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Short communication

Magnetic beads-based electrochemical immunosensor for detection of pseudorabies virus antibody in swine serum

Fang Li[a,b], Rui Zhou[b], Kaihong Zhao[b,c], Huanchun Chen[b], Yonggang Hu[b,c,*]

[a] School of Environmental Science and Engineering, Huazhong University of Science and Technology, Wuhan 430074, China
[b] State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan 430070, China
[c] College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, China

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ABSTRACT

A novel magnetic electrochemical immunosensor has been developed for the detection of pseudorabies virus antibody in swine serum. The magnetic glass carbon electrode was fabricated to manipulate magnetic beads for the direct sensing applications. Magnetic beads were employed as the platforms for the immobilization and immunoreaction process, and gold nanoparticles were chosen as electroactive labels for the electrochemical detection. The parameters concerning the assay strategy were carefully investigated. Under the optimal conditions, the linear response range of pseudorabies virus antibody dilution ratio (standard positive serum) was 1:250 to 1:1000 with a detection limit of 1:1000. Finally, this developed immunoassay method was successfully applied in the detection of pseudorabies virus antibody in swine serum, and had a good diagnostic accordance in comparison with ELISA.

1. Introduction

Special attention has been paid in magnetic beads (MBs)-based electrochemical immunoassay in the recent years [1–9]. As an ideal platform candidate, MBs allow for quick and efficient purification, and concentration from crude samples, thereby eliminating the need for most of the pre-treatment steps as well as the matrix effect from the samples. Additionally, MBs can provide a low detection limit since MBs have large surface area to immobilize biomolecules. More importantly, MBs make the possible of immobilization and immunological reaction events to be performed away from the electrode surface [9], effectively reducing the complexity and time required for sensing application. One major obstacle for MBs-based electrochemical immunoassay is the lack of uncomplicated, low cost and practical magnetic electrode for MBs manipulation and the direct sensing [9,10].

Pseudorabies, or designated as Aujeszky's disease (AD), is a serious acute infectious disease for livestock and pets [11,12]. It has the high death rate and strong infection capability, contributes to significant economic losses in animal husbandry worldwide. Swine is considered as the natural host of pseudorabies virus (PRV) and play an important role in transmission. Therefore, development of a rapid, simple, sensitive, low cost, and reliable assay is crucial for the clinical diagnosis as well as early prevention of further spread. Herein, we introduced a simple procedure to prepare magnetic glass carbon electrode (MGCE), and used the MGCE to construct a novel MBs-based immunosensor. To demonstrate this concept, this sensor was employed for the detection of PRV antibody in swine serum. Gold nanoparticles (AuNPs), which is stability, easy preparation, and has good biological compatibility, excellent conductivity, were selected as the electroactive labels for the electrochemical detection. The schematic design is depicted in Fig. 1. The PRV antigen was attached onto the MBs surface, and then respectively incubated with the PRV antibody in swine serum and the second antibody that modified with AuNPs (AuNPs-Ab2) (Fig. 1(A)). After the immunological reaction, immunocomplex-coated magnetic beads (IMBs) carried the relevant information were captured and focused on the detection surface with help of MGCE (Fig. 1(B)). MGCE was immersed in 0.1 mol/L HCl solution for the electrochemical detection, in which AuNPs can be electro-oxidized to produce $AuCl^{4-}$ [13]. Finally, this strategy was applied in the detection of PRV antibody in swine serum.

2. Experimental

2.1. Apparatus and electrodes

Cyclic voltammograms (CVs) and differential pulse voltammograms (DPVs) were performed in a CHI 660 electrochemical analyzer (Shanghai Chenhua Instruments, Shanghai, China). All

* Corresponding author at: State Key Laboratory of Agricultural Microbiology, College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, China. Tel.:+86 027 87280670; fax: +86 027 87280670.
E-mail address: yongganghu@mail.hzau.edu.cn (Y. Hu).

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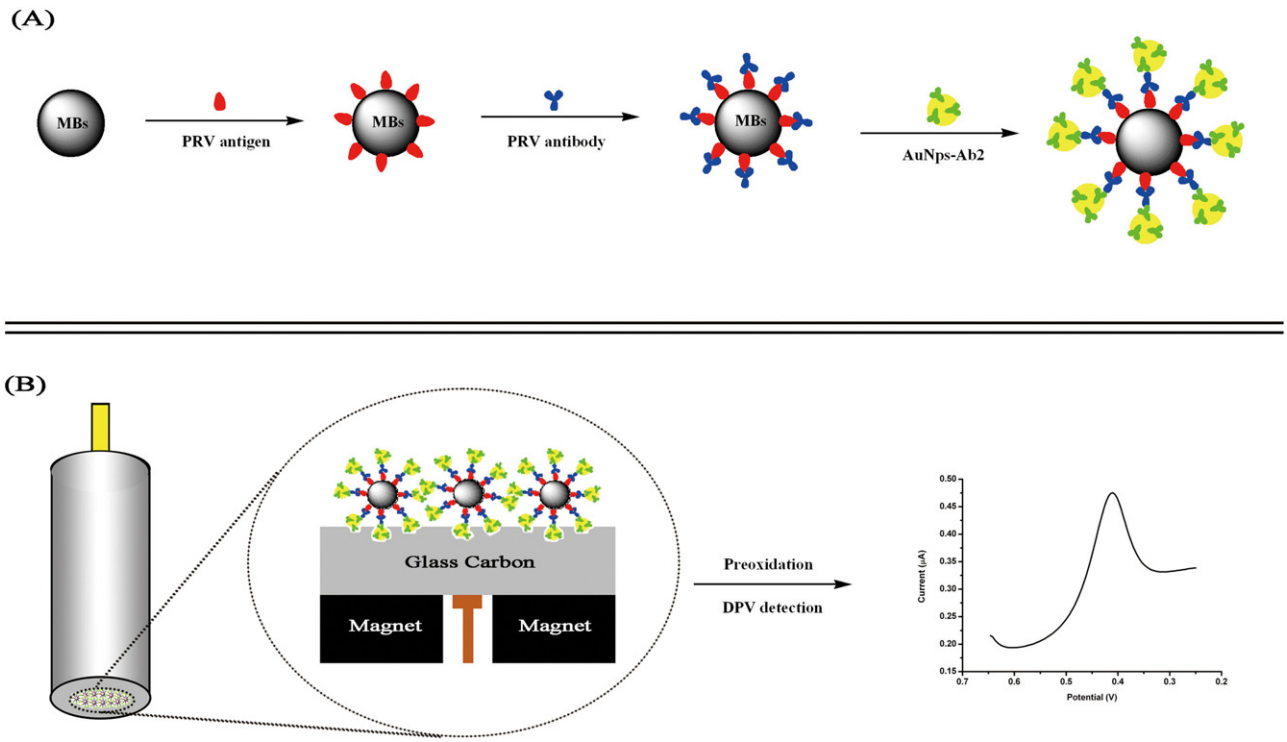

Fig. 1. (A) Schematic diagram of the immunological reaction procedure. (B) Schematic diagram of the electrochemical detection procedure (not in scale).

measurements were carried out at room temperature (25 ± 1 °C) in a 2 mL electrochemical cell with a normal three-electrode configuration. A platinum wire counter electrode and a saturated calomel reference electrode (SCE) were used in this three electrodes configuration. A homemade MGCE was used as working electrode. After the detection, the MGCE surface was rubbed with alcohol pledget, washed with ultrapure water. Then, MGCE was cleaned in 0.5 mol/L H_2SO_4 by performing CVs from −1.0 to 1.0 V (vs. SCE) with a scan rate of 100 mV/s for 20 cycles. After thoroughly rinsed with water and dried with high purity nitrogen gas (99.999%), MGCE was used to capture IMBs for the subsequent detection. Transmission Electron Microscope (TEM) H-7650 (HITACHI, Japan) and UV–vis spectrometer (DU 800, Bekman, Japan) were used to characterize the AuNPs.

2.2. Reagents and materials

PRV antigen, standard positive and negative serum, ELISA Kit for the detection of PRV antibody, the clinical serum specimens collected from swine were all supplied by Wuhan Keqian Animal Biological Products Co., Ltd. (Wuhan, China). The standard positive serum was anti-serum from a pig experimentally infected with PRV. The standard negative serum was obtained from a PRV-free herd. Classical swine fever virus (CSFV), Japanese encephalitis virus (JEV) and porcine parvovirus (PPV) were acquired from State Key Laboratory of Agricultural Microbiology Huazhong Agricultural University (Wuhan, China). Rabbit anti-pig IgG (the second antibody, Ab2) was purchased from Beijing Biosynthesis Biotechnology Co. Ltd. (Beijing, China). Bovine serum albumin (BSA) was supplied by German Roche Co. Ltd. (Germany). COOH-coated MBs 3.0 μm sized were obtained from Shaanxi Lifegen Co. Ltd. (Sian, China). 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide hydrochloride (EDC), and N-hydroxysuccinimide (NHS) were purchased from Aladdin Reagent Co. Ltd. (Shanghai, China). Hydrogen tetrachloraurate (III) trihydrate ($HAuCl_4 \cdot 3H_2O$, 99.9%), other reagents and chemicals were all supplied by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The solutions were prepared using ultrapure water, which was obtained through a Cascada Lab Water System (USA). The phosphate buffer solution (PBS) consisted of 0.01 mol/L phosphate buffered saline, 0.137 mol/L NaCl and 0.003 mol/L KCl (pH = 7.4). Prior to electrochemical detection, the solutions were piped with high purity nitrogen gas for half an hour.

2.3. Methods

2.3.1. Synthesis of AuNPs and preparation of AuNPs-Ab2

AuNPs were synthesized by reducing tetrachloroauric acid with trisodium citrate [14]. All glassware was cleaned in aqua regia, rinsed with ultrapure water, and dried prior to use. A volume of 200 mL of 0.01% $HAuCl_4$ solution was boiled with vigorous stirring, and then 5 mL of 1% trisodium citrate solution was rapidly added, which result in a change in solution color from faint yellow to wine-red within 1 min. Boiling continued for an additional 5 min, after that the solution was left stirring and cooling down. AuNPs was stored in 4 °C and used to prepare AuNPs-Ab2 as soon as possible. The AuNPs were characterized by TEM that indicated the particle size was about 13 ± 2 nm and UV–vis spectrometer that showed an absorption peak at 520 nm.

The conjugation of AuNPs-Ab2 was performed according to the following procedure: pH-adjusted AuNPs solution (pH = 9.0) was mixed with 100 μL of 100 μg/mL Ab2 and incubated at room temperature for 30 min. The antibody was absorbed onto the surface of AuNPs through a combination of ionic and hydrophobic interactions. After that, a blocking step with 1% BSA, incubating at room temperature for 30 min was performed. Finally, the mixture solution was purified by a centrifugation at 12,000 rpm for 30 min, and then the soft sediment was resuspended in PBS containing 1% BSA.

2.3.2. Preparation of PRV-coated MBs [15]

5 mg (400 μL from the store solution) of MBs were placed in a tube and were separated from the suspension media after

placement on a magnetic rack (Lifegen, Sian, China). Subsequently, the MBs were washed twice with 2-morpholino-ethanesulfonic acid solution (MES) (0.1 mol/L, pH = 5.5) and suspended in MES solution containing 10 mg EDC and 5 mg NHS. The activated MBs were washed twice with coupling buffer (0.01 mol/L PBS, pH = 7.4) after 30 minutes. PRV antigen was added to the activated MBs, and the mixture was incubated with gentle mixing at room temperature for 4 h. After washed with PBS–Tween (0.01 mol/L PBS containing 0.05% Tween-20) three times, the PRV-coated MBs were resuspended in PBS containing 5% BSA to block any remaining active surface and then incubated at room temperature for 1 h. Finally, the resulting MBs were washed three times with PBS–Tween and resuspended in a final volume of 10 mL with PBS containing 0.1% BSA.

2.3.3. Immunoassay procedure

A series of dilutions of the serum (100 μL, anti-PRV) was added into the 50 μg of PRV-coated MBs (MB/PRV). The mixture was incubated with rotation at 37 °C for 30 min. After the washing steps with PBS–Tween five times, the MB/PRV/anti-PRV were incubated with 100 μL of the previously synthesized AuNPs-Ab2 at 37 °C for 30 min. Finally, the IMBs (MB/PRV/anti-PRV/AuNPs-Ab2) were washed with PBS–Tween for five times.

2.3.4. Electrochemical detection

The electrode was used to capture IMBs for the subsequent detection. After that 1.5 mL of 0.1 mol/L HCl was added into the electrochemical cell to perform the electrooxidation of AuNPs at a constant potential of +1.3 V for 150 s, and immediately the DPV

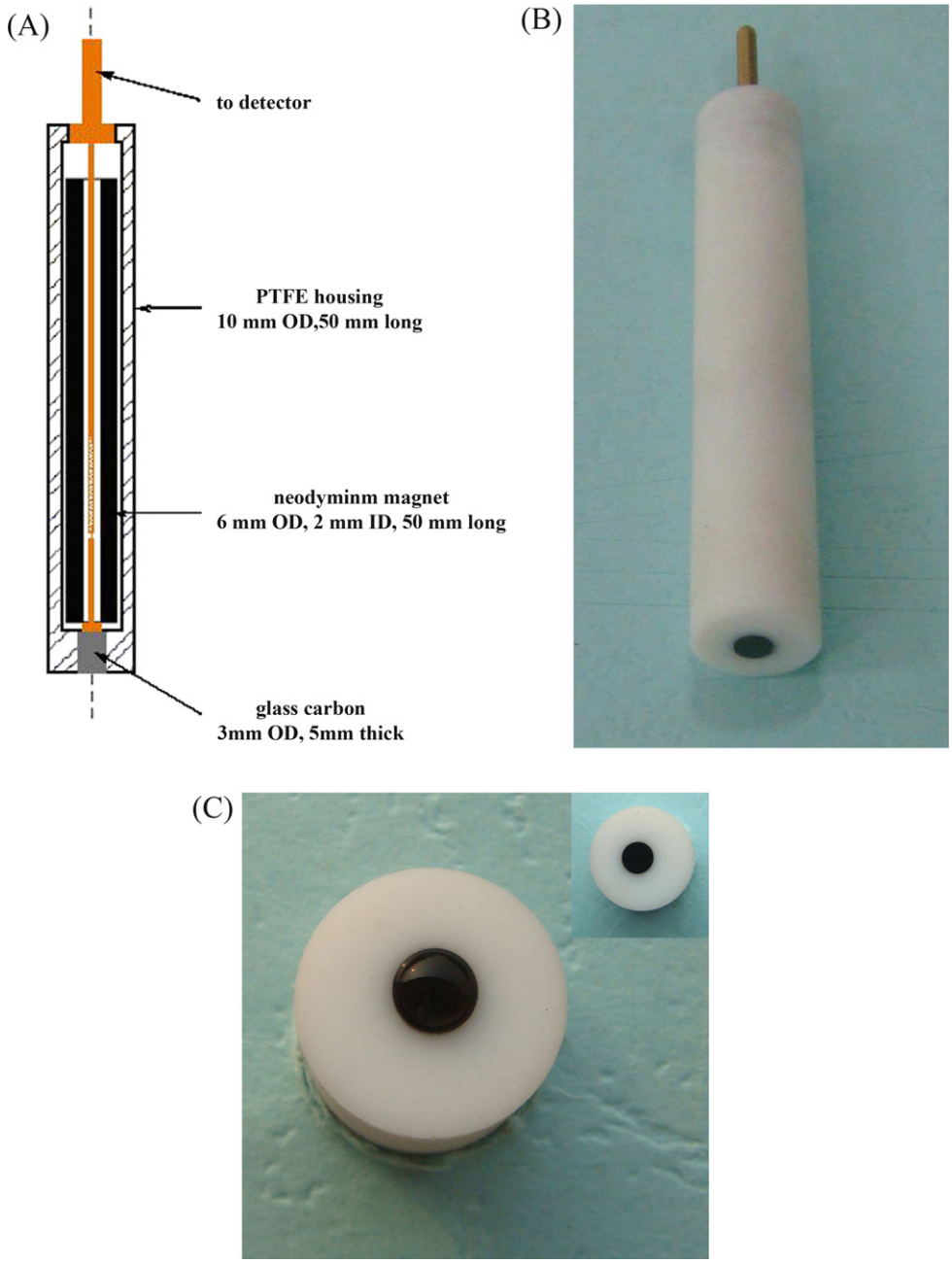

Fig. 2. Schematic diagram (A) and photograph (B) of MGCE, and (C) the photograph of MGCE that confined the IMGs on the detection surface (Inset is the image of bare MGCE).

detection from +0.65 to 0.25 V, with a step potential of 4 mV, a pulse amplitude of 50 mV, and a pulse period of 0.2 s. CVs were performed in 0.01 mol/L PBS solutions (pH = 7.4) containing 0.025 mol/L $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ and recorded at a potential range from −0.1 to 0.6 V (vs. SCE) with a scan rate of 100 mV/s.

3. Results and discussion

3.1. Characterization of MGCE

Glass carbon, possess the smooth surface, the large electrochemical window, the high hydrogen overpotential and the excellent chemical stability, has been one of the most widely used carbon material in electro-analytical applications. Owing to these, the MGCE can be used for the sensing both of metal-based tag (e.g., AuNPs) and enzymatic tracer [16], extending the application area of MBs-based electrochemical immunoassay. Fig. 2(A) illustrated schematic diagram of the MGCE and Fig. 2(B) is the photograph of MGCE. It consists of a neodymium hollow cylinder magnet (6 mm diameter × 52 mm length) that was press-fitted into a PTFE cylindrical tube (10 mm diameter × 65 mm length). One end of the PTFE cylindrical tube was packed with glass carbon to about 5 mm depth and 3 mm diameter. A copper connector with a spring was designed to combine the glass carbon with electrochemical analyzer because the wire (e.g., copper wire or silver wire) cannot be weld with glass carbon. It was inserted into the central of the neodymium hollow cylinder magnet, and connected with the glass carbon with help of the spring.

The internal resistances of MGCE and the normal glass carbon electrode were, respectively, tested by an electronic multimeter. A minute difference between the two electrodes was found, indicating that MGCE had efficient electrical conductivity. The surface magnetic field distribution for the electrode was also investigated. The electrode yielded magnetic field intensities of 73.6, 70.8, 68.2, 55.0, and 36.9 mT, measured at radius of 0, 1.5, 3.0, 4.0, and 5.0 mm of the electrode surface, respectively. The result shows that the maximum magnetic field intensity is obtained in the centre of the electrode surface, and its magnetic field intensity is twice larger than that of magnetic gold electrode [16]. The dispersive IMBs, therefore, could be well captured and focused on the detection surface (Fig. 2(C)). The IMBs can be easily removed from the electrode surface without the need for the magnet to be taken out from the PTFE tube. The regeneration procedure is simple, allowing the electrode reused for a long time while its cost increases only $6 in comparison with the normal glass carbon electrode. Furthermore, the MGCE can be integrated in the traditional electrochemical system without making the device too complicated and expensive. These factors demonstrated that the homemade MGCE was inexpensive, robust, user-friendly, and had a commercial promise for electrochemical detection.

The electrochemical characterization of the immunosensor was tested by cyclic voltammetry of electro-active species in a conducting aqueous solution. The immobilization of the PRV antigen and antibody onto the MBs surfaces significantly induced the decrease of the peak current of redox couple ($K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$). The peak current of redox couple slightly enhanced after AuNPs-Ab2 immunologically attached on the MBs surfaces. These results demonstrate that the proposed strategy can be successfully used for immunoassay, which further confirmed the success in the immunosensor design.

3.2. Optimization of the experimental conditions

Oxidation potential was crucial since too low potential would not be able to oxidize gold in HCl to $AuCl^{4-}$, while too high potential would cause damage of the MGCE surface and further affect electrochemical signals. The oxidation potential from +1.2 to +1.6 V was investigated with DPV detection after the preoxidation of the captured AuNPs in 0.1 mol/L HCl, and +1.3 V was selected for the subsequent detection. Oxidation time in the range of 50–250 s was also examined, and 150 s was evaluated to be the optimum time.

The effect on the sensitivity of the amount of IMBs immobilized on the electrode surfaces was investigated from 10 to 150 μg. An increase in the sensitivity was observed up to 50 μg. The large amount of IMBs did not improve the sensitivity for electrochemical detection, which is probably due to the excess IMBs blocking the mass transformation, minimizing the electric conductivity. For this reason, an immobilized IMBs amount of 50 μg was chosen for further work.

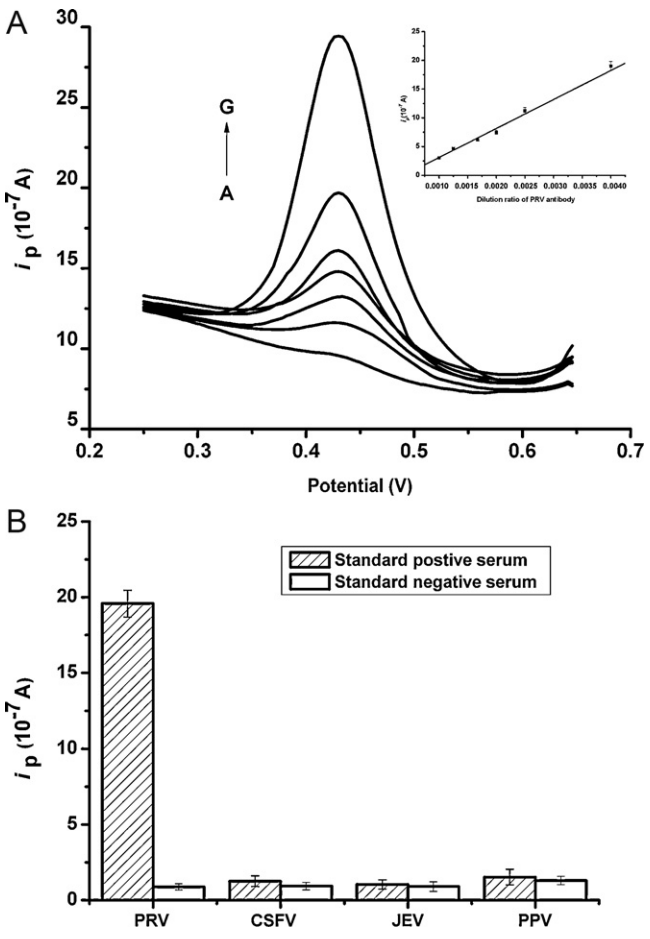

Fig. 3. (A) DPV responses of the magnetic electrochemical immunoassay using AuNPs-Ab2 conjugations in 0.1 mol/L HCl with different dilution ratio of RPV antibody standard positive serum from (A) to (G) (0, 1/1000, 1/800, 1/600, 1/500, 1/400, 1/250). Insets: corresponding calibration curves. (B) Specificity of the magnetic electrochemical immunoassay. (pseudorabies virus (PRV), classical swine fever virus (CSFV), Japanese encephalitis virus (JEV) and porcine parvovirus (PPV)).

3.3. Performance of the sensor

Under the optimal conditions, the sensitivity of the electrochemical immunoassay was investigated by varying dilution ratio. Different peak currents obtained in the DPV response of gold tag after immunological reactions was tested. As shown in Fig. 3(A), the reduction current of gold was linear with respect to the dilution ratio over the range from 1:250 to 1:1000. The regression equation was $Y = 5345.1X - 2.4499$ (Y is the peak current and X is the dilution ratio of PRV antibody) with the regression correlation coefficient of

0.9942. The detection limit of this method was estimated as 1:1000 (S/N = 3), which is lower than that of ELISA (1:300). This indicates the proposed immunosensor can successfully detect PRV antibody with a high sensitivity and low detection limit. A batch of three electrodes prepared in the same manner was used to evaluate the electrode fabrication reproducibility. A value of 3.31% for overall standard deviation (n = 9, three times per electrode for three electrodes) was obtained by analyzing 1:400 PRV standard positive sera. In addition, three series of five measurements of 1:500 PRV standard positive sera with three independent electrodes yielded reproducible signals with relative standard deviations of 4.85%, 3.08% and 5.26%, respectively.

In order to detect the specificity of the present immunosensor, MBs modified with PRV, CSFV, JEV, PPV were incubated with standard positive and negative serum, respectively, and the subsequent detection is followed the present immunosensor procedure. As shown in Fig. 3(B), the peak currents obtained from MBs modified with CSFV, JEV and PPV incubated with positive or negative serum were all similar to that obtained from PRV-MBs incubated with negative serum, indicating that the proposed immunosensor had sufficient specificity for the diagnosis of PRV antibody.

3.4. Application of immunosensors for real samples

The proposed method was further validated by using fifty-two swine serum samples with reference to commercialized ELISA kit. The results obtained from the present method verified that 50 samples were positive and the other samples were negative, consistent with the results of ELISA. The efficiency value of 100% was calculated from the equation [17]: Efficiency = (TP + TN) × 100/Total (TP, True Positive; TP = 50; TN, True Negative; TN = 2; Total = 52). Such result demonstrated that the proposed method provided assay performance comparable to the commercialized ELISA kit, and might promise to apply in clinical diagnosis for the detection of PRV antibody.

4. Conclusion

In summary, we have developed a novel MBs-based electrochemical immunoassay for the detection of PRV antibody based on the homemade MGCE for the capture and direct sensing, which effectively reduces the complexity and time required for sensing applications. The present method was applied for the detection of PRV antibody in serum samples, and had a good diagnostic agreement with the results from ELISA. This sensitivity, high specificity and low-cost electrochemical immunoassay provides a new concept for capture and direct sensing, and has a great promise for clinical diagnosis, environmental monitoring and food analysis.

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