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Short Communication

Fabrication of an Electrochemical Immunosensor Containing Au–Ag Alloy for the Detection of Alpha Fetoprotein

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This study presented the enzyme-free determination of alpha fetoprotein (AFP) using a new electrochemical immunosensor. Our proposed immunosensor consisted of a sandwich system involving catalytic Au–Ag nanocrystals. The determination of AFP in the absence of an enzyme was achieved through the generation of signals by the remarkable Au–Ag alloy-induced catalysis of hydrogen peroxide reduction and the increase in sensitivity by enhanced charge transfer. Our developed immunosensor exhibited a linear range as broad as 0.05–30 ng/mL and a limit of detection (LOD) as low as 0.007 ng/mL. This immunosensor was found to be sensitive for clinical determination due to its simplicity and the involvement of catalytic Au–Ag nanoparticles.

Keywords: Electrochemical immunosensor; Enzyme-free; Au–Ag; Alpha fetoprotein; Clinic detection

1. INTRODUCTION

The highly selective and sensitive determination of protein biomarkers has been of great importance in numerous fields, such as in the development and biodefence applications of new drugs, immunology, disease vector elucidation, and medical diagnosis [1, 2]. In particular, the clinical analysis of cancer biomarkers, along with proteomics research, has been considered important for the detection of cancer at an early stage by facilitating the understanding of basic biological processes

during disease progression and monitoring patient responses to therapy methods [3-5]. Hence, substantial attention has been recently paid to the highly sensitive determination of tumour markers.

The traditional immunoassay techniques for the detection of cancer biomarkers primarily consist of the immune-polymerase chain reaction (PCR) assay [6], fluorescence immunoassay [7], chemiluminescence assay [8], electrophoretic immunoassay [9], mass spectrometric immunoassay [10], and enzyme-linked immunosorbent assay (ELISA) [11]. Despite several advantages of the above techniques, there are also certain disadvantages. For instance, the analysis time and labelling require a significant amount of time, and complex instrumentation and/or qualified personnel are usually needed, which limit the application of these methods [12]. Hence, it is necessary to develop new detection techniques for the sensitive and early analysis of cancer biomarkers (typical of point-of-care cases).

In recent years, increasing attention has been paid to the fabrication of electrochemical immunosensors because they are cost effective, highly specific and sensitive; have fast detection and low manpower requirements; and utilize facile instrumentation [13, 14]. As nanoscience and nanotechnology have developed, nanomaterial-involved electrochemical immunosensors have been considered as potential devices for analysis. Substantial studies have been performed on the selective and sensitive detection of various proteins based on the fabrication of nanoparticle-involved electrochemical immunosensing platforms using combined electrochemical strategies that involve functional nanomaterials, including carbon nanostructures [12], graphene nanosheets [15], quantum dots [12, 16] and noble metal nanoparticles [17]. For a majority of the immunosensors that are based on multienzyme probes, the sensitivity of detection could be improved using enzyme-functionalized nanoparticles in which the enzyme loading causes an increase in the immunological reaction [18]. Unfortunately, the above probes have limited applications because they suffer from the disadvantages of enzyme leakage and denaturation [19]. In addition, they are costly and time-consuming in their fabrication and cleaning processes [20]. Hence, there have been many studies on the development of more cost-effective and facile sensing systems for specific and sensitive detection.

In recent years, there have been reports on catalytic systems in which Au–Ag nanoparticles are highly reactive and the reduction of hydrogen peroxide could be remarkably enhanced [21-23]. Similar to hydrogen peroxidase, Au–Ag nanoparticles exhibit excellent catalytic activity in hydrogen peroxide reduction, suggesting that they have potential to be used in the development of new immunosensors for the enzyme-free detection of biomarkers.

Based on these previous studies, this study presented a new enzyme-free electrochemical immunosensor for the detection of AFP. The fabrication of our proposed immunosensor involved Au–Ag nanoparticles (instead of traditional hydrogen peroxidase) in a sandwich-like system, and amplification of the signal was achieved by incorporating catalytic Au–Ag nanoparticles. Using the amidation of graphene-containing carboxylic groups and the remaining amino groups of AFP, the selected AFP primary antibody was attached onto graphene sheets. This work aimed to confirm the reliable, fast and enzyme-free determination of AFP based on this new, facile immunosensor.

2. EXPERIMENTS

2.1. Chemicals

AFP and anti-AFP were purchased from Wang Er Biochemical Reagents (Beijing, China). $HAuCl_4 \cdot 4H_2O$ and $AgNO_3$ were obtained from Shanghai Chemical Reagents Co. All other reagents were of analytical grade and used as received.

2.2. Fabrication of colloidal Au–Ag alloy NPs

The following procedures describe the preparation of the Au–Ag alloy NPs. Prior to reaction, the culture medium was prepared by boiling and subsequently cooling 40 mL of a sucrose solution (50 mg/mL) to 30 °C. After the addition of instant dry yeast (600 mg), the obtained mixture was maintained at 30 °C for 24 h to cultivate the yeast cells. The as-prepared yeast cells were then centrifuged for 3 min at 2500 rpm and collected followed by three cycles of washing with sterile water and re-suspension in sterile water at a concentration of 10^6 cells/mL. This yeast cell solution (3 mL) was then treated with $AgNO_3$ and $HAuCl_4$ solutions of a given volume to achieve a final concentration of 1.0 mg/mL. After sealing, the as-prepared mixture was kept below 30 °C for 24 h to obtain the colloidal Au–Ag alloy NPs.

2.3. Fabrication of the immunosensor

The immunosensor was prepared by first immobilizing a primary antibody (Ab_1, 5 μL) on a glassy carbon electrode (GCE) (denoted GCE/Ab1). After blocking the nonspecific interaction with 1 wt% BSA (denoted GCE/Ab_1/BSA), varying concentrations of AFP were introduced to the as-prepared GCE and reacted for 3 h. This was followed by dropping the obtained Au–Pd–Ab_2 solution onto the surface of the electrode (denoted GCE/Ab1/BSA/antigen/Au–Ag–Ab_2), which was then allowed to react for another 1 h. Finally, the electrodes were washed and cleaned before use.

2.4. Instruments

An electrochemical workstation (CHI 760D, China) was used throughout the electrochemical experiments. An IM6e workstation from ZAHNER Elektrik (Germany) was used for the electrochemical impedance spectra (EIS) characterization. EDX was collected on an SEM (Agilent 8500 FE-SEM) coupled to an EDX analyser. Optical characterization was performed on a UV-vis spectrophotometer in the wavelength range 350 to 700 nm. X-ray photoelectron spectroscopy (XPS) was performed on a Kratos AXIS Nova (UK) spectrometer. A JSM-6700F microscope (Japan) was used to record SEM images. A traditional triple-electrode configuration was used in which the working, counter and reference electrodes were a 4 mm GCE, Pt and Ag/AgCl, respectively.

3. RESULTS AND DISCUSSION

The Au–Ag NPs on the bare GCE was characterized via the EDX (Fig. 1A), and the primary elements on the electrode surface were Ag, Au and C, suggesting the co-existence of Ag and Au in the specimen. The Au–Ag alloy NP suspension with varying reaction time was characterized by collecting UV–vis absorption spectra (Fig. 1B). A gradual shift in the absorption peak between 541 and 445 nm was observed with varying reaction time, which is associated with an increase in the Ag mole fraction. All peaks were observed in the range 545 nm - 413 nm, with only one adsorption peak recorded.

As indicated in Fig. 1C and D, a drop-coated film of the Au–Ag alloy NPs was chemically analysed via XPS measurement. Two spin–orbit components were observed in the Ag 3d spectrum (Fig. 1D). The Ag $3d_{3/2}$ and $3d_{5/2}$ peaks were observed at a BE of 373.4 and 367.4 eV, respectively. On the other hand, two spin–orbit components were recorded in the Au 4f spectrum (Fig. 1C). The Au $4f_{5/2}$ and $4f_{7/2}$ peaks were observed at binding energies (BE) of 87.7 and 84.1 eV, respectively. Thus, Ag and Au existed in only one form in solution, Ag^0 and Au^0. These XPS results indicated the reduction of all silver and gold ions during the preparation of the nanoparticles. In addition, these ions all existed in the metallic form. As predicted, the immunosensor achieved a sandwich-like structure in which catalytic Au–Ag nanoparticles and highly conductive graphene sheets were combined for signal amplification [24].

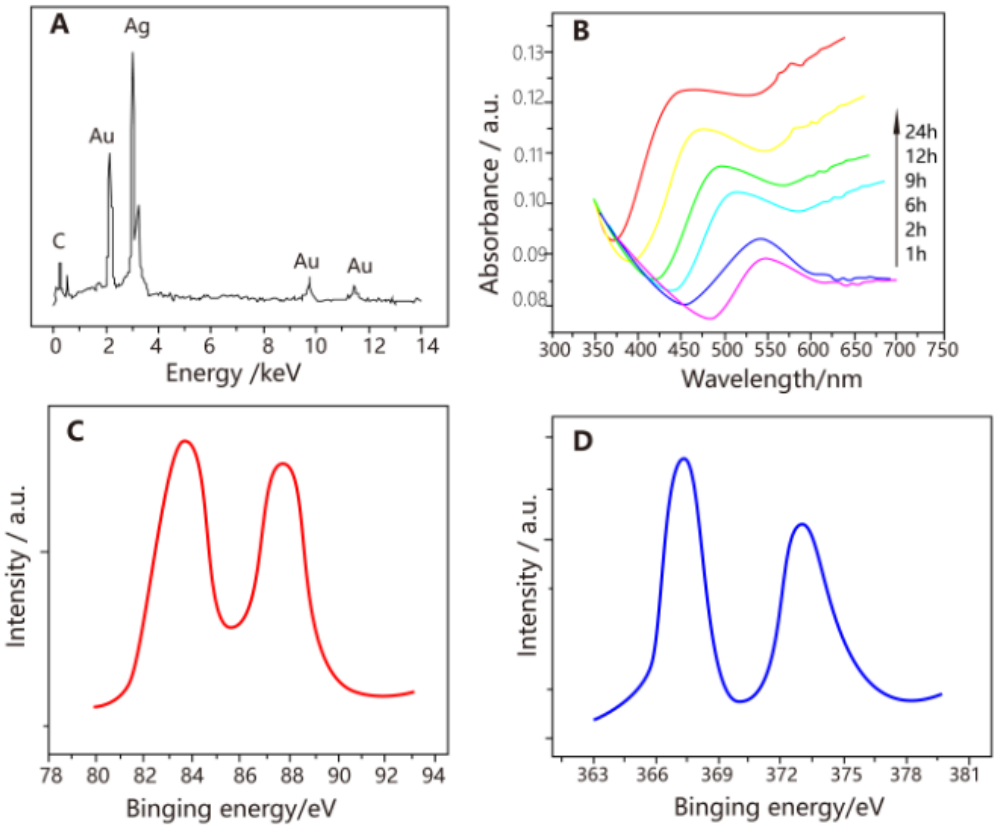

Figure 1. (A) EDX profile of the Au–Ag alloy nanoparticles on a GCE, (B) UV-vis absorption spectra of the Au–Ag alloy nanoparticles with varying reaction time, (C) Au 4f and (D) Ag 3d core-level spectra for a drop-coated Au–Ag alloy nanoparticle.

The obtained Au–Ag nanoparticles were applied to the catalysis of hydrogen peroxide reduction, as indicated in Fig. 2. We employed the Ab_2-anchored Au–Ag nanoparticles due to the comparable conditions created by anchoring the second antibody (Ab_2) on the surface of the Au–Ag nanoparticles with respect to our proposed immunosensor. The current peak of the Au–Ag nanoparticles that decorated electrode was observed to be strong. Nevertheless, this electrode showed no electroactivity to water under comparable conditions. The Au–Pd nanoparticles showed the ability to promote the reduction of hydrogen peroxide and therefore generated electrochemical signals [25].

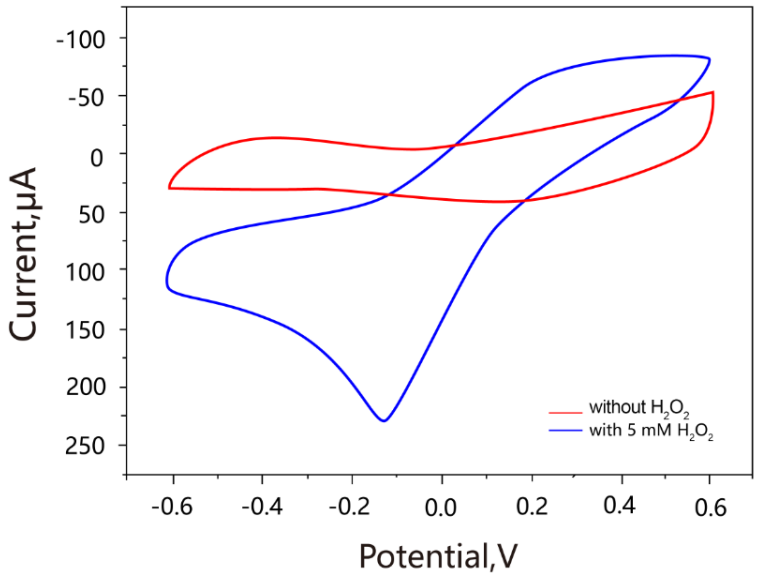

Figure 2. CVs of the Au–Ag nanoparticles on the decorated electrode in nitrogen-saturated PBS before and after the addition of H_2O_2 (5 mM).

Fig. 3 shows the EIS spectra of our proposed immunosensor in the process of preparation and assembly. It is known that changes in electrochemical impedance reflect the chemical processes occurring in electrochemical sensors [26]. The spectra were resolved into two sections, a semicircle section and a linear section, which correspond to the electrochemical reaction involving charge transfer and the diffusion-limited electrochemical performance. Note that the diameter of the semicircle section was associated with the charge transfer resistance. A comparative study was achieved by examining each step of the immunosensor preparation, including its stepwise assembly and coating. A seemingly linear GCE was observed in the Nyquist profiles. The aforementioned semicircle sections were enhanced after the electrode was coated with the antigens BSA and Ab_1, which became clearer upon further incubation with Au–Ag–Ab_2. This result suggested that the aforementioned electrodes were successfully modified. Charge transfer was impeded after access to the electrode surface was decreased by the Ab_2, antigen, BSA, and Ab_1 coatings. Electron transfer decreased with consecutive incubations with Ab_1, BSA, antigen and Ab_2. It is therefore clear that the assembly of these biomolecules on the surface of the electrode to form an immunosensor was successful [27].

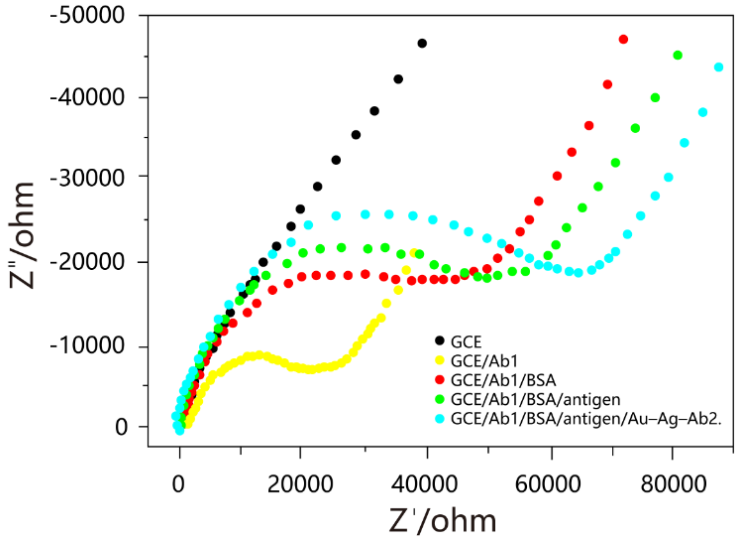

Figure 3. EIS for the response of our proposed immunosensor to AFP (10 ng/mL) at 0.1 - 10^5 Hz for the bare GCE, GCE/Ab_1, GCE/Ab_1/BSA, GCE/Ab_1/BSA/antigen and GCE/Ab_1/BSA/antigen/Au–Ag–Ab_2 in pH 7.4 PBS + 1 mM $K_3Fe(CN)_6$.

The determination and investigation of AFP (0.05 - 30 ng/mL) with our proposed immunosensors were characterized via Fig. 4. As the concentration of AFP increased, an increase in the amperometric signal was observed. The linear range for our proposed immunosensor was as broad as 0.05–30 ng/mL. Compared with other electrochemical immunoassays, our proposed immunosensors exhibited a low limit of detection (LOD) of 0.007 ng/mL, which was calculated as the ratio of signal to noise, revealing desirable sensitivity [28-32]. The calibration curve was found to exhibit a linear relationship, having a correlation coefficient of 0.997. Hence, our proposed immunosensor exhibited desirable sensitivity towards the detection of AFP. To allow for comparison to previous reports, the characteristics of different electrochemical sensors for AFP are summarized in Table 1.

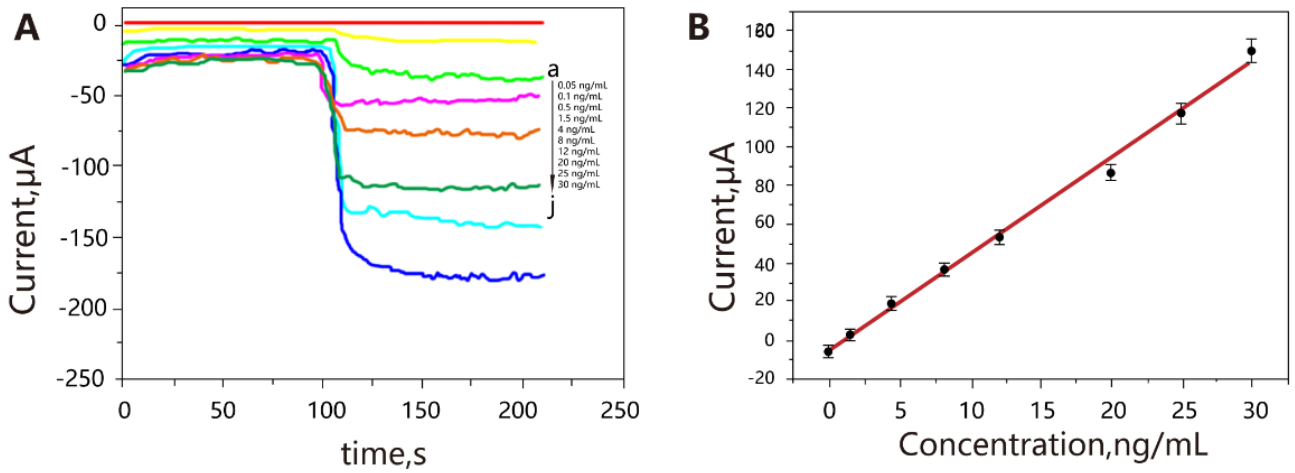

Figure 4. (A) Amperometric response of our proposed immunosensor to various concentrations of AFP in PBS containing 5 mM H_2O_2; the concentration of AFP was 0.05, 0.1, 0.5, 1.5, 4, 8, 12, 20, 25 and 30 ng/mL. (B) Calibration curve of our proposed immunosensor.

Table 1. Comparison of the major characteristics of electrochemical sensors used for the detection of AFP.

Electrode	Linear detection range	Detection limit	Reference
Au–PDA–THi–GO NCs	0.1 - 150 ng/mL	0.03 ng/mL	[33]
graphene/SnO_2/Au	0.02 - 50 ng/mL	0.01 ng/mL	[34]
Multi-walled carbon nanotubes/silica	0.1 - 30 ng/mL	0.018 ng/mL	[35]
GCE/Ab_1/BSA/antigen/Au–Ag–Ab_2	0.05 - 30 ng/mL	0.007 ng/mL	This work

We fabricated five electrodes under comparable conditions and used them to investigate the reproducibility with an AFP concentration of 2 ng/mL. The reproducibility was confirmed to be satisfactory as indicated by a standard deviation of 5.1% for the five investigations. Furthermore, 89% of the initial value was maintained after our proposed immunosensor was stored in pH 7.4 PBS at 4 °C for 8 weeks, as shown in the investigation of the stability of our proposed immunosensor. The sensor therefore has potential for application based on the desirable stability and reproducibility. As shown in the calibration profile, the practical determination of 4 serum specimens was performed. The standard ELISA technique was also used, and its performance was compared with that of our proposed immunosensor. It can be seen from Table 2 that the performance of our proposed immunosensor was comparable with the standard ELISA method with a relative error below 5.3%. This result also confirmed that the proposed immunosensor has potential for practical application.

Table 2. Determination of serum specimens with our proposed immunosensor and the standard ELISA technique (ng/mL).

Sample	1	2	3	4	5
The immunosensor	1.65	2.58	5.04	10.57	25.66
ELISA	1.69	2.60	4.95	10.51	26.07
RSD (%)	4.8	2.1	5.3	2.7	4.4

4. CONCLUSIONS

This work proposed the sensitive determination of AFP using a nonenzyme-based immunosensor. The determination of AFP using this developed immunosensor (where Au–Ag nanoparticles replaced the traditional enzyme) was found to be sensitive and reliable, with a linear range as broad as 0.05–30 ng/mL and an LOD as low as 0.007 ng/mL. Our proposed immunosensor was confirmed to be applicable based on the catalysis of hydrogen peroxide reduction by the Au–Ag nanoparticles along with the subsequent generation of a signal. The graphene sheet was highly conductive, leading to an increase in charge transfer and a further decrease in LOD and increase in sensitivity. Combined with the facile preparation, our proposed immunosensor is ideal for clinical study as a common method. In addition, with respect to the up-and-coming sensor-involved fields, the

strategies used for the preparation of our proposed immunosensors could be considered an excellent reference.

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