An electrochemical immunosensor comprising thionin/silver nanoparticles decorated KIT-6 for ultrasensitive detection of squamous cell carcinoma antigen

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An ultrasensitive electrochemical immunosensor is designed and constructed for the quantitative detection of squamous cell carcinoma antigens (SCCAs). Silver nanoparticles (AgNPs) hybridized and 3-aminopropyltriethoxysilane functionalized graphene sheets (Ag@APTES-GS) serve as the platform to immobilize primary antibodies (Ab1). The mesoporous AgNPs-decorated silica KIT-6 (Ag@KIT-6) with a large specific surface area is utilized to label the electron mediator thionin (TH) and secondary antibodies (Ab2) by physical adsorption and chemical bonding. Carboxymethyl chitosan (CMC) doped with ionic liquids (ILs) is used to facilitate electron transfer and prevent leaking of TH resulting in multiple signal amplification. In addition to the excellent detection limit (17 fg mL⁻¹, S/N = 3) and wide linear range (5 × 10⁻⁵–10⁷ ng mL⁻¹), the immunosensor shows good selectivity, reproducibility, and stability. Providing quantitative detection of SCCA in serum, the sensor has great potential in clinical and diagnostic applications.

1. Introduction

Cervical cancer is second to breast cancer in occurrence in women and poses a serious health threat. The squamous cell carcinoma antigen (SCCA) is a specific antigen for diagnosis and monitoring of squamous cell carcinomas in the cervix. SCCA is a sub-fraction of the tumor antigen TA-4 and belongs to the ovalbumin family of serine proteinase inhibitors, a glycoprotein with isoforms ranging from 45 to 55 kDa. The serum levels of SCCA have been observed to increase as tumors grow and compared to normal women, 98% of women with cervical cancer show the increase and so timely and accurate monitoring is crucial to diagnosis and treatment.

Analytical methods that have been developed for the determination of SCCA include the chemiluminescence immunoassay, enzyme-linked immune-sorbent assay, radio-immunoassay, and electrochemical immunosensing. Among these methods, electrochemical immunosensing based on the highly-biologically-specific recognition interactions between antigens and the corresponding antibodies is suitable for the quantitative detection of tumor makers because of the high sensitivity, cost effectiveness, reliability, fast response, miniaturization, and disposable nature. In electrochemical immunosensors, material with good conductivity are critical to the success and different types of nanomaterials have been proposed for signal amplification. For example, Merkoçi et al. developed an electrochemical immunoassay using Ag nanoparticles as the label for detection of SCCA and Zhou et al. used nanosilver-doped bovine serum albumin microspheres with a large ratio of horseradish peroxidase as labels for quantitative monitoring of SCCA achieving a detection limit of 5.0 pg mL⁻¹.

Silver nanoparticles (AgNPs), KIT-6, ionic liquids (ILs), graphene sheets (GS), thionin (TH), and carboxymethyl chitosan (CMC) all have unique properties. AgNPs have excellent electron transfer properties facilitate the process from the redox center to the electrode surface. KIT-6 is a mesoporous silica material with large and tunable pores, thick pore walls, high hydrothermal stability, large pore volume, as well as a bi-continuous cubic structure with the iα3d symmetry in the interpenetrating cylindrical porous system. The unique three-dimensional (3D) channels constitute an open porous platform enabling easy and direct access of guest species thereby facilitating diffusion through the channels and mitigating pore blockage. In addition, KIT-6 with good adsorption properties is a promising carrier for loading of nanomaterials and envisioned to be a superior mesoporous structure containing 3D channels bonding well for dispersion of catalysts and diffusion of reactants. Ionic liquids (ILs) consist of ions in the liquid state at room temperature and have high thermal stability, negligible volatility, low toxicity, low melting temperature, high...
conductivity, and good electrochemical stability. ILs have been used to fabricate electrochemical biosensors and it has been observed that an enzyme can maintain the high activity and stability of the suitable ILs. Graphene sheets (GSs) comprise a single layer of carbon atoms in a hexagonal lattice and have been used in the fabrication of immunosensors because of their excellent electronic properties and large surface area. AgNPs decorated GS (Ag@GS) with good conductivity, biocompatibility, and large specific surface area have been prepared to immobilize the primary antibody (Ab1). The enhanced sensitivity arises from the large surface area of GS facilitating Ab1 loading. The high conductivity of GS promotes electron transfer and the high catalytic activity and large surface area of AgNPs accelerate the reduction of H2O2. TH, a small planar molecule having two –NH2 groups symmetrically distributed on each side, has good electrochemical reversibility and stability as well as fast electron transfer ability. It has been introduced into Ag@KIT-6 to produce electrochemical signals. In addition, ILs doped CMC has been employed to prevent leaking of TH to enhance electron transfer. Therefore, signal amplification and high sensitivity can in principle be achieved by using TH/Ag@KIT-6/CMC/ILs as the transducing materials in electrochemical immunosensors for the quantitative detection of SCCA in human serum.

2. Materials and methods

2.1. Reagents and apparatus

2.1.1 Reagents. Anti-SCCA and SCCA were purchased from Shanghai Line-Bio Science Co., Ltd., China and KIT-6 was obtained from Nanjing XF NANO Co., Ltd., China. TH was bought from Sinopharm Chemical Reagent Co., Ltd. in Shanghai, China and 1-(3-dimethylamino)-propyl)-3-ethylcarbodiimide hydrochloride (EDC, 98.5%), N-hydroxy-succinimide (NHS, 98%) and 3-aminopropyltriethoxysilane (APTES, 98%) were purchased from 3-aminopropyltriethoxysilane (APTES, 98%) was purchased from Shanghai Aladdin Chemistry Co., Ltd. in China. 1-Butyl-pyridine tetrafluoroborate (>99%) was purchased from Lanzhou Institute of Chemical Physics and CMC was obtained from Nantong Lvshen Bioengineering Co., Ltd., China. The phosphate buffered saline (PBS, 0.1 mol L⁻¹ Na2HPO4 and KH2PO4) was used as an electrolyte in the electrochemical measurement. All other reagents were of analytical grade and ultrapure water (18.25 MΩ cm, 24 °C) was used throughout the experiments.

2.1.2 Materials characterization. Transmission electron microscopy (TEM) was conducted on the Hitachi H-600 microscope (Japan), scanning electron microscopy (SEM) was carried out on the Hitachi H-600 microscope (Japan), scanning electron microscopy (SEM) was conducted on the Quanta FEG250 field-emission environmental SEM (FEI, United States) at 4 kV, and X-ray diffraction was performed on the INCA Energy (Oxford, England). The electrochemical measurements were performed on a CHI760D electrochemical workstation (Chenhua Instrument Shanghai Co., Ltd., China).

2.2. Preparation of the Ag@GS

The APTES modified graphene sheet (APTES-GS) was synthesized by a modified method according to the previous report. In brief, 10 mL of ethanol containing 0.1 g of graphene oxide (GO) were mixed with 0.2 mL of APTES at 70 °C for 1.5 h and then 0.1 mL of 80 wt% hydrazine hydrate was added at 95 °C for another 1.5 h. The powdery APTES-GS was obtained after washing and drying in vacuum at 35 °C.

The AgNPs were prepared according to the published method. Briefly, AgNO3 (1 mL, 50 mmol L⁻¹) and trisodium citrate (1 mL, 5% (w/v)) were mixed with ultrapure water under vigorous stirring and 5 mg of NaBH4 were added to the solution. After stirring for 10 min, the mixture changed to brown-yellow indicative of the formation of AgNPs and the solution was vigorously stirred until the color did not change. The GS (10 mg) was dispersed in 10 mL of ultrapure water ultrasonically for 1 h and then introduced into 50 mL of the AgNPs solution under stirring. The Ag@GS was obtained by mild centrifugation and vacuum drying at 35 °C.

2.3. Preparation of the Ag@KIT-6

The 3-aminopropyl-functionalized KIT-6 (NH2-KIT-6) was synthesized by a modified method according to the literature. Briefly, 0.1 g of KIT-6 was dispersed in 10 mL of anhydrous toluene with 0.1 mL of APTES at 70 °C for 1.5 h. The powders were obtained by mild centrifugation and drying at 110 °C. The NH2-KIT-6 contained amino groups (–NH3) based on the ninhydrin test. Ultrapure water (10 mL) containing 10 mg of NH2-KIT-6 were treated ultrasonically for 1 h to disperse the materials and then introduced to 40 mL of the AgNPs solution under stirring. The Ag@KIT-6 was obtained by mild centrifugation and vacuum drying at 35 °C.

2.4. Preparation of the TH/Ag@KIT-6/CMC/ILs-Ab2 labels

Fig. 1A shows the preparation procedures of the TH/Ag@KIT-6/CMC/ILs-Ab2 labels. Ab2 (1 mL, 10 μg mL⁻¹) was added to 1 mL (1 mg mL⁻¹) of the TH/Ag@KIT-6 solution and stirred for 12 h at 4 °C. After centrifugation, 1 mL of EDC/NHS (10 mmol L⁻¹/2 mmol L⁻¹) and 1 mL of the TH solution (2 mg mL⁻¹) were added to obtain the precipitate and also stirred for 12 h at 4 °C. After centrifugation, 0.5 mL of 1-butyl-pyridine tetrafluoroborate (10 mg mL⁻¹) as a kind of ILs dissolved in 1 wt% CMC were added to the mixture and stirred for 1 h at 4 °C. After centrifugation, the TH/Ag@KIT-6/CMC/ILs-Ab2 labels were dispersed in 0.5 mL of PBS at pH 7.4 and stored at 4 °C.

2.5. Fabrication of the immunosensor

The schematic diagram of the stepwise self-assembly procedures to prepare the sandwich-type immunosensor is illustrated in Fig. 1B. A glassy carbon electrode (GCE, 4 mm) was polished with 1.0, 0.3, and 0.05 μm alumina (Al2O3) powders sequentially and cleaned thoroughly before used. 6 μL of Ag@GS (3 mg mL⁻¹) were added to the pretreated GCE and then dried. After washing, 6 μL of the Ab1 solution (10 μg mL⁻¹) were added to the GCE. After incubating at 4 °C for 1 h and washing, 3 μL of bovine serum albumin (BSA, 1 wt%) were added to eliminate nonspecific binding sites. The GCE was then washed and incubated with different concentrations of SCCA for 1 h at room temperature and then the GCE was
washed extensively to remove unbounded SCCA molecules. Finally, the TH/Ag@KIT-6/CMC/ILs-Ab2 buffer solution was added to the surface of the electrode for 1 h at room temperature to complete the immune reaction. The GCE was washed thoroughly prior to measurement.

2.6. Detection of SCCA

A conventional three-electrode system was employed in the electrochemical measurements. The modified GCE with a diameter of 4 mm served as the working electrode, a saturated calomel electrode (SCE) as the reference electrode, and platinum wire electrode as the auxiliary electrode. Square wave voltammetry (SWV) was performed in PBS (pH = 7.4) by scanning the potential from −0.8 V to 0.2 V.

3. Results and discussion

3.1. Characterization of GO, Ag@GS, KIT-6 and Ag@KIT-6

Fig. 2A and B depict the SEM images of the GO and Ag@GS, Fig. 2B shows that many AgNPs are linked to the surface of GS by Ag–N bonding and Ag (10%), C (47%), O (23%), N (5%), and Si (15%) exist in Ag@GS as determined by EDS (Fig. 2C) indicating success of graphene amination. Fig. 2D shows the TEM image of KIT-6 with the ordered silica channels and cubic structure. The mesoporous KIT-6 is highly interconnected comprising two continuous interpenetrating subnetworks of channels separated by a silica wall (Fig. 2D). The SEM image (Fig. 2E) of the Ag@KIT-6 reveals that KIT-6 has a size of approximately 5 μm and Ag NPs are incorporated into the
surface of KIT-6. The EDS spectrum of Ag@KIT-6 in Fig. 2F shows the existence of O (27%), Si (57%) and Ag (15%) indicating AgNPs are linked to NH₂-KIT-6 by Ag–N bonding.

3.2. Characterization of TH/Ag@KIT-6/CMC/ILs-Ab₂ labels

Ab₂, a protein containing –NH₂, can be conjugated with Ag@KIT-6 by Ag–N bonding. Using EDC/NHS as the coupling agent, TH can be conjugated to Ab₂ by the chemical reaction between –NH₂ and –COOH. In our experiments, an excessive amount of TH is added to the precipitate so that more TH is available for conjugation on Ab₂.

The main disadvantage of organic dyes-based biosensors is leaking of the electron mediator from the electrode surface. In our design, CMC offers enough adsorption groups such as –NH₂, –COOH, and –OH to improve the adsorption capacity to TH and prevent leaking. As an amphoteric polyelectrolyte, CMC has different structures in the acidic and alkaline media. When the pH of the solution is less than 7.2, the CMC surface is positively charged and when at a pH larger than 7.2, it is negatively charged. Here, PBS with a pH of 7.4 is used to disperse the TH/Ag@KIT-6/CMC/ILs-Ab₂ and so the CMC surface is negative. Taking into account that TH exists in the cationic form, the attractive force between the CMC surface and TH increases TH adsorption.

The AC impedance is a suitable parameter to monitoring surface changes in the assembly process. Fig. 3 displays the Nyquist plots of the GCE layer-by-layer recorded from 1 to 10³ Hz at 0.24 V in PBS containing 0.1 M KCl and 2.5 mM Fe(CN)₆³⁻/Fe(CN)₆⁴⁻. The plots show a semicircular part at high frequencies and linear part at low frequencies. The former is related to the electrode transfer limited process. In the redox probe Fe(CN)₆³⁻/Fe(CN)₆⁴⁻, the semicircle diameter is equal to the resistance which is an important parameter of the electrochemical immunosensor. The Warburg line in the low frequency region corresponds to diffusion in the overall process. Rₑ can be estimated from the diameter of the semicircular part at high frequencies in the Nyquist plot. The bare GCE possesses a small resistance (curve a) and a smaller resistance (curve b) is observed due to the good electron transfer ability of Ag@APTES-GS. When Ab₁ (curve c), BSA (curve d), and SCCA (curve e) are added, the efficiency of electron transfer between the working electrode and counter electrode is hindered implying successful capture. When TH/Ag@KIT-6/CMC/ILs-Ab₂ (curve f) is modified

![Fig. 3](image_url) Nyquist plots obtained by the AC impedance method: (a) GCE, (b) Ag@APTES-GS/GCE, (c) Ab₁/Ag@APTES-GS/GCE, (d) BSA/Ab₁/Ag@APTES-GS/GCE, (e) SCCA/BSA/Ab₁/Ag@APTES-GS/GCE, and (f) TH/Ag@KIT-6/CMC/ILs-Ab₂/SCCA/BSA/Ab₁/Ag@APTES-GS/GCE.

![Fig. 4](image_url) Effects of (A) solution pH, (B) concentration of Ag@APTES-GS, (C) Au@KIT-6, and (D) ILs on the SWV peak current response of the immunosensor for the detection of 10 ng mL⁻¹ of SCCA (error bar = 5%).
on the electrode, a smaller resistance is observed suggesting that ILs facilitate electron transfer. The resistance of the whole immunosensor is small also because of the good electron transfer ability of Ag@APTES-GS.

3.3. Optimization of experimental conditions

In order to obtain the best electrochemical signal, optimization of the experimental conditions is necessary. The solution pH has a large impact on the electrochemical properties of the immunosensor because the activity of the antigen and antibody is influenced by the surroundings. To optimize the pH, a series of PBS buffer solutions with pH from 4.49 to 9.18 is tested. Fig. 4A shows that the current increases from pH of 5.0, reaches a maximum at pH of 7.4, and then decreases until pH of 9.18. Hence, considering the activity of biological materials (Ag, BSA, and SCCA), pH 7.4 is chosen.

The relationship between the current and concentration of Ag@APTES-GS is presented in Fig. 4B. The current increases due to enhanced immobilization of Ab1 for binding more antigens and signal tags. The peak current decreases slowly as the concentration of Ag@APTES-GS is increased from 2.0 to 3.0 mg mL⁻¹ due to the higher interface electron transfer resistance making electron transfer more difficult. Based on the experimental results, the concentration of Ag@APTES-GS is chosen to be 2.0 mg mL⁻¹. In addition, the influence of the concentrations of Ag@KIT-6 and ILs is assessed as shown in Fig. 4C and D which show that 2.0 mg mL⁻¹ of Ag@KIT-6 and 10 mg mL⁻¹ of ILs are optimal.

3.4 Calibration of the immunosensor

The TH/Ag@KIT-6/CMC/ILs and Ag@APTES-GS are used as signal amplification labels and basic materials to determine different concentrations of SCCA. A linear relationship between the SWV peak currents and logarithmic values of SCCA concentrations is obtained in the concentration range between 5 × 10⁻⁵ ng mL⁻¹ and 10⁻³ ng mL⁻¹ (Fig. 5). The linear regression equation of the calibration curve is derived to be \( I = -10.38 - 1.86 \log C \). The detection limit is 17 fg mL⁻¹ (S/N = 3) which is lower than those reported previously (Table 1).

3.5. Selectivity, reproducibility, and stability

To evaluate the reproducibility of the immunosensor, five electrodes are prepared for the detection of 1 ng mL⁻¹ of SCCA. The relative standard deviation (RSD) of the measurements conducted on the five electrodes is less than 4.28% (Fig. 6A) and so the reproducibility is quite good.

To investigate the selectivity, interference experiments are performed in the presence of alpha fetoprotein (AFP), carcinoembryonic antigen (CEA), glucose, and vitamin C. The 1 ng mL⁻¹ of SCCA solution containing 100 ng mL⁻¹ of the interfering substances is analyzed by the immunosensor and the results are depicted in Fig. 6B. Compared to SCCA, the SWV current variation after introducing the four proteins is less than 5% and so the selectivity is acceptable as well.

The stability of the immunosensor is examined by monitoring the current responses periodically. When the immunosensor is not used, it is stored at 4 °C. The immunosensor is measured every week and each reading represents the average of five assays (Fig. 6C). After 2 and 3 weeks, the SWV current response does not change appreciably and after 4 weeks, the current response retains 91.2% of the initial value. Hence, good stability is demonstrated.

**Table 1** Comparison of the linear ranges and detection limits between this and other studies

<table>
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<th>Methods</th>
<th>Linear ranges</th>
<th>Detection limits</th>
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<tr>
<td>(1) Electrochemical immunosensor based on electron transfer mediated by graphene oxide initiated silver enhancement</td>
<td>0.01–100 ng mL⁻¹</td>
<td>5 pg mL⁻¹</td>
<td>36</td>
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<tr>
<td>(2) Controlled release system-based homogeneous immunoassay</td>
<td>0.004–4.0 ng mL⁻¹</td>
<td>0.3 pg mL⁻¹</td>
<td>37</td>
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<tr>
<td>(3) Magneto-controlled electrochemical immunosensor</td>
<td>2.5–15 ng mL⁻¹</td>
<td>1.0 pg mL⁻¹</td>
<td>38</td>
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<tr>
<td>(4) Cathodic electrochemiluminescence immunosensor</td>
<td>0.025–10 ng mL⁻¹</td>
<td>8.5 pg mL⁻¹</td>
<td>39</td>
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<tr>
<td>(5) Ultrasensitive electrochemical immunosensor using dumbbell-like Pt–Fe₃O₄ nanoparticles for signal amplification</td>
<td>0.05–18 ng mL⁻¹</td>
<td>15.3 pg mL⁻¹</td>
<td>3</td>
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<tr>
<td>(6) Ultrasensitive non-enzymatic immunosensor based on bimetallic gold–silver nanoclusters</td>
<td>0.005–20 ng mL⁻¹</td>
<td>1.3 pg mL⁻¹</td>
<td>4</td>
</tr>
<tr>
<td>(7) Electrochemical immunosensor using thionin/silver nanoparticles decorated KIT-6 as signal amplification labels</td>
<td>5 × 10⁻⁵ to 100 ng mL⁻¹</td>
<td>17 fg mL⁻¹</td>
<td>This work</td>
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3.6. Real-sample analysis

In order to evaluate the feasibility of the immunosensor in practical applications, it is used to determine the SCCA concentration in serum samples by the standard addition method. Table 2 shows the experimental results indicating RSDs between 2.55% and 4.52%. The recovery is in the range between 97% to 102% and the immunosensor is acceptable in real-sample analysis.

4. Conclusion

An ultrasensitive sandwich-type immunosensor composed of Ag@APTES-GS as the platform and TH/Ag@KIT-6/CMC/ILs for multiple signal amplification is designed and fabricated for the determination of the squamous cell carcinoma antigen (SCCA). The immunosensor provides quantitative detection of SCCA with a linear response in a wide range, a low detection limit, as well as acceptable reproducibility, selectivity, and stability. The immunosensor which can be prepared by simple procedures have large potential in clinical serological applications.

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