Label-Free Electrochemical Immunosensor Based on Ionic Liquid Containing Dialdehyde As a Novel Linking Agent for the Antibody Immobilization

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ABSTRACT: Demand for label-free electrochemical immunosensor has resulted in extensive research in improving the conductivity of a sensing interface and antibody immobilization. In this paper, an electrochemical immunosensor for prostate specific antigen based on dialdehyde-functionalized ionic liquid used as a novel linking reagent to replace glutaraldehyde for the antibody immobilization is described. The novel linking reagent enhanced the conductivity of the sensing interface. Thus, the proposed immunosensor had a wider linear range of 0.05–30 ng mL⁻¹, with a lower detection limit of 0.04 ng mL⁻¹ compared with the immunosensor based on glutaraldehyde for the antibody immobilization.

1. INTRODUCTION

Up to now, many electrochemical immunosensors have been developed because they possess some attractive properties such as sensitivity, ease of use, and simplicity. Antibody is usually used as a capture element in immunosensors, and therefore the antibody immobilization is very important. One of the most common methods of antibody immobilization favors premodifying of electrode with some materials containing carboxylic acid or amine groups, followed by cross-linking of antibody to the modifying material. For example, Chit is usually used as a matrix to modify electrode due to its excellent film-forming ability and abundant reactive amine groups and then glutaraldehyde (GA) is used as a linking agent for antibody immobilization. But after glutaraldehyde was introduced, the electrical conductivity of the electrode surface decreased. Therefore, seeking a novel cross-linking agent to immobilize antibody and simultaneously improve the conductivity of a sensing interface is of great significance for the immunosensors based on Chit and other modifying materials with amino groups.

Ionic liquids (ILs) have been used as the modifier or the supporting electrolyte in the electroanalysis field because of their high ionic conductivity and biocompatibility. ILs were also incorporated into conventional matrices, including biopolymers, cellulose, metal nanoparticles, and sol–gel-based silica matrices to form stable composite materials for the fabrication immunosensor. Due to the high ionic conductivity and biocompatibility, ILs-containing modifying films provided a good microenvironment to entrap proteins and enhanced the conductivity of the electrode surface. In our previous work, we developed an electrochemical immunosensor based on ionic liquid functionalized with aldehyde. But a molecule of aldehyde-functionalized ionic liquid (DIL) contains only one aldehyde group that is used to capture antibody. It was modified on the electrode surface through noncovalent interaction, which is not favorable for the stability of the immunosensor due to the leakage of IL. Thus, this work focused on the use of dialdehyde-functionalized ionic liquid (DIL) as a linking agent to the fabricated immunosensor. The one aldehyde group was used to covalently interact with the amino group of Chit, which introduced DIL onto the electrode surface. This covalent interaction prevented the leakage of DIL from the electrode surface to electrolyte solution. The other aldehyde group was used to capture antibody. To the best of our knowledge, few electrochemical immunosensors based on DIL were reported.

Prostate specific antigen (PSA) is a marker related to prostate cancer or other prostate disorders. The determination of PSA is of great significance in clinical diagnosis and postcure monitoring. In this work, DIL was successfully synthesized. It was introduced on the electrode surface through covalent interaction between aldehyde group of DIL and the amino group of Chit, which introduced DIL onto the electrode surface.

Supporting Information

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group of Chit that was modified on the electrode surface in advance. Herein, DIL not only links a linking agent to immobilize antibody but also can improve the conductivity of the sensing interface. Thus, the immunosensor based on DIL is simple, sensitive, and stable. We hope this strategy would provide a new platform for the detection of PSA and other cancer markers.

2. EXPERIMENTAL SECTION

2.1. Reagents and Apparatus. PSA antigen (PSA), anti-PSA antibody (Ab), and bovine serum albumin (BSA) were provided by Beijing Dingguo Biotechnology Company (Beijing, China). 4,4′-Bipyridine and 4-(bromomethyl)benzaldehyde were purchased from Sigma-Aldrich. Phosphate buffer solution (PBS, 0.1 M, pH 7.0) was obtained with Na2HPO4 and KH2PO4. Chit solution (1%) was prepared by fully dissolving chitosan in acetic acid solution by sonication. Electrochemical experiments were performed on a CHI 660E electrochemistry workstation (Shanghai CH Instruments, China) with a standard three-electrode.

2.2. Preparation of Dialdehyde Ionic Liquid (DIL). 4,4′-Bipyridine (0.156 g, 1 mmol) and 4-(bromomethyl)benzaldehyde (0.498 g, 2.5 mmol) were added to acetonitrile (20 mL) and refluxed overnight. The mixture was cooled to room temperature, and then the precipitate was filtered to afford a yellow solid of 0.42 g (76%). The synthesis route for dialdehyde-functionalized ionic liquid is shown in Figure 1A.

2.3. Fabrication of the Immunosensor. Prior to preparing the immunosensor, the gold electrode was polished repeatedly with 0.3 and 0.05 μm alumina slurries and washed with doubly distilled water. The cleaned electrode was modified with Chit by drop-coating 10 μL of 1% Chit solution directly onto the electrode surface and dried in the air. Then, 10 μL of 3 mg mL−1 DIL aqueous solution was dropped on the Chit film, resulting in the formation of Chit/DIL film. One hour later, this was washed with doubly distilled water and dried in the air. Then, the Chit/DIL film-modified electrode was incubated with 10 μL of 50 μg mL−1 antibody solution for 40 min at 37 °C and washed carefully with PBS. Then, the Chit/DIL/Ab-modified electrode was incubated with 10 μL of BSA (2.0 wt %) for 30 min at 37 °C to block nonspecific active sites and washed with PBS. Finally, the electrode blocked with BSA was incubated with 10 μL of antigen solution with different concentration for 40 min at 37 °C, followed by washing with PBS and then measuring the electrochemical signals. The schematic illustration of the immunosensor is shown in Figure 1B.

2.4. Electrochemical Measurements. The used three-electrode system includes a Pt electrode (counter electrode), a saturated calomel electrode (reference electrode), and a gold electrode (Au) (working electrode). After BSA-blocked electrodes were incubated with 10 μL of PSA solution with different concentrations, the electrodes were dipped in a 5 mM Fe(CN)63−/4− solution and measured with differential pulse voltammetry (DPV). The peak current of DPV decreases with the increase of the PSA concentration. Thus, the quantitative detection of PSA can be achievable. DPV measurements were carried under the following conditions: the potential range is from 0.2 to 0.8 V, pulse amplitude is 0.05 V, pulse width is 0.05 s, sample width is 0.02 s, and scan rate is 100 mV s−1.

3. RESULTS AND DISCUSSION

3.1. NMR Characterization of DIL. 1H NMR characterization of DIL/CHO (500 MHz, DMSO-d6) δ (ppm): 1H NMR (500 MHz, DMSO-d6) δ (ppm): 10.06 (s, 2H), 9.60 (d, J = 5.5 Hz, 4H), 8.840 (d, J = 5.5 Hz, 4H), 8.01 (d, J = 7.5 Hz, 4H), 7.83 (d, J = 7.5 Hz, 4H), 6.13 (s, 4H); 13C NMR (125 MHz, DMSO-d6) δ (ppm): 193.23, 149.78, 146.48, 140.61, 137.10, 130.62, 130.00, 127.80, 63.24. The NMR graphene was shown in Figures S1 and S2 (see the Supporting Information).

3.2. Electrochemical Characterization of the Modified Electrode. Cyclic voltammograms (CV) was a convenient method for probing the feature of the modified electrode surface. In this work, CV measurements were performed in 0.1 M PBS (pH 7.0) containing 5 mM K3[Fe(CN)6]/K4[Fe(CN)6] at a scan rate of 100 mV s−1 from −0.2 to 0.6 V. Figure 2A showed the cyclic voltammograms of bare electrode, Chit-modified electrode, Chit/DIL-modified electrode, Chit/DIL/Ab-modified electrode, Chit/DIL/Ab/BSA-modified electrode, and Chit/DIL/Ab/BSA/PSA-modified electrode. A couple of well-defined redox peaks could be observed at bare Au electrode (Figure 2A,a). The peak current of Chit-modified electrode decreased (Figure 2A,b), whereas for the DIL modified on the Chit film, the peak current at the Chit/DIL-modified electrode increased (Figure 2A,c) obviously as compared with that at the Chit-modified electrode and even bare electrode. The enhancement can be ascribed to the high conductivity of DIL. After the Chit/DIL-modified electrode was incubated with Ab solution, the current response decreased (Figure 2A,d), demonstrating that antibody was immobilized on the electrode. When BSA was used to block the nonspecific active sites, a further decrease of the peak current of CV was observed (Figure 2A,e). After Chit/DIL/Ab/BSA-modified electrode was incubated with antigen solution, a decrease in peak current was obtained (Figure 2A,f). This is because the formed antibody—antigen immunocomplex on the electrode surface hindered the electron transfer.
Electrochemical impedance spectroscopy (EIS) was also used to monitor the changes of interfacial properties at electrode surfaces. The EIS curves resulted from 0.1 M PBS (pH 7.0) containing 5 mM K$_3$[Fe(CN)$_6$]/K$_4$[Fe(CN)$_6$] are presented in Figure 2B. The semicircle diameter is equal to the electron-transfer resistance ($R_{et}$). Curve a represents the $R_{et}$ of the bare electrode (Figure 2B,a). When the bare electrode was modified with Chit, the $R_{et}$ increased (Figure 2B,b). However, when DIL/CHO was modified on the Au/Chit electrode, $R_{et}$ decreased (Figure 2B,c), which proved that DIL/CHO improved the conductivity of electrode surface. After the electrode was modified stepwise with anti-PSA antibody (Figure 2B,d), BSA (Figure 2B,e), and PSA (Figure 2B,f), the $R_{et}$ was gradually increased. The reason is that the protein layer obstructed the interfacial electron transfer, leading to an increase in $R_{et}$.

The effect of the scan rate on performance of the proposed immunosensor was also investigated. Figure 3 showed that the peak current increased with the increase of the scan rate at the range of 40 to 200 mV s$^{-1}$. Moreover, a linear relationship between the peak current and the square root of the scan rate was observed (Figure 3, Inset). These results demonstrated that the redox reaction was controlled by a diffusion process. 20,21

3.3. Comparison of Glutaraldehyde with Dialdehyde-Functionalized Ionic Liquid As a Linking Agent. In the fabrication of electrochemical immunosensors, Chit was usually used as a coating material to functionalize the electrode surface, followed by cross-linking of antibody to it by using glutaraldehyde as a linking agent. Here, dialdehyde-functionalized ionic liquid replaced glutaraldehyde as a linking agent for attaching antibody to the Chit film. Under the same concentration of 0.3% (w/w), the comparison of glutaraldehyde with dialdehyde-functionalized ionic liquid as a linking agent was carried out. As shown in Figure 4, curve a represented the CV of Chit-modified electrode. When dialdehyde-functionalized ionic liquid was modified on the Chit film, the peak current of CV increased (Figure 4 curve b), indicating that the introduction of DIL improved the conductivity of the sensing interface. When glutaraldehyde was modified on the Chit film, the peak current of CV decreased (Figure 4 curve c), indicating that the use of glutaraldehyde obstructed the electronic transfer of the sensing interface.

3.4. Optimization of the Experimental Conditions.

The reaction time of Chit with DIL was discussed at room temperature. Figure 5A shows the peak current of DPV of the immunosensor decreasing as reaction time increases until a

Figure 2. CV (A) and electrochemical impedance spectroscopy (EIS) (B) profiles of the stepwise preparation of the immunosensor: (a) Au bare electrode, (b) Chit/Au, (c) DIL/Chit/Au, (d) Ab/DIL/Chit/Au, (e) BSA/Ab/DIL/Chit/Au, and (f) PSA/BSA/Ab/DIL/Chit/Au. The concentration of PSA is 10 ng mL$^{-1}$.

Figure 3. Cyclic voltammograms of the developed immunosensor at different scan rates in 0.1 M PBS (pH 7.0) containing 5 mM Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$. From (a) to (i): 40, 60, 80, 100, 120, 140, 160, 180, and 200 mV s$^{-1}$. Inset: plots of the peak currents vs square root of the scan rate.

Figure 4. Comparison of cyclic voltammograms of different linking reagents: (a) Chit/Au, (b) DIL/Chit/Au, and (c) glutaraldehyde/Chit/Au.
platform appeared at 40 min. Thus, the choice of reaction time was 40 min.

The influence of the concentration of DIL on the response of the immunosensor was investigated. Figure 5B shows when the concentration of DIL increased from 0.1 to 0.3% (w/w), the peak current of DPV decreased. When the concentration of DIL was higher than 0.3%, the peak current of DPV was almost a constant value. As a result, 0.3% of DIL was selected.

The influence of the antibody concentration on the response of the immunosensor was investigated from 25 to 100 μg mL\(^{-1}\) at room temperature. These antibodies of different concentrations reacted with the same PSA target concentration of 10 ng mL\(^{-1}\). Figure 5C shows that the antibody concentration of 50 μg mL\(^{-1}\) was an optimal selection.

### 3.5. Detection of PSA

After different concentrations of PSA were modified on the electrode, the DPV curves were recorded. The linear relationship of the proposed immunosensor between the peak current and the concentration of PSA was obtained in the range of 0.05−30 ng mL\(^{-1}\) (shown as the inset of Figure 6A), with a detection limit of 0.04 ng mL\(^{-1}\). The equation was \(I (\mu A) = 49.5−1.16 \times c \text{ (ng mL}^{-1}\text{)}\) \((R = 0.9972)\). However, the linear range of the immunosensor based on glutaraldehyde (GA) as a linking reagent was 0.1−25 ng mL\(^{-1}\) (shown as the inset of Figure 6B), with a detection limit of 0.08 ng mL\(^{-1}\). The equation was \(I (\mu A) = 29.7−0.62 \times c \text{ (ng mL}^{-1}\text{)}\) \((R = 0.9932)\).

The detection limit was calculated based on 3σ (where σ is the standard deviation of a blank solution). These results demonstrated that the immunosensor based on DIL had a wider linear range and lower detection limit than that of the immunosensor-based GA. The proposed immunosensor was compared with other PSA immunosensors. As shown in Table 1, the immunosensor fabricated by us is compared with other immunosensors.

### 3.6. Reproducibility, Specificity, and Stability of the Immunosensor

Using inter-and intra-assay \((n = 5)\) at 10 ng mL\(^{-1}\) PSA, we investigated the reproducibility of the proposed immunosensor. As a result, the coefficients of variation of inter- and intra-assay were 8.3 and 6.8%, respectively, suggesting the developed immunosensor possessed good reproducibility.

To evaluate the specificity of the proposed immunosensor, carcinoembryonic antigen (CEA), human IgG, and α-fetoprotein (AFP) were used as potential interfering materials instead of PSA, followed by measuring the DPV. Figure 7 showed that the peak current of DPV toward a higher

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**Table 1. Comparison of the Immunoassay and Other PSA Methods**

| modifying materials | linear range (ng mL\(^{-1}\)) | detection limit (ng mL\(^{-1}\)) | references |
|---------------------|-----------------------------|-------------------------------|------------|
| gold nanoparticles  | 0.1−100                     | 0.001                         | 22         |
| functionalized peptide | 0.5−40                   | 0.2                           | 23         |
| graphene/gold composites | 0−10                     | 0.59                          | 24         |
| ionic liquid/carbon nanotubes | 1−40                    | 0.02                          | 25         |
| AuPd@Au nanocrystals | 0.1−50                      | 0.078                         | 26         |
| DIL                 | 0.05−30                     | 0.04                          | this work  |

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Figure 5. (A) Effect of reaction time between DIL and Chit on the peak current of the immunosensor. (B) Effect of the concentration of DIL on the peak current of the immunosensor. (C) Effect of the concentration of antibody on the peak current of the immunosensor. The concentration of PSA is 10 ng mL\(^{-1}\).

Figure 6. (A) DPV responses of the DIL-based immunosensor to different concentrations of PSA (inset: calibration curve of the DIL-based immunosensor to different concentrations of PSA). From (a) to (g): 0.05, 1, 5, 10, 15, 20, and 30 ng mL\(^{-1}\). (B) DPV responses of the GA-based immunosensor to different concentrations of PSA (inset: calibration curve of the GA-based immunosensor to different concentrations of PSA). From (a) to (g): 0.1, 1, 5, 10, 15, 20, and 25 ng mL\(^{-1}\). Error bars represent the standard deviation, \(n = 3\).

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concentration (100 ng mL\(^{-1}\)) of interfering substances was close to the response toward blank solution. The peak current of DPV toward PSA (10 ng mL\(^{-1}\)) was much lower than that of interfering substances. However, the signal of the mixture materials of CEA, IgG, AFP, and PSA is close to the response toward PSA alone. These results indicated that the specificity of the fabricated immunosensor was acceptable.

To estimate the stability of the immunosensor, the initial DPV was measured. The modified electrode was stored at 4 °C when it was not in use. After 7 days, the DPV current response was measured as decreased by 9.6%. After 21 days, the DPV current response decreased by 9.6%. The results demonstrated the designed immunosensor has satisfactory stability.

3.7. Real Sample Analysis. To further investigate the practical application of the proposed immunosensor in a clinical assay, we analyzed four undiluted human serum samples obtained from real patients. The assay results were compared with those resulted from enzyme-linked immunosorbent assay (ELISA) method and are represented in Table 2.

| Sample | ELISA (ng mL\(^{-1}\)) | this method (ng mL\(^{-1}\)) | Relative deviation (%) |
|--------|---------------------|----------------------|-----------------------|
| 1      | 2.86                | 2.98                  | 4.4                   |
| 2      | 4.32                | 4.01                  | −7.2                  |
| 3      | 11.65               | 12.27                 | 5.3                   |
| 4      | 6.81                | 6.98                  | 2.5                   |

By comparing experimental results, we can see no significant differences of two methods, indicating a good correlation between ELISA and this proposed method. The proposed immunosensor was reliable for PSA detection.

4. CONCLUSIONS

Here, dialdehyde-functionalized ionic liquid was prepared and replaced glutaraldehyde as a novel linking regent for the fabrication of label-free electrochemical immunosensors toward PSA. The use of dialdehyde-functionalized ionic liquid can improve the conductivity of the sensing interface. The developed immunosensor exhibited good reproducibility, specificity, and stability, which provided a promising potential for accurate clinic immunoassays of PSA as well as other tumor markers.
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