Simple and Effective Approach to Prepare an Epoxy-Functionalized Polymer and Its Application for an Electrochemical Immunosensor

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ABSTRACT: An epoxy-functionalized polymer based on a new skeleton has been prepared via an efficient method and it combined with aminated carbon nanotubes to form a new composite material. This new composite material was applied for the fabrication of an electrochemical immunosensor with good performance. The inexpensive and easily available IgG was used to test the performance of the prepared composite material. The levels of IgG were quantitatively analyzed using a differential pulse voltammetry detection system and the lowest detection limit was calculated to be 0.05 ng/mL. The detection system can also respond to IgG in the concentration range from 0.1 to 25 ng/mL.

1. INTRODUCTION

Over the past few years, the design and synthesis of biocompatible polymers with reactive functional groups for fabricating chemical sensors have been a highly topical field.1 The epoxy group, as a highly reactive functional group, facilitates the covalent attachment of proteins, enzymes, cells, and peptides via nucleophilic ring-opening reactions with amines at moderate reaction conditions.2 Given the advantage of epoxy-functionalized polymers in chemical sensors, research into the synthesis of these polymers bearing epoxy groups has become a key area of interest. For example, Mehlhase et al. have developed an efficient approach for creating core/shell architectures with a large number of epoxy moieties at the surface.3 Şenel et al. reported that a new polymer containing a high number of epoxy- and ferrocene-functionalized groups can be prepared by free-radical copolymerization.4 The synthesis of polymers bearing epoxy groups can also be achieved through rhodium-catalyzed polymerization of glycidyl 2-diazoacetate5 and so on.6 The varied synthetic approaches have accelerated the preparation of epoxy-functionalized polymeric materials; however, utilization of commercial polymers as an initiator for the fabrication of well-defined molecular weight polymers containing epoxy groups can be regarded as one of the best options since this approach does not need particular catalysts or monomers with special structures and only needs simple operations and moderate reaction conditions. Moreover, this approach will be more favorable to industrial production. In this regard, poly(vinylbenzyl chloride) (PVBC) is a good candidate for producing functional materials because (a) it has good solubility in common organic solvents, good film formation, and biocompatibility and (b) it can be easily modified by nucleophilic reagents without requiring harsh reaction conditions.8 Linear PVBC has been used to synthesize a series of alkaline anion-exchange membranes for comb-like amphiphilic co-polyelectrolyte alkaline,9 alkaline anion-exchange membranes,10 bactericidal surfaces,11 organic anode-active materials,12 and so on.13 However, until now, linear PVBC as a superior reagent for synthesizing an epoxy-functionalized polymer has not been investigated.

Electrochemical immunosensors are currently considered powerful tools for the detection of biomolecules, owing to their rapid detection, high sensitivity, simple instrumentation, miniaturization, and low cost.14,15 Many factors can affect the performance of electrochemical immunosensors including selection of electrode materials, modification methods of the electrode surface, and immobilization technology. Nowadays, because biomolecule immobilization on matrixes plays essential roles in preparing immunosensors with good performance, immobilization technology is emerging in this area.16 Covalent immobilization is the method most commonly encountered and of considerable interest, mainly owing to the stability with which biomolecules can be bound.17,18 For effective immobilization of biomolecules onto the electrode surface, biocompatible polymers containing reactive groups such as hydroxyl, carboxylic acid, and amine groups have been widely utilized.19,20 For practical operation, polymers with epoxy groups are more attractive, mainly because the reaction
between the epoxy groups and amino groups of biomolecules is efficient and no additive chemical agents such as 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC-HCl),21 N,N′-carbonyldimidazole (CDI),22 and high-concentration glutaraldehyde solution23 are required during the reaction.

Carbon nanotubes (CNTs) are one of the most popular conducting materials due to their great electrical conductivity, high thermal stability, good biocompatibility, and strong absorbability.24 Owing to their distinctive advantages, CNTs have been used in different fields, especially for electrochemical sensors.25 Moreover, the use of polymer–CNT composites for the development of electrochemical sensors has been extensively investigated. A large number of polymers have been used for the preparation of CNT composites, such as natural polymers,26 Nafton,27 and conducting polymers.28 However, as far as we know, epoxy-functionalized polymer–CNT composites are still very rare to date, and only a few reports have focused on electrochemical immunosensors based on them.

One purpose of this study is two-fold: (1) to present a versatile and experimentally simple technique for the synthesis of a well-defined epoxy-functionalized polymer by the use of commercial linear PVBC as an initiator (Scheme 1); (2) and to combine the advantages of epoxy-functionalized polymers and aminated carbon nanotubes to design a disposable and sensitive label-free electrochemical immunosensor, for which human IgG was chosen as template biomolecules (Scheme 2).

2. RESULTS AND DISCUSSION

2.1. Design and Characterization of Polymer EFP. We envisioned that polymer EFP could be designed with commercial linear PVBC as the skeleton of the polymer and a small organic molecule containing both a phenolic hydroxyl group and an epoxy group as graft objects. From this, we begin to consider whether a simple synthetic method of new compound 1 can be established. Compound 1 could be prepared via the chemical oxidation of allyl 4-hydroxybenzoate, and this method is described for the first time (Scheme 1). To ascertain whether the epoxy groups were successfully grafted onto the PVBC, infrared (IR) spectra of polymer EFP (a), compound 1 (b), and PVBC (c) were investigated. The spectrum showed typical features of polymer EFP and compound 1 with an ester carbonyl peak at 1722 cm\(^{-1}\) (Figure 1). In addition, two distinct bands measured at 910 and 853 cm\(^{-1}\) have appeared in the sample of polymer EFP (c) and compound 1, which are ascribed to the bending vibration of the epoxy groups. To our delight, we found that a distinct band at 1264 cm\(^{-1}\) in the spectrum of PVBC associated with a chloromethylen unit has disappeared in the sample of polymer EFP. Therefore, we confirmed that epoxy groups were successfully introduced into PVBC and polymer EFP containing a large number of very stable epoxy groups.

As can be seen from the thermogravimetric analysis (TGA) weight loss curve (Figure 2a), PVBC underwent two-step weight loss from 350 to 800 °C. The first step of the weight loss commenced between 350 and 450 °C and the second degradation step occurred above 450 °C. The TGA weight loss curve of polymer EFP (Figure 2b) indicates that the weight loss temperature is about 350 °C, which is similar to the weight loss temperature of PVBC (Figure 2a). This finding indicates that the two polymers have the same skeleton. However, polymer EFP underwent one-step weight loss, which obviously occurred between 350 and 800 °C. These results clearly

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indicated that polymer EFP possessed good thermal stability, which was beneficial for preservation and actual usage.

2.2. SEM Images of the Aminated Carbon Nanotubes and the Polymer EFP–CNT Composite. Aminated carbon nanotubes and the polymer EFP–CNT composite were characterized by scanning electron microscopy (SEM). Figure 3A–C shows the SEM image of aminated carbon nanotubes, which is measured at different magnifications. From the SEM images of the polymer EFP–CNT composite (Figure 2D–F), it is clear that the form of carbon nanotubes has not been changed and is well encapsulated by polymer EFP. The specific morphology indicates that the polymer EFP–CNT composite can be easily made into a film.

2.3. Electrochemical Characterization of Modified Electrodes. After the structure and thermal stability of polymer EFP as well as the morphology of the polymer EFP–CNT composite were confirmed, we begin to evaluate the performance of the composite in electrochemical immunosensors. The stepwise modification of the immunosensor electrode was first investigated by electrochemical impedance spectroscopy (EIS), which is a good and simple method for characterizing the interfacial properties of surface-modified electrodes. According to literature methods, the semicircle diameter of the Nyquist plot of EIS represents the electron transfer resistance, $R_e$. The Nyquist plots of a bare electrode and an electrode modified with different substances in a 10 mM K$_3$Fe(CN)$_6$/K$_4$Fe(CN)$_6$ solution are summarized in Figure 4. Curve a represented the EIS of the bare glassy carbon electrode (GCE) and had a very small semicircle diameter, implying low electron transfer resistance. The semicircle diameter of curve b was obviously larger than that of the bare GCE due to the introduction of the polymer EFP–CNT composite film on the surface of the electrode. Similarly, after the electrode was further modified with an antibody (Figure 4, curve c), bovine serum albumin (BSA) (Figure 4, curve d), and an antigen (Figure 4, curve e), the corresponding semicircle diameter increased stepwise, which suggested that electron transfer barriers increased.

CV was also used to evaluate the electron transfer property of the modified electrodes using 10 mM K$_3$Fe(CN)$_6$/K$_4$Fe(CN)$_6$ as a redox probe. Besides, the concentrations of the antibody and the antigen were 150 μg/mL and 10 ng/mL, respectively. Figure 5 displays the electrochemical behavior of the electrodes after each step of modification. As depicted in Figure 5 (curve a), the bare electrode possessed an obvious couple of reversible redox peaks. Consistent with expectations, when the GCE was stepwise modified with the polymer EFP–CNT composite (curve b), antibody (curve c), BSA (curve d), and antigen (curve e), the peak currents of CVs decreased gradually, demonstrating that the enhanced electron transfer barriers were introduced by stepwise modifications.

2.4. Optimization of Analytical Conditions. The effect of the concentration of the antibody from 50 to 250 μg/mL on the response of the immunosensor was investigated. When the concentration of the antibody increased to 150 μg/mL, there was a significant decline in the peak current and it reached a plateau (Figure 6A). The experiment data suggested that a 150 μg/mL antibody solution in phosphate-buffered saline (PBS) (0.1 M, pH 7.2) chosen for this work was appropriate. Additionally, the influence of the reaction time of the antibody with the polymer EFP–CNT composite on the performance of the immunosensor was also investigated over the range 30–90 min. At room temperature, the differential pulse voltammetry (DPV) response of the immunosensor decreased obviously with the immobilization time up to 60 min (Figure 6B). Thus, a reaction time of 60 min was selected for this work.

2.5. Detection of IgG with the Electrochemical Immunosensor. Under the above optimal conditions, we continued to investigate the performance of the electrochemical immunosensor based on the polymer EFP–CNT composite.

![Figure 2](image2.png)

**Figure 2.** Thermogravimetric analysis of PVBC (a) and polymer EFP (b).

![Figure 3](image3.png)

**Figure 3.** SEM images of aminated carbon nanotubes: $\delta = 3 \, \mu m$ (A), $\delta = 1 \, \mu m$ (B), and $\delta = 500 \, nm$ (C) and the polymer EFP–CNT composite: $\delta = 3 \, \mu m$ (D), $\delta = 1 \, \mu m$ (E), and $\delta = 500 \, nm$ (F).
composite toward different IgG concentrations. Upon increasing the concentration of IgG, the peak current gradually decreased, as depicted in Figure 7A. Additionally, as shown in Figure 7B, the good linear response of the peak current toward [IgG] was obtained in the IgG concentration range of 0.1−25 ng/mL, with a low detection limit (LOD) of 0.05 ng/mL (a widely used method based on a signal-to-noise ratio of 3). The linear regression equation was $Y (\mu A) = 30.8795 - 0.9145X (\text{ng/mL})$, with a linear regression coefficient of 0.9841. In addition, we compared the performance of the IgG electrochemical immunosensor based on some new materials (Table 1). These data suggested that the linear range and low detection limit of the immunosensor based on the polymer EFP−CNT composite, the same result can be achieved.

2.6. Specificity, Reproducibility, and Stability of the Immunosensor. To further validate the specificity of this system for IgG detection, common interfering proteins including alpha-fetoprotein (AFP), Human Serum Albumin (HAS), immunoglobulin M (IgM), and immunoglobulin E (IgE) were chosen as reference substances. The peak current signals of the developed immunosensor incubated with 10 ng/mL IgG, the mixture of IgG and AFP, HAS, IgM, or IgE (the concentrations of IgG, AFP, HAS, IgM, and IgE are 10 ng/mL, 1 μg/mL, 1 μg/mL, 1 μg/mL, and 1 μg/mL, respectively) was obtained under the same experimental conditions (Figure 8). Upon inspection of Figure 8, it is clear that nearly no or little changes in the peak current signals were obtained after interfering proteins (AFP, HAS, IgM, and IgE) were added into IgG. All these observations indicate that the developed immunosensor can identify IgG with an acceptable specificity. Then, to illustrate the good reproducibility of the electrochemical immunosensor, an experiment has been performed under the optimized conditions. The immunosensors were prepared with five individual electrodes and were used to detect a sample with a fixed concentration of IgG (10 ng/mL). The coefficient of variation of the five immunosensors was 4.95%, which indicated that the proposed electrochemical immunosensor can be used for detecting biomolecules with good reproducibility. Furthermore, we examined the immunosensor stability. The electrode was stored in the fridge at 4 °C when not in use. After a storage period of four weeks, the electrochemical immunosensor retained 89.5% of its initial peak current value of DPV for 10 ng/mL IgG. This result indicates that the electrochemical immunosensor has good stability, which may be ascribed to the good biocompatibility of the polymer EFP−CNT composite. Moreover, three human serum samples were tested by the electrochemical immunosensor based on the polymer EFP−CNT composite and also by the enzyme-linked immunosorbent assay (ELISA). The relative errors (%) between the developed immunosensor and ELISA ranged from 4.8 to 6.3% (Table 2). By contrasting the
HAS, IgM, and IgE are 10 ng/mL, 1 μg/mL, 1 μg/mL, 1 μg/mL, and 1 μg/mL, respectively. Error bars represent standard deviation, n = 3.

**Table 1. Comparison of the Modified Electrode Materials for IgG Detection**

| modified electrode materials | linear range (ng/mL) | LODs (ng/mL) | refs |
|------------------------------|----------------------|--------------|------|
| nickel nanoparticles         | 0.3–400              | ~0.3         | 30   |
| ZnO–chitosan                 | 2.5–500              | 1.2          | 31   |
| ferrocenyldendrimer          | 2–50                 | 2            | 32   |
| labeled nanogold             | 5–500                | 1.1          | 33   |
| polymer EFP–CNTs             | 0.1–25               | 0.05         | this work |

Figure 7. (A) DPV recordings for IgG concentrations between 0.1 and 25 ng/mL; (B) calibration curve of the immunosensor for the detection of different concentrations of IgG. Error bars represent standard deviation (n = 3).

Figure 8. Specificity of the immunosensor to IgG, IgG + AFP, IgG + HAS, IgG + IgM, and IgG + IgE. The concentrations of IgG, AFP, HAS, IgM, and IgE are 10 ng/mL, 1 μg/mL, 1 μg/mL, 1 μg/mL, and 1 μg/mL, respectively. Error bars represent standard deviation, n = 3.

data, the developed immunosensor was suitable for offering evidence for clinical diagnosis.

**Table 2. Determination of IgG in the Serum Samples (n = 5)**

| samples | immunosensor (ng/mL) | ELISA* (ng/mL) | relative errors (%) |
|---------|----------------------|----------------|---------------------|
| 1       | 6.83                 | 6.47           | 5.6                 |
| 2       | 18.92                | 18.05          | 4.8                 |
| 3       | 23.58                | 22.19          | 6.3                 |

*The IgG concentration of the human serum specimens was diluted to the detection range of the electrochemical immunosensor using PBS buffer (0.01 M, pH 7.2). *Enzyme-linked immunosorbent assay method.

3. CONCLUSIONS

A well-defined composite containing both epoxy groups and CNTs can be easily obtained and used for the construction of an electrochemical immunosensor. The main advantage of the surface of the composite lies in its immobilized antibodies that can efficiently and directly link nucleophilic ring-opening reactions with amino groups of antibodies. The results showed that the label-free electrochemical immunosensor based on the polymer EFP–CNT composite possesses high sensitivity and good reproducibility and storage stability. Besides, polymer EFP is an attractive carrier for enzyme immobilization.

4. EXPERIMENTAL SECTION

4.1. Reagents and Materials. All of the reagents and solvents used in this work were of analytical grade and allyl 4-hydroxybenzoate, m-chloroperoxybenzoic acid (m-CPBA, 85%), Na3HPO4, NaH2PO4, dimethylformamide (DMF), dichloromethane, and ethanol were purchased from Tokyo Chemical Industry (Shanghai). Poly(vinylbenzyl chloride) (PVBC, a 60/40 mixture of 3- and 4-isomers with average molecular weight Mn ca. 55 000 and Mw ca. 100 000), human immunoglobulin G (IgG), goat anti-human immunoglobulin G antibody (anti-IgG), and bovine serum albumin (BSA) were obtained from Sigma-Aldrich. Amino-modified multiwalled carbon nanotubes (~NH2 content: 0.45 wt %, length: ~50 um, outer diameter: 8–15 nm) were purchased from Nanjing XFNANO Materials Tech Co., Ltd. Double distilled water was used in this work. Phosphate-buffered saline (PBS, 0.1 M, pH 7.2) was prepared using Na2HPO4 and NaH2PO4. Three human serum samples were donated by the Brain Hospital of Hunan Province, China.

4.2. Apparatus. All electrochemical investigations including electrochemical impedance spectroscopy (EIS), cyclic voltammetry (CV), and differential pulse voltammetry (DPV) were conducted on a CH 660E electrochemistry workstation (Shanghai CH Instruments, China). A conventional three-electrode cell, consisting of a glassy carbon electrode (GCE) modified with an epoxy-functionalized polymer–CNT composite film as the working electrode, a Pt electrode as the counter electrode, and a saturated calomel electrode (SCE) as the reference electrode, was used. A Nicolet FT-170SX instrument was used for recording FT-IR spectra, using KBr discs. 1H NMR and 13C NMR measurements were performed on a Bruker AVB-500 MHz NMR spectrometer (Bruker BioSpin, Switzerland). A TGA-50 thermalgravimetric analysis (TGA) instrument (Shimadzu Corporation) was used for the characterization of the polymer. Electrospray ionization high-resolution mass spectra (ESI-HRMS) were recorded on a Bruker P-SIMS-Gly FT-ICR mass spectrometer.

4.3. Preparation of Compound 1 and Polymer EFP. Syntheses of compound 1 and polymer EFP are outlined in Scheme 1. Compound 1 was prepared by a simple and one-step chemical oxidation of allyl 4-hydroxybenzoate. Briefly, allyl 4-hydroxybenzoate (1.78 g, 10 mmol) was dissolved in anhydrous dichloromethane (15 mL) at room temperature, and then m-chloroperoxybenzoic acid (3.23 g, 15 mmol) was added. The solution was stirred at 25 °C for 12 h. After the...
The polymer containing epoxy groups (polymer EFP) was facilely prepared from compound 1 by a nucleophilic substitution reaction. To a stirred solution of poly(vinylbenzyl chloride) (305 mg, 2 mmol chloromethyl groups) in dry DMF (10 mL), compound 1 (398 mg, 2 mmol hydroxyl groups) and potassium carbonate (276 mg, 2 mmol) were added, and then the mixture was stirred at 60 °C for 12 h. After cooling to room temperature, the reaction mixture was poured into 100 mL of water and stirred for 30 min. The precipitate was collected by filtration and washed with ethanol and water several times until compound 1 could not be detected in the washing solution via thin-layer chromatography (TLC). After drying under vacuum at 60 °C for 24 h, polymer EFP was obtained as an off white solid (430 mg).

4.4. Construction of the Electrochemical Immunosensor. Our strategy to fabricate the electrochemical immunosensor can be seen in Scheme 2. First, the bare GCE surface (3 mm in diameter) was polished repeatedly with alumina powder (particle sizes of 0.3 and 0.05 μm in turn), followed by successive sonication for 5 min in doubly distilled water and 5 min in ethanol, respectively. The polished GCE was dried in air at room temperature and then 10 μL of a 0.1% polymer EFP–carbon nanotube composite (the mass ratio of polymer EFP/carbon nanotubes is 1:1) solution diluted in DMF was dropped on the GCE surface and dried in air at room temperature for 5 h. After drying, 10 μL of the antibody solution (150 μg/mL) was dropped onto the modified electrode and incubated for 1 h, followed by washing with ultrapure water to remove unspecific physical adsorption. Subsequently, 10 μL of 1.0 wt % BSA was dropped onto the modified electrode with the antibody and incubated for 30 min at room temperature to eliminate a nonspecific binding effect and blocking the remaining active sites. Finally, antigen solutions with different concentrations were dropped onto the electrode. The reaction time between the antibody and the antigen was 30 min. Moreover, the as-prepared immunosensor (designed as anti-IgG/polymer EFP–CNT composite/GCE) was stored at 4 °C for further detection of the IgG analyte.

4.5. Electrochemical Measurements. All electrochemical experiments were carried out in a conventional electrochemical cell containing a three-electrode arrangement. CV, EIS, and DPV measurements were performed in a 10 mM K$_{3}$Fe(CN)$_{6}$/K$_{4}$Fe(CN)$_{6}$ solution. The CV measurements were carried out at a scanning rate of 100 mV s$^{-1}$ from −0.2 to 0.6 V relative to the saturated calomel electrode. EIS measurements were carried out in the frequency range 10$^{-1}$ to 10$^{3}$ Hz under an open potential. The amplitude of the alternative voltage was 5.0 mV. DPV measurements were carried out as follows: the potential range was from −0.1 to 0.6 V, the pulse amplitude was 0.05 V, the pulse width was 0.05 s, and the sample width was 0.02 s.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c05183.

$^{1}$H NMR, $^{13}$C NMR, and HRMS spectra of compound 1 (PDF)

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Notes

The authors declare no competing financial interest.

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