimens with our proposed electrochemical sensor and a standard immunochromatography kit (U/mL).

Sample	1	2	3
Added	5 U/mL	10 U/mL	20 U/mL
The immunosensor	5.08	9.98	19.85
Immunochromatography kit	5.07	10.11	20.07
RSD (%)	2.11	3.24	5.01

The determination of CA 153 in serum specimens was also investigated. The specimen was a mixture of CA 153-free human blood serum and CA 153 (Table 2). When the CA 153 level exceeded the range of the calibration curve, the serum specimens were diluted using CA 153-free human blood

serum. For the concentrations of 5 U/mL, 10 U/mL and 20 U/mL, the relative standard deviations (RSD) were obtained as 2.11%, 3.24% and 5.01%, respectively. The CA 153 recoveries were found to be 96.3%, 101.3% and 98.2%, respectively. Thus, our developed immunosensor has potential for clinical study and diagnosis applications.

4. CONCLUSIONS

The present study presented the preparation of an electrochemical immunosensor based on PGA/CNTPE for a label-free and sensitive method for the determination of the level of CA 153. The results showed that our proposed immunosensor was highly sensitive to the level of CA 153 because of the significant charge transfer. Our developed immunosensor showed a desirable performance with a broad linear range of 0.1–30 U/mL and a low LOD of 0.025 U/mL. The cooperative effect of a facile preparation method and a direct measurement protocol is desirable. Furthermore, it would not only benefit clinical studies but also promote a series of novel immunodiagnostic apparatuses for diagnostic use in breast cancer.

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