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Development of Disposable Immunosensors for Rapid Determination of Sildenafil and Vardenafil in Functional Foods

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ABSTRACT - We introduced disposable amperometric immunosensors for the detection of Sildenafil and Vardenafil (SDF/VDF) based on screen printed carbon electrodes. The developed immunosensors were used as a non-competitive sandwich-type enzyme immunoassay with a horseradish peroxidase label. The sensors were constructed on screen printed carbon electrodes by the simple electrochemical deposition of a reduced graphene oxide and chitosan (ErGO-CS) composite. To evaluate the sensing chemistry and optimize the sensor characteristics, a series of electrochemical experiments were carried out including electrochemical impedance spectroscopy, cyclic voltammetry and amperometry. The sensors showed a linear response to SDF/VDF concentrations in a range from 100 pg/mL to 300 ng/mL. The lower detection limit was calculated to be 55 pg/mL, the sensitivity was calculated to be 1.02 μAng/mL/cm^2, and the sensor performance exhibited good reproducibility with a relative standard deviation (RSD) of 7.1%. The proposed sensing chemistry strategy and the sensor format can be used as a simple, cost-effective, and feasible method for the in-field analysis of SDF/VDF in functional or health supplement food samples.

Key words : Disposable sensor, Functional foods, Immunosensor, Sildenafil/Vardenafil

Sildenafil (SDF) and Vardenafil (VDF) are inhibitors of the human phosphodiesterase type 5 enzymes (PDE5Is). SDF and VDF were approved for use in Viagra drugs (Food and Drug Administration (FDA), United States), which are used to treat male erectile dysfunction and to improve breathing efficiency in pulmonary hypertension[1,2]. Taking a medicine should only be done under a doctors' supervision because overdoses of these drugs may create various problems such as headaches, flushing, dizziness, blurred vision and an increased risk of death for those with cardiovascular problems and diabetes[3]. Some recent reports showed that there may be contaminants in these drugs and some health supplement foods produced in poor conditions (*news.joins.com/article/18694079*). This poses a health threat to patients who may unwittingly take unsafe drugs.

Mass spectrometry and chromatographic analytical methods have been reported for the detection of SDF/VDF[4,5]. However, these methods have several drawbacks when they are applied to 'in field' sample analysis because they require expensive equipment and time consuming operating processes. To overcome these limitations, electrochemical sensor systems have attracted considerable attention due to their intrinsic advantages including portability, low cost, simple instrumentation, fast turn-around time and high sensitivity[6,7]. Carbon-based electrodes have been widely used to develop highly sensitive and selective electrochemical sensors; these carbon materials include carbon paste, glassy carbon, carbon nanotubes, graphene and graphite electrodes with modified redox mediators. Among these carbon materials, graphene has received attention as an electrode material due to its unique properties, which include excellent electrical conductivity, high stability and a large specific surface area[8]. In addition, screen printed carbon electrodes (SPCEs) have also been the subjects of sensor-based electro-analytical studies because of their convenient and cheap manufacturing processes, disposability and simplicity[9-10]. Several research groups (including the authors of the present study) have reported disposable electrochemical sensor systems based on screen printed carbon electrodes[11-14].

In the present system, new SPCEs and a simple one-step procedure for the co-deposition of electrochemically reduced graphene oxide (ErGO) and a protein modifier have been

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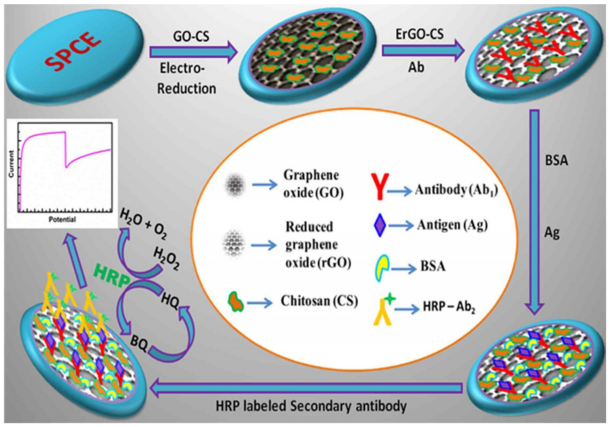

Fig. 1. Schematic diagram of immunosensor fabrication process and sensing chemistry.

developed to construct disposable electrochemical immunosensors for SDF/VDF. To immobilize antibodies onto the modified SPCEs, a bio-polymer, chitosan (CS), was chosen as a protein modifier because of the polymer's good biocompatibility, film-forming properties, adhesion properties and robustness[15]. As shown in Fig. 1, ErGO and the chitosan layer was constructed by using cyclic voltammetry to effectively immobilize and capture antibodies. After capturing SDF/VDF, horse radish peroxidase (HRP) labelled secondary antibodies (HRP-Ab_2) were applied. The enzymatic reaction of HRP with hydroquinone (HQ) in the presence of hydrogen peroxide produces benzoquinone (BQ). BQ is then subsequently reduced back to HQ at the electrode. The current produced from the BQ reduction is proportional to the concentration of the targets. We therefore hypothesized that the synergistic combination of the electrochemically deposited ErGO-CS and the HQ-BQ redox couple could improve the sensor characteristics and could be used for the 'in-field' analysis of SDF/VDF in functional or health supplement food samples.

Materials and Methods

Reagents and Materials

Sildenafil/Vardenafil (SDF/VDF), SDF/VDF antibody (Ab_1), and HRP labeled SDF/VDF secondary antibody (HRP-Ab_2) were supplied as an ELISA kit from ID Labs (London, Ontario, Canada). Graphite powder with 98% purity, hydroquinone, bovine serum albumin (BSA), Na_2HPO_4, NaH_2PO_4, H_3PO_4, NaOH, $K_3[Fe(CN)_6]$, and H_2O_2 (30%) were purchased from Sigma (St. Louis, MO, USA). Graphene oxide (GO) was synthesized from graphite by using a modified Hummers and Offeman method[14,15]. All the chemicals used were analytical reagent grade and all aqueous solutions were prepared with Milli-Q filtered ultrapure water (18 MΩ cm^{-2}).

Instrumentation

Cyclic voltammetry and amperometry were performed with a Compactstat Electrochemical Analyzer (Ivium Technology, Eindhoven, Netherlands). Electrochemical impedance spectroscopy (EIS) was carried out by using an electrochemical workstation (VersaSTAT, Princeton Applied Research, OakRidge, TN, USA).

Fabrication of Electrochemical Immunosensor

Screen printed carbon electrodes (SPCEs) were prepared by an 'in-house' procedure as reported previously by authors of the present study[15]. This procedure consisted of a carbon working electrode (diameter = 3 mm), a carbon counter electrode and a Ag/AgCl reference electrode. A 5 μL aliquot of a graphene oxide (GO, 0.5 mg/mL) dispersion was dropped onto the working electrode in the SPCE and was dried for 2 hours under ambient conditions. After drying, a 3 μL aliquot of a 0.05% chitosan solution prepared in PBS (pH 7.4) was placed onto the surface of the GO modified electrode and was dried for 2 hours. The GO-CS modified SPCE was then transferred into an electrochemical cell containing a 1 mL volume of N_2 saturated 100 mM PBS (pH 5.0). Cyclic voltammetry was carried out to deposit reduced GO-CS (ErGO-CS) by using a potential sweep from −1.4 to 0 V vs. Ag/AgCl for 15 cycles at a scan rate of 50 mV/s. The ErGO-CS modified SPCEs were then rinsed with deionized water and dried at room temperature for 2 hours. A 5 μL aliquot of SDF/VDF antibody (Ab_1) was then dropped onto the ErGO-CS. It was then incubated at room temperature for 30 min and dried in a moisture-saturated environment at 4°C for 12 hours. Subsequently, the antibody modified SPCEs were incubated for 1 hours in a 3% BSA solution prepared in PBS (pH 7.4) to block the remaining bare ErGO-CS site and to avoid any possible nonspecific adsorption of SDF/VDF during the measurement. All prepared immunosensors were gently rinsed with PBS and were stored in the dark at 4°C prior to use.

Electrochemical Measurement Procedure

To construct a calibration curve of SDF/VDF, an immunosensor prepared was immersed in a SDF/VDF sample solution for 15 min. The sensor then removed from the sample solution and was rinsed gently with PBS (pH 7.4). The HRP labeled secondary antibody (HRP-Ab_2) solution was applied, and the sensor was incubated for 10 min. Finally, the immunosensor was transferred to an electrochemical cell containing a 0.9 mL aliquot of 0.5 mM HQ in 100 mM PBS (pH 7.5). The immunosensor was set up using an electrochemical workstation, and amperometric measurements were performed. A fixed potential of 0.2 V vs. Ag/AgCl was applied, and a baseline current was

recorded. After achieving a stable baseline, a 0.1 mL aliquot of a 1 mM hydrogen peroxide solution prepared in 100 mM PBS (pH 7.5) was added. After this addition, an immediate current change was observed, and the current change was recorded for 3 min. Electrochemical impedance spectroscopy (EIS) was also performed with the modified SPCEs in a 5 mM $[Fe(CN)_6]^{3-}$ solution containing 0.1 mM KCl at a frequency range from 0.1 Hz to 100 kHz at an applied DC potential of 0.25 V (the formal potential of $[Fe(CN)_6]^{3-}$ redox couple) and an AC amplitude of 5 mV.

Results

Electrochemical Immunosensor Construction

The electrochemically reduced graphene oxide-chitosan (ErGO-CS) composite layers were deposited onto the SPCEs by using cyclic voltammetry. The applied potential sweep with the GO-CS modified SPCE caused the reduction of GO. A representative cyclic voltammogram of the electrochemical reduction of GO is shown in Fig. 2. In this first segment, a large and broad cathodic peak appeared at −1.23 V with an onset potential of −0.7 V. In subsequent cycles, cathodic peaks decreased significantly and almost disappeared after 15 cycles.

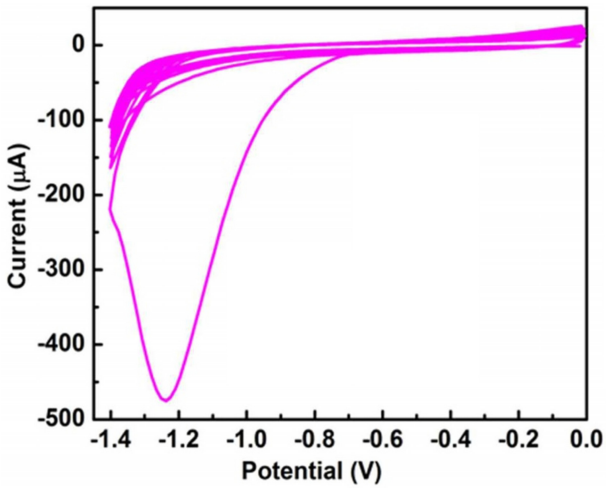

Fig. 2. Cyclic voltammograms of electrochemical reduction of GO-CS/SPCE in N_2 saturated 100 mM PBS (pH 5.0) with a scan rate of 50 mV/s.

To investigate each immobilization step during sensor fabrication, a series of cyclic voltammetry measurements were carried out with the modified SPCEs in the presence of a 5 mM $[Fe(CN)_6]^{3-}$ solution prepared in 0.1 M KCl. As shown in Fig. 3A, a well-defined redox peak of $[Fe(CN)_6]^{3-}$ was clearly observed on the bare SPCE (curve a). After GO-CS modification, the redox peak disappeared (curve b). Fig. 3B depicts the EIS of a bare SPCE and the modified SPCEs. The GO-CS modified SPCE (curve b) showed a much larger charge transfer resistance than that of the bare SPCE (curve a). The resistance of the redox probe decreased after reduction of GO-CS (curve c). Obvious resistance increases were observed after additional steps with antibody, BSA and SDF/VDF (curves e-f).

Immunosensor Optimization

The effect of applied potentials and the composition of HQ and H_2O_2 solutions on the electrochemical behavior of SDF/VDF in the constructed immunosensors were investigated. A series of amperometric measurements were carried out as descried in the measurement procedure section above. The measurements were carried out with standards with fixed concentrations of SDF/VDF in PBS containing different concentrations of HQ and H_2O_2. The measurements were also repeated at different applied potentials ranging from −0.4 V to 0.2 V. As can be seen in Fig. 4, the current response of SDF/VDF at 2.0 V in 0.5 mM HQ and 1.0 mM H_2O_2 solutions (curve a) showed the maximum current response. The effect of pH on the immunosensor was also

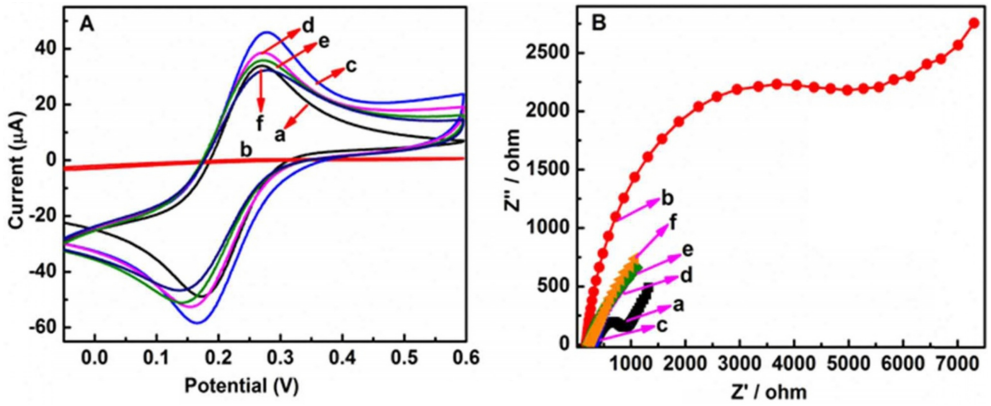

Fig. 3. (A) Cyclic voltammograms and (B) EIS of (a) SPCE, (b) GO-CS/SPCE, (c) ErGO-CS/SPCE, (d) Ab_1-ErGo-CS/SPCE, (e) BSA-Ab_1-ErGO-CS/SPCE, and (f) Ag-BSA-Ab_1-ErGO-CS/SPCE in 5 mM $K_3Fe(CN)_6$ and 0.1 M KCl. Scan rate: 50 mV/s.

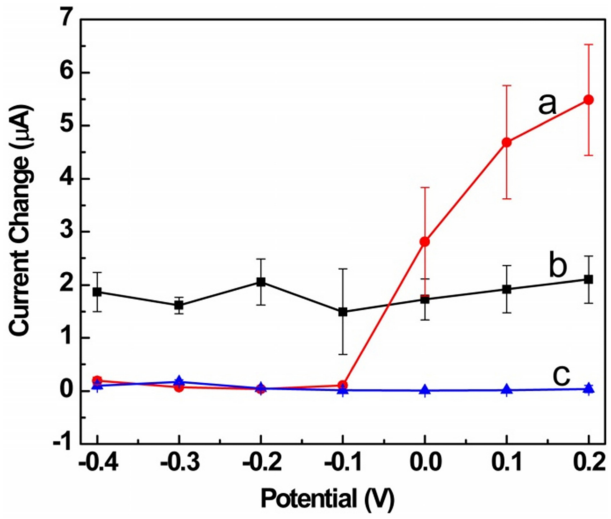

Fig. 4. Effects of applied potential on immunosensors in SDF/VDF sample measured in (a) 0.5 mM HQ + 1.0 mM H_2O_2, (b) 0.5 mM HQ only, and (c) 1.0 mM H_2O_2 only.

investigated. Repeatable amperometric measurements with SDF/VDF were carried out in PBS at in a pH range from 4.0 to 8.0. The maximum current response was observed at pH 7.5 with 0.5 mM HQ and 1.0 mM H_2O_2. Therefore, this composition was selected as the optimum operating condition and was used in subsequent experiments.

Immunosensor Characteristics and Calibration Curves

Fig. 5 shows representative calibration curves obtained from the results of amperometric measurements of SDF/VDF samples by using Ab-ErGO-CS-SPCEs under optimized experimental conditions. Aliquots of SDF/VDF samples were prepared in 100 mM PBS by the dilution of the SDF/VDF standard solution from the ELISA kit, and the sample concentrations ranged from 5 pg/mL to 1000 ng/mL. Fig. 5B shows a logarithmic calibration curve of SDF/VDF, and each error bar indicates a standard deviation obtained from five measurements for each point. In the constructed curve, a linear range was calculated from 100 pg/mL to 300 ng/mL, and the current varied as i (μA) = 0.072 (μA) × SDF/VDF concentration (log ng/mL^{-1}) + 0.058 (μA) with a correlation coefficient (R^2) of 0.972. The lower detection limit was calculated to be 55 pg/mL from a blank test. The sensitivity of Ab-ErGO-CS-SPCEs was also calculated to be 1.02 μAngmL^{-1}cm^{-2}, and the sensor performance exhibited good reproducibility with a relative standard deviation (RSD) of 7.1%.

To investigate and validate the applicability of the developed immunosensor, three different functional or health supplement food samples were analyzed by using a standard addition method. The food samples, two red ginseng drinks and an energy drink (product name not shown), were all liquid products and were purchased from a local supermarket. After repeatable amperometric measurements under the optimized operating conditions, there were no detectable current responses to SDF/VDF from the food samples after 1:10 dilution with PBS. Three different food samples were then randomly spiked by the addition of the SDF/VDF standard to obtain final concentrations of 10, 50 and 100 ng/mL. The recovery rates were calculated to be 63%, 76% and 71%, respectively, for these concentrations.

Discussion

Each step of the sensor construction process was studied to validate the construction of the immunosensor. As can be seen in Fig. 3A, the ErGO-CS composite layer was successfully deposited onto the SPCEs by using cyclic voltammetry. The large and broad cathodic peak (curve a in Fig. 3A) produced by the first potential sweep was attributed due to the reduction of oxygen functional groups present on the GO surface such as epoxide, carboxyl and hydroxyl

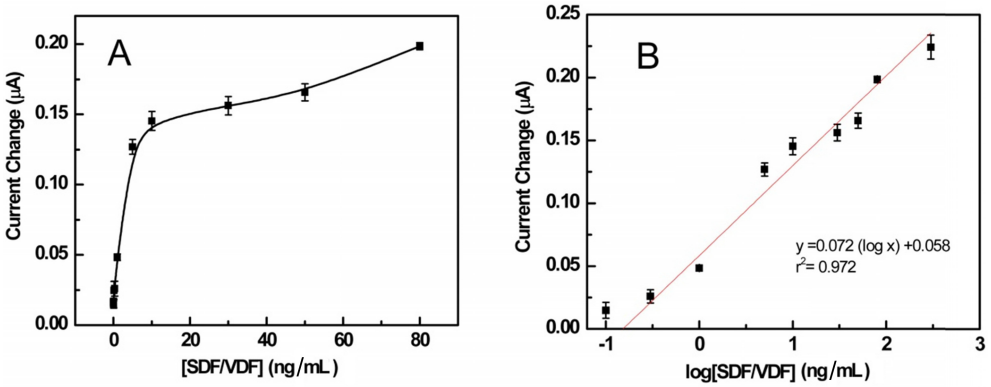

Fig. 5. Calibration curves for SDF/VDF in 100 mM PBS using (A) BSA-Ab$_1$-ErGO-CS/SPCEs and (B) the same results from sample concentrations in logarithmic form.

groups. After the first potential sweep, the cathodic peaks significantly decreased for the subsequent potential sweepings. These results clearly indicate that the reduction of GO occurred quickly and irreversibly under these experimental conditions[15,16], and the co-immobilized CS did not affect the characteristics of the electrochemical reduction process of GO. It is obvious that the unreduced GO-CS could act as an insulating layer that makes the electron transfer process difficult at the electrode surface (curve b in Fig. 3A). As shown in ErGO-CS (curve c in Fig. 3A), however, the redox peak current greatly increased because the large surface area of ErGO enhanced the redox reaction of $[Fe(CN)_6]^{3-}$. Similarly, the subsequent modification with antibody, BSA and SDF/VDF resisted the electron transfer process due to electrochemical insulation by the immobilized proteins (curves d-f in Fig. 3A). Electrochemical impedance spectra (EIS) are an efficient tool for evaluating the interface properties of surface modified electrodes. EIS can provide information on the impedance changes of the electrode interface. The changes in charge transfer resistance of the modified SPCEs (curves a-f in Fig. 3B) were in good agreement with the results obtained from voltammetry measurements (Fig. 3A). These results indicated the successful immobilization of immune-proteins on the surface of the SPCEs.

According to an internet based drug index, RxList, the average maximum Sildenafil plasma concentrations after a single oral dose of 100 mg to a healthy adult male is about 450 ng/mL, and the lower therapeutic concentrations after a 25 mg single oral dose are about 7 ng/mL[17]. This index data indicated that the present immunosensor system can be applied to develop a clinical SDF/VDF sensor kit because of the observed lower detection limit with a short turnaround time (approximately 30 min for each measurement). The simple screen-printing technique and the effective modification procedure using direct electrochemical deposition of antibodies onto the ErGO-CS modified electrodes result in inexpensive and disposable amperometric SDF/VDF immunosensor fabrication.

In conclusion, we introduced a new disposable electrochemical immunosensor system based on SPCEs for the detection of SDF/VDF. The combination of a simple electrochemical co-deposition of ErGO and CS was successfully integrated for the antibody immobilization. We suggest that further research could be required to investigate the possible interferences and improve the sensor characteristics for the 'in field' analysis in functional or health supplement food samples. To the best of our knowledge, there has been no report on the strategies of electrochemical rGO-CS based immunosensors with HRP-Ab_2 to detect Sildenafil and Vardenafil.

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국문요약

본 연구의 목적은 screen printed 탄소계 전극에 기반하여 기능성 식품중에 포함된 소량의 Sildenafil과 Vardenafil (SDF 및 VDF)을 쉽게 검출하기 위한 전류계 면역센서를 개발하고자 하였다. 본 연구에서 개발한 면역센서는 horseradish peroxidase로 labeling 시킨 후 비경쟁적 샌드위치 ELISA 측정법에 의한 원리를 이용하였다. 개발된 센서는 그래핀 산화물 및 키토산(ErGO-CS) 복합체를 단순한 전기화학적 증착에 의해 screen printed 탄소계 전극을 이용하는 원리이며, 감지된 화학물질을 평가하고 센서특성을 최적화하기 위해 전기 화학적 임피던스분광법, 순환 전압전류법 등을 포함한 일련의 전기화학적 실험방법에 기초하여 완성되었다. 본 연구에서 개발된 센서는 100 pg/mL ~ 300 ng/mL 농도의 SDF 및 VDF에 대하여 직선상의 농도-의존적인 반응을 나타내었다. 또한 최저 검출 한계는 55 pg/mL이었으며, 센서의 민감도는 1.02 μAng/mL/cm^2로 산출되었다. 센서의 성능은 7.1%의 상대 표준편차로 매우 우수한 재현성을 나타내었다. 본 연구에서 개발된 센서의 전략적 가치는 향후 건강기능식품중에 함유된 SDF 및 VDF과 같은 의약품의 미량을 현장에서 쉽게 분석 할 수 있어서 비용-효과적 측면에서 그 가치가 우수하다는 것을 제시한다.

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