

Electrochemical sandwich immunoassay for human epididymis-specific protein 4 using a screen-printed electrode modified with graphene sheets and gold nanoparticles, and applying a modular magnetic detector device produced by 3D laser sintering

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Abstract The authors describe an electrochemical sandwich immunoassay for the human epididymis-specific protein 4 (HE4). A commercially available electromagnetic detector device and a screen-printed electrode (SPE) modified with graphene sheets and gold nanoparticles were used to fabricate the detector. First, a nanocomposite suspension consisting of graphene sheets and gold nanoparticles was deposited onto the SPE. This results in an enlarged electrochemically active area and improves electron transfer. Next, biotinylated monoclonal antibody against HE4 (anti-HE4) was bound to streptavidin-modified magnetic beads via biotin-avidin binding. Nonspecific binding sites were blocked with bovine serum albumin. Stepwise changes in the microscopic features of the surfaces and electrochemical properties upon the formation of each layer were studied by scanning electron microscopy, transmission electron microscopy and cyclic voltammetry. After magnetic separation and washing, the biotinylated anti-HE4 beads were deposited on the SPE, which then was inserted into the electromagnetic modular detector. Following the immunoreaction with HE4, a sandwich-type immunoassay

was performed on the SPE using horseradish peroxidase (HRP)-labeled HE4 as a tracer label. Electrochemical detection was carried out after addition of H₂O₂ as a substrate for HRP. Under optimal conditions, differential pulse voltammetry can detect HE4 in the 0 to 400 pM concentration range with a detection limit of 0.5 pM (at an S/N ratio of 3). The system was applied to the determination of HE4 with good accuracy and selectivity.

Keywords Electromagnetic detection · Magnetic beads · HE4 · Scanning electron microscopy · Differential pulse voltammetry · Ovarian cancer

Introduction

Ovarian cancer is the sixth most common cancer among women in the European Union, with an incidence of 18 per 100,000 women; this cancer also represents the first and third most common causes of death from gynecological cancer and oncologic diseases, respectively [1–3]. Presurgical differentiation of benign and malignant pelvic mass and early stage intervention play important roles in improving the survival rate of ovarian cancer patients. Human epididymis protein 4 (HE4), a new ovarian biomarker initially identified in the epithelium of the distal epididymis, is a new tumor marker developed to improve ovarian carcinoma diagnosis [4–8]. It is important to highlight that HE4 is not only expressed in the early stages of the disease, but also an early indicator of disease recurrence. The American Food and Drug Administration recently approved the use of HE4 for monitoring the recurrence or progression of epithelial ovarian cancer [9]. Therefore,

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determination of HE4 plays an important role in early clinical diagnosis of ovarian cancer.

Compared with the traditional detection method of disease biomarkers, immunosensor shows more sensitivity and convenience [10–12]. Antibody immobilization is a key step of immunosensor fabrication because the captured amount of antibody labeled by nanomaterials often influences not only the immunoassay sensitivity but also the reliability of the immunosensor [13]. Nanomaterials are frequently used to fabricate various bioelectronic devices because of their distinct properties, which include rapid electron transportation, high conductivity, and thermal stability. Au nanoparticles (AuNPs) are the most promising nanomaterials widely used for immobilization of antibody owing to their large surface-to-bulk ratio and good biocompatibilities [14, 15]. AuNPs can not only firmly adsorb antibodies and enzymes but also retain their biological activity. Graphene sheets (GS), which are a flat monolayer of carbon atoms closely packed into a 2D honeycomb lattice, exhibit large surface area and high conductivity [16–18]. Immunofunctionalized magnetic beads have drawn extensive attention because of the excellent properties, which include specific preconcentration of diluted target biomolecules and separation of the desired constituents from complex sample matrices. More importantly, biotinylated monoclonal antibody can be firmly absorbed onto streptavidin-modified magnetic beads (MBs) by a biotin–avidin system. Herrasti et al. [19] developed an immunosensor based on magnetic microparticles and disposable carbon screen-printed electrodes.

Our group recently developed an ultrasensitive electrochemical method to detect HE4 based on rolling circle amplification. However, complicated procedures and strict experimental conditions were necessary to support the sensitivity and reproducibility of this method [20]. To develop a convenient electrochemical method for HE4 using simple procedure, a modular detector device produced via 3D laser sintering is introduced. This device consists of a SPE, a magnet switch, and electrical connectors. By turning the magnet switch on, biotinylated HE4 monoclonal antibody (anti-HE4) and streptavidin-modified MBs could be confined over the working electrode by a magnetic and biotin–avidin system. Use of the streptavidin-modified MBs as a signal amplification device offers a large ratio of antigen–antibody binding sites to HE4 and increased sensitivity. Barallat et al. [21] successfully used a customized modular detector device produced by 3D laser sintering based on immobilization of anti-HE4 antibodies onto MBs to detect myeloperoxidase in human plasma.

We developed an electrochemical immunosensor based on an AuNP–GS nanocomposite suspension-modified SPE and an electromagnetic modular detector device. AuNPs and GS-modified SPEs were designed to develop immunosensors with enhanced electrochemical active area and improved electronic transfer properties. Biotinylated HE4 antibody was

immobilized onto the SPE by a biotin–avidin system, an electromagnetic modular detector device, and streptavidin-modified MBs. A sandwich-type HE4 antigen and antibody immunoreaction was performed. The electrochemical signal of H_2O_2 catalyzed by HRP was used to evaluate HE4 levels. The electrochemical immunoassay exhibited high sensitivity and selectivity when it was used to determine HE4.

Experimental

Reagents and materials

Streptavidin-modified magnetic beads (Dynabeads M-280 streptavidin, $2.8 \mu\text{m}$ ϕ , 10 mg mL^{-1}) were supplied by Invitrogen (Life Technologies, U.K., <http://www.thermofisher.com>). GS were purchased from Pioneer Nanotechnology Co. (Nanjing, China, <http://graphene.cn.china.cn>). Anti-HE4, chloroauric acid, sodium citrate, and bovine serum albumin (BSA, 96–99 %) were obtained from Sigma (St. Louis, MO, USA, <http://www.sigmaaldrich.com>). Streptavidin was obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (China, <http://www.sangon.com>). An HE4 ELISA kit was obtained from Fujirebio Diagnostics Inc. (Malvern, PA, <http://www.fdi.com>). *N,N*-dimethylformamide (DMF) was purchased from Shanghai Sinopharm Group Chemical Reagent Co., Ltd. (Shanghai, China). Standard HE4 was diluted into different concentrations by phosphate-buffered saline (PBS). PBS at various pH (4.5–7.5) were prepared using $0.1 \text{ M NaH}_2\text{PO}_4$, $0.1 \text{ M Na}_2\text{HPO}_4$, and 0.9% NaCl. Double distilled water from a Milli-Q system (Millipore, Billerica, MA) was used throughout this study. All reagents were of analytical grade unless otherwise stated.

Apparatus

A CHI 6043D workstation (Shanghai Chenhua Instruments, China, <http://www.instrument.com.cn>) was employed in all electrochemical measurements. The modular detector device and SPE, which were supplied by DropSens (DropSens, Oviedo, Spain, <http://www.dropsens.com>). The magnetic switch holds a neodymium magnet 1.5 mm in diameter. The SPE consisted of a carbon working electrode, a carbon counter electrode and a silver reference electrode. Electrochemical characterizations and measurements on the modified electrode were carried out by using cyclic voltammetry from -0.2 to 0.6 V (versus Ag/AgCl) in 0.1 M PBS (pH 7.0) containing 0.1 M KCl and $5 \text{ mM Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$. Scanning electron microscopy (SEM) was carried out using a FEI Nova-400 SEM (FEI Nova-400, USA). Transmission electron microscopy (TEM) was carried out using a Tecnai G2 F30 S-Twin (Philips-FEI, Netherland).

Preparation of AuNPs and AuNP–GS nanocomposite suspension

The AuNPs were prepared according to Frens's method [22]. 1 mL of 1.0 wt.% HAuCl₄ solution was initially added to 99 mL water and then heated until boiling. 2.5 mL of 1.0 wt.% sodium citrate solution was injected into the boiling solution quickly. The mixture was heated for 15 min continuously until the solution color became claret. The obtained gold colloids were cooled at room temperature and stored at 4 °C when not in use.

About 0.5 mg of GS was dispersed in 4 mL N, N-dimethylformamide (DMF) containing 1 mL as-prepared AuNPs solution. After sonication for 2 h, well-dispersed GS–AuNP nanocomposite suspensions were obtained; these suspensions were kept at 4 °C until use.

Preparation of the magnetic beads -biotinylated anti-HE4 conjugates

MBs-biotinylated anti-HE4 conjugates were prepared according to the literature [10]. Approximately 5 μL of the MBs suspension was transferred into a 1.5 mL plastic tube, followed by two cycles of washing with 100 μL of PBS. Afterward, the sodium azide preservative was removed. The MBs were resuspended in 50 μL of a 50 μg mL⁻¹ biotinylated anti-HE4 solution in PBS, and the resulting mixture was shaken at 37 °C for 30 min at 600 rpm. The streptavidin-modified MBs and biotinylated anti-HE4 were firmly conjugated by a biotin–avidin system. Subsequently, the tube was placed in the magnetic separator, the supernatant was removed, and the beads were washed two times with PBST. Finally, the prepared MBs-biotinylated anti-HE4 conjugates were resuspended in 50 μL of PBS and stored at 4 °C for further use.

Immunosensor assembly and electrochemical detection of the HE4

A nitrogen gas environment was maintained on the electrode surface before electrode modification during the reaction to prevent critical oxidation. About 5 μL of the GS–AuNP

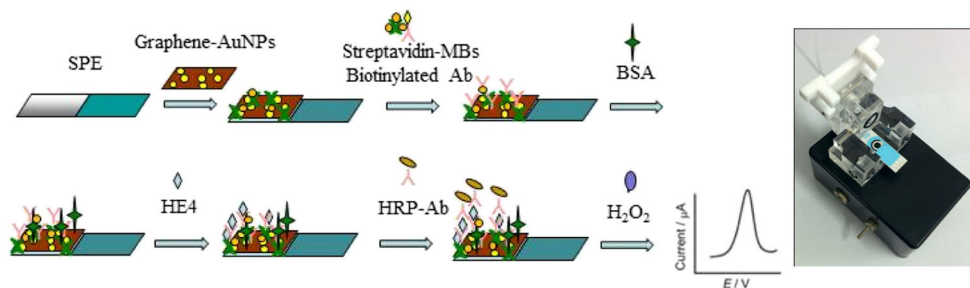
nanocomposite suspension was coated onto the screen-printed carbon electrode, and the electrode was kept at room temperature to dry. Approximately 5 μL of streptavidin-modified MBs-biotinylated anti-HE4 conjugates was dropped onto SPE. Turning on the electromagnetic switch of the modular detector device, the streptavidin-modified MBs-biotinylated anti-HE4 was firmly immobilized on the SPE. To retain the consistence of the electrode modification from batch to batch, we set the binding time at 5 min. After that, the electrodes were rinsed with PBS thoroughly to remove the possible nonspecific binding. The modified electrode was then incubated into 2.5 wt.% BSA–PBS (pH 7.0) solution for 60 min at room temperature to block the remaining active sites of AuNPs. Electrochemical measurements of the immunosensor toward the HE4 antigen were carried out using a sandwich-type immunoassay with HRP labeled anti-HE4 and H₂O₂ as trace label and enzyme substrate, respectively. Different concentrations of the standard HE4 antigen were incubated with the prepared immunosensor. After each step, the electrodes were rinsed with PBS thoroughly. Finally, about 20 μL PBS solution containing of 0.75 mM H₂O₂ was dropped onto the electrode surface of the SPE. The electrochemical signal of H₂O₂ catalyzed by HRP was measured by differential pulse voltammetry (DPV) to evaluate the HE4 level. The as-prepared immunosensor was stored at 4 °C when not in use. Scheme 1 schematically illustrates the fabrication procedure of the immunosensor.

Results and discussion

SEM and TEM characterization of different modified SPEs

The surface morphologies of different modified SPEs were studied by SEM. As shown in Fig. 1a, the SPE surface was mostly covered with homogenous GS. Figure 1b shows many spherical AuNPs on the surface of the GS film, thus confirming a homogenous dispersion AuNP–GS nanocomposition film on the electrode surface. Figure 1c shows individual AuNPs. The mean diameter of AuNP was about 20 nm estimated from TEM.

Scheme 1 Stepwise fabrication of the immunosensor; Inset: the image of the magnetic modular detector device



Cyclic voltammetric characterization of different modified SPEs

The electrochemical behaviors of the different modified SPEs were investigated by cyclic voltammetry. Figure 2a shows a pair of well-defined peaks for the bare SPE, which reflect the reversible redox reaction of ferricyanide ions on the electrode. After GS-AuNP nanocomposite suspension was coated onto

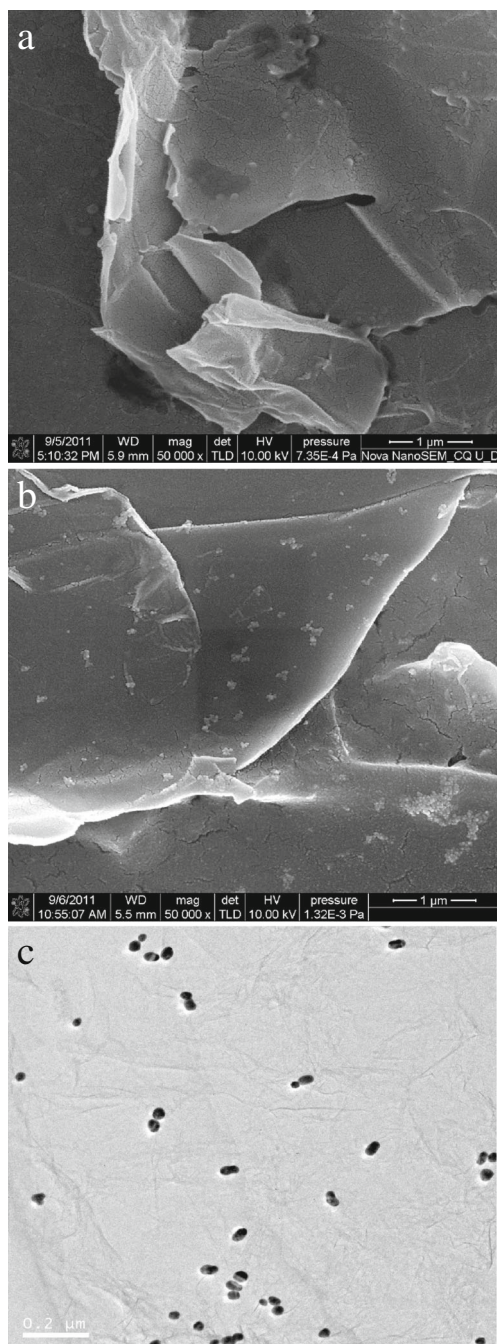


Fig. 1 **a** The SEM image of SPE electrode covered with GS; **b** The SEM image of SPE electrode covered with GS-AuNP composite film; **c** The TEM image of GS covered with AuNPs

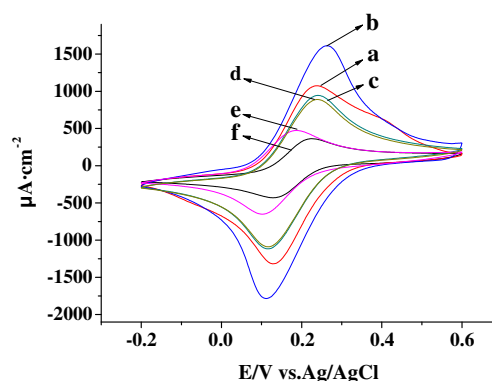


Fig. 2 CVs of different electrodes in 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (pH 7.0) solution containing 0.1 M KCl at a scan rate of 50 mV s^{-1} : **a** bare SPE; **b** GS-AuNP/SPE; **c** Biotinylated Ab1/GS-AuNP/SPE; **d** BSA/Biotinylated Ab1/GS-AuNP/SPE; **e** HE4/BSA/Biotinylated Ab1/GS-AuNP/SPE; **f** HRP Ab2/HE4/BSA/Biotinylated Ab1/GS-AuNP/SPE

the SPE, an obvious increased redox peak can be observed, as shown in Fig. 2b; this figure demonstrates the efficient electroactive performance and conductivity of GS-AuNP. When the MBs-biotinylated anti-HE4 was immobilized on the surface of GS-AuNP/SPE, the peak currents of $[Fe(CN)_6]^{3-/4-}$ decreased (Fig. 2c). Anti-HE4 is a type of protein that hinders electron transfer to a certain extent. After the remaining active sites of the nano-Au were blocked with BSA, the peak current further decreased in the same manner (Fig. 2d). After successively immobilizing HE4 (Fig. 2e) and HRP-labeled HE4 antibodies (Fig. 2f) onto the electrode surface, the peak currents further decreased. This observation indicated that formation of the antigen–antibody complex results in a significant decrease in the background current.

Comparison of this protocol with control experiment

In order to make a contrast of this immunosensor, an immunoassay without the addition of H_2O_2 was performed to prove the effect of H_2O_2 . Figure 3 shows that when detecting the

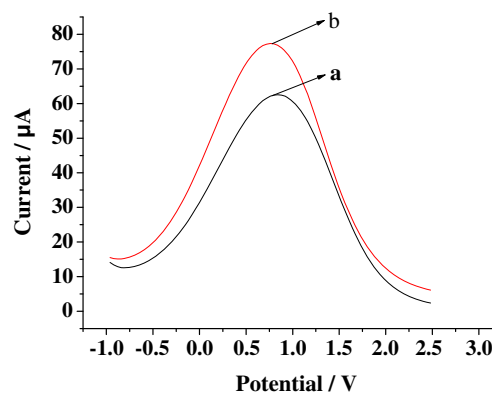


Fig. 3 DPVs response of this immunoassay when detecting 200 pM HE4 in 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (pH 7.0) solution containing 0.1 M KCl without (**a**) and with (**b**) the addition of 0.75 mM H_2O_2

same concentration of HE4 (200 pM), the peak current of the developed immunoassay (curve b) was larger than that of the immunoassay without the addition of H_2O_2 (curve a). Consequently, the detection signal was greatly increased. The reason can be explained as follows: The immunocomplex hydrophobic protein layer inhibited the electron transfer of the electrochemical probe (curve a). After adding 0.75 mM H_2O_2 , an obvious catalytic characteristic of detection appeared with a dramatically increased oxidation current (curve b). The function of HRP-catalyzed oxidation of H_2O_2 resists that of the inhibition of the immunocomplex hydrophobic protein layer towards the electron transfer.

Optimization of the experimental parameters of the immunosensor

To select the optimum concentration of anti-HE4 solution, the standard anti-HE4 was diluted into different concentrations. As the concentration of anti-HE4 solution increased, more HE4 can be immobilized on the electrode surface. When the anti-HE4 concentration reached $1 \mu\text{g mL}^{-1}$, the amount of HE4 immobilized on the electrode surface showed no further increases (Fig. S1A). Diffusion of ferricyanide toward the

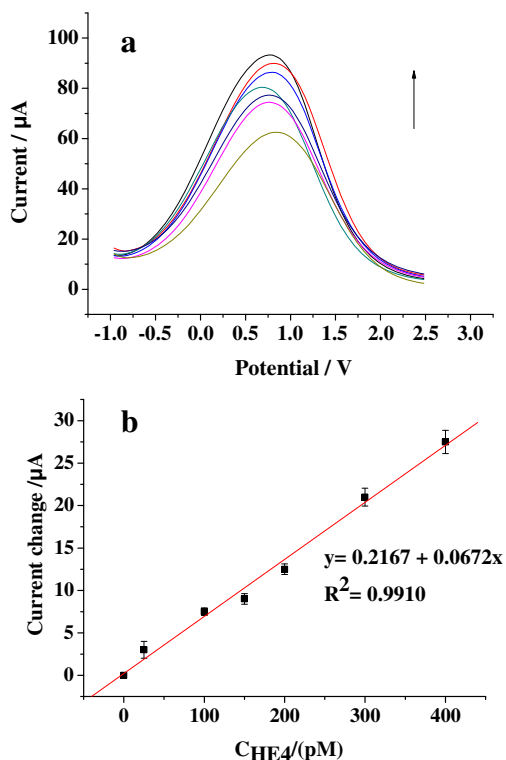


Fig. 4 **a** The DPV responses of different concentrations of HE4 determined by the developed immunosensor (from inner to outer: 0, 25, 100, 150, 200, 300, 400 pM) in 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (pH 7.0) solution containing 0.1 M KCl; **b** The calibration curve of the peak current response vs. logarithm of HE4 concentration with the developed immunoassay

Table 1 Specificity of the immunosensor

Sample	Current(μA)	$(i_b - i_a) \cdot 100 / i_a$ (%)
HE4	85	–
HE4(CA125)	80	–5.9
HE4(CA242)	90	5.9
HE4(CEA)	89	4.7
HE4(AFP)	87	2.4

ia: Amperometric response to the standard 100 pM HE4 solution

ib: Amperometric response to the solutions composed of 100 pM HE4 solution and 100 pM interference respectively

electrode surface was considerably hindered, causing the most obvious current change. Thus, we selected $1 \mu\text{g mL}^{-1}$ as the optimum concentration of anti-HE4 solution. The effect of pH of the wash buffer solution on the current responses of the fabricated immunosensor was also studied (Fig. S1B). Highly acidic or alkaline surroundings can damage the bioactivity of the immobilized antibody. The current change increased with the increasing pH from 5.0 to 7.0 and then decreased. When the pH of the buffer solution was adjusted to 7, the maximum amount of the anti-HE4/HE4 immunocomplex was obtained. Therefore, we established the optimum pH to be 7.0. The influence of the antigen–antibody incubation temperature on the amperometric response was also studied. When the temperature was set at 37°C , we obtained the most obvious current change (Fig. S1C). Hence, the optimal temperature was 37°C .

Amperometric response and calibration curve

Under optimized experimental conditions, different concentrations of the HE4 antigen standard solution were incubated with the immunosensor. The detection principle was based on the change of oxidation peak current response (ΔI_{pa}) before and after the antibody–antigen reaction, which was evaluated as the following equation: $\Delta I_{pa} = I_n - I_0$, where I_0 was the response current before the immunoreaction and I_n was the response current after the immunoreaction. As shown in Fig. 4a, the peak currents gradually decreased with the

Table 2 Recovery of the prepared immunosensor

Sample	Added(pM)	Founded(pM) (mean \pm RSD, $n = 3$)	Recovery(%)
1	25.0	24.8 ± 2.60	99.2
2	50.0	50.4 ± 1.58	100.8
3	75.0	71.9 ± 0.51	95.9
4	100.0	102.0 ± 11.60	100.2
5	150.0	146.0 ± 9.90	97.3

incubation amount of HE4. The electrochemical signal was recorded by differential pulse voltammetry (DPV). Seen from Fig. 4b, the current changes (before and after the HE4 incubation) were proportional to HE4 concentrations. The immunosensor showed broad linear responses to the HE4 concentration within the range of 0–400 pM with a detection limit of 0.5 pM. The regression equation was $\Delta I (\mu\text{A}) = 0.2167 + 0.0672 \log C_{\text{HE4}} (\text{pM})$, $R^2 = 0.9910$. According to Radi's method [23], the estimated limit of detection (defined as $\text{LOD} = 3S_B/m$, where m is the slope of the corresponding calibration curve, and S_B is the standard deviation of the blank, $n = 10$) was 0.5 pM.

Reproducibility and specificity

The repeatability and reproducibility of the developed immunoassay were investigated by the variation coefficients (CVs) of intra- and inter-assays. By making 5 successive measurements of three HE4 concentration levels using the same immunosensor, the CVs of the intra-assay were determined as 4.3, 5.6, and 6.2 % at 50, 100, and 150 pM HE4, respectively. Similarly, the inter-assay CVs of five immunosensors independently used were 2.8, 3.1, and 6.7 % at 50, 100, and 150 pM HE4, respectively. These results confirmed that the immunosensor exhibited acceptable precision and fabrication reproducibility.

To investigate the sensor's specificity, the immunosensor was incubated with a 100 pM HE4 solution containing one of the following interferences: CA125 (100 pM), CA242 (100 pM), CEA (100 pM), and AFP (100 pM). The results are shown in Table 1. This result indicates that the selectivity of the immunosensor based on the highly specific antigen–antibody immunoreaction is satisfactory.

The recovery of the prepared immunosensor

To investigate the accuracy of the immunosensor, a recovery test was carried out. The samples were prepared using

standard HE4 solution. The immunosensor was incubated with five different HE4 concentrations (25, 50, 75, 100, and 150 pM) in the working buffer for 20 min each. Each solution was detected three times per run and the experimental results are listed in Table 2. The recovery was within the range of 95.9–100.8 %, which indicates that the immunosensor is applicable for HE4 detection in the working buffer.

The analytical performance of our immunosensor was compared with those reported previously in Table 3. It was clear that the developed immunosensor displayed an accepted performance compared with other methods in analytical range and detection limit.

Conclusion

An electrochemical immunosensor based on an AuNP–GS nanocomposite suspension-modified SPE and an electromagnetic modular detector device was reported. In contrast to our previous study, this work does not require complicated conjugation chemistry or cascading signal amplification. The application of screen-printed electrodes and the electromagnetic modular detector device improve the convenience and precision of HE4 determination by the immunosensor. The streptavidin-modified MBs absorb more biotinylated anti-HE4 via the magnetic and biotin-avidin system. This results in a large number of antigen-antibody binding sites and increases the sensitivity of the assay. In contrast to the characteristics of previous well-established methods, this immunosensor showed a broad dynamic range and acceptable reproducibility. When the concentration of HE4 is above 400 pM, it needs to be diluted. Furthermore, the immunosensor exhibited the best performance only if the immunosensor was under the optimal detection conditions (pH, temperature, etc.). The HE4 electrochemical immunosensor allows identification of patients at risk of ovarian cancer, thereby providing an approach for early diagnosis in daily clinical practice.

Table 3 Compare of merit of recently reported methods for determination of human epididymis-specific protein 4

Method	Analytical range	Detection limit	Reference
ECL ^a	15.0–1500 pM	–	[24]
SERS ^b	–	21 fM	[25]
Microfluidic Platform	0–200 pM	2.3 pM	[26]
Electrochemical immunosensor (based on ELISA ^c)	23 fM–400 pM	6.8 fM	[27]
Electrochemical immunosensor (based on chitosan-TiC nanocomposite suspension)	3–300 pM	0.06 pM	[20]
Electrochemical immunosensor (based on AuNP–GS nanocomposite suspension)	0–400 pM	0.5 pM	This study

^a Electrochemiluminescence immunoassay

^b Plasmon length-based surface enhanced Raman scattering

^c Enzyme linked immunosorbent assay

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