Short communication

Facile fabrication of magnetic gold electrode for magnetic beads-based electrochemical immunoassay: Application to the diagnosis of Japanese encephalitis virus

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\textbf{A B S T R A C T}

A novel magnetic beads-based electrochemical immunoassay strategy has been developed for the detection of Japanese encephalitis virus (JEV). The magnetic gold electrode was fabricated to manipulate magnetic beads for the direct sensing applications. Gold-coated magnetic beads were employed as the platforms for the immobilization and immunoreaction process, and horseradish peroxidase was chosen as an enzymatic tracer. The proteins (e.g., antibodies or immunocomplexes) attached on the surface of magnetic beads were found to induce a significant decline in their electric conductivity. Multiwalled carbon nanotubes were introduced to improve sensitivity of the assay. The envelope (E) protein, a major immunogenic protein of JEV, was utilized to optimize the assay parameters. Under the optimal conditions, the linear response range of E protein was 0.84 to 11,200 ng/mL with a detection limit of 0.56 ng/mL. When applied for detection of JEV, the proposed method generated a linear response range between $2 \times 10^{3}$ and $5 \times 10^{5}$ PFU/mL. The detection limit for JEV was $2.0 \times 10^{3}$ PFU/mL, which was 2 orders of magnitude lower than that of immunochromatographic strip and similar to that obtained from RT-PCR. This method was also successfully applied to detect JEV in clinical specimens.

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1. Introduction

Japanese encephalitis virus (JEV), which contributes to huge economic losses in animal industry in southern and eastern Asia and threatens public health, is a single-strand positive-sense RNA virus\textsuperscript{4} (Wang et al., 2009; Misra and Kalita, 2010; Van den Hurk et al., 2009). The diagnosis of JEV is generally carried out by reverse transcriptase polymerase chain reaction (RT-PCR). However, RT-PCR is expensive and may not be appropriate for the developing countries. In addition, RT-PCR method has the risk of releasing amplified nucleic acids into the environments, thereby contaminating the subsequent analysis. Development of a rapid, cost-effective, sensitive and specific antigen detection method is, therefore, urgent for early and reliable clinical diagnosis as well as effective surveillance of new JEV infection.

Immunassay, quantitative analysis of antibody or antigen concentrations based on the highly biospecific recognition interactions, is of paramount importance for clinical diagnosis (Aytur et al., 2006; Du et al., 2010; Fu et al., 2008; Kerman et al., 2007). Special attention has been paid to electrochemical detection method due to its high sensitivity, simple instrumentation, excellent compatibility, low cost and power requirements (De la Escosura-Muniz et al., 2010; Du et al., 2010; Nie et al., 2009; Zhong et al., 2009). Typically, the electrochemical immunoassay involves two processes: the recognition elements (antibodies or antigens) are fixed on the electrode surface to react with target analytes, and then the immunological responses are converted into a measurable electrical signal. These two different events are performed at the same electrode surface. Therefore, it is not surprising that the properties of the optimum surface for immunoreaction can hardly be the same as those for electrochemical detection (Palecek and Fojta, 2007), thereby causing high nonspecific binding, poor reproducibility, et al.

One major emerging strategy is the incorporation of magnetic beads (MBs) into electrochemical immunoassay (Campuzano et al., 2010; De la Escosura-Muniz et al., 2010; Liebana et al., 2009; Hervas...
et al., 2009; Palecek and Fojta, 2007; Zacco et al., 2006). This can circumvent the problems in generic technique discussed above, by allowing the immobilization and immunoreaction to be performed away from the electrode surface. As an ideal platform candidate, MBs can automatically purify and concentrate target analytes by a magnet and effectively minimize the matrix effect from the samples. Additionally, MBs provide a high surface area to immobilize the biomolecules as many as possible, leading to a lower detection limit. Ideally, after the process of immunoreaction, MBs carried the relevant information would be captured by the magnetic electrode and focused on the detection surface for the direct sensing without the need for further processing. This concept effectively reduces the complexity and time required for the sensing application (Goon et al., 2010). Thus, the development of a magnetic electrode to manipulate MBs is a key step to achieve the direct capturing and sensing.

A gold electrode, which has a wide potential window in the cathode region and efficient electric conductivity, is considered to be a major metallic electrode. In the last decade, it has been widely explored as a transducer in electrochemical immunoassay (Chaki and Vijayamohanan, 2002; Lucarelli et al., 2004). To our best knowledge, the fabrication of a magnetic gold electrode and its subsequent applications has not been reported in the literature. In the present work, we first and foremost fabricated magnetic gold electrodes, and then developed a novel MBs-based electrochemical immunoassay strategy for the detection of JEV. As shown in Fig. 1, gold-coated magnetic beads (GMBs) were chosen as the platform for the immobilization and the selective capture (step 1–3). After the immunoreactions, multiwalled carbon nanotubes (MCNTs) were mixed with immunocomplex-coated GMBs (IGMBs) to improve the performance of the assay (step 4). Then, the mixture was captured and focused on the detection surface with help of magnetic gold electrode, and the electrochemical detection was carried out with the use of H₂O₂-hydroquinone-HRP system (step 5). Finally, this assay was applied in clinical diagnosis of JEV.

2. Experiments

The magnetic electrode (Supplementary Fig. S1) was developed in our lab for the assay. It consists of a neodymium hollow cylinder magnet (6 mm diameter x 52 mm length) that was press-fitted into a PTFE cylindrical tube (10 mm diameter x 65 mm length). One end of the PTFE cylindrical tube was packed with gold to about 5 mm depth and 3 mm diameter. A silver wire was then fitted through the neodymium magnet hole and was connected with the gold for electric conductivity. The apparatus, reagents and methods are described in detail in the Supplementary Content section.

3. Results and discussion

3.1. Characterization of the magnetic gold electrode

The internal resistances of the magnetic gold electrode and a normal gold electrode were examined, respectively. The difference
was found not to be significant, which indicates that this magnetic gold electrode had good electric conductivity. The magnetic field strengths measured at 0, 1.5 and 5 mm from the centre of the electrode surface were 35.9, 34.7, and 22.3 mT, respectively. The strength of the central magnetic field was moderate, and higher than that of the surrounding field. This fact allowed IGMBs to be well captured and focused on the detection surface (Supplementary Fig. S2), making the process of collection and detection readily available and simultaneously performed on the same platform. In addition, electrode regeneration can be easily achieved, i.e., removing IGMBs away from the detection surface without the need for the magnet to be taken out from the PTFE tube. These factors demonstrate that the magnetic gold electrode was robust, user-friendly, and show a commercial promise for electrochemical immunoassay.

3.2. Optimization of the experimental conditions

Taking account of biosafety, the envelope (E) protein, a major immunogenic protein of JEV, was utilized to produce monoclonal antibodies (2A2 and 4D1) (Li et al., 2010), optimize the assay parameters and evaluate the performance of this assay. Due to the inert nature of most MBs, 10–75 μg of GMBs were selected and examined as the platform. As expected, the sensitivity rose with an increase of the GMBs and up to 40 μg. Excess GMBs was not necessary, because it was not in close contact with the electrode surface, even minimized the electric conductivity of the assay. The amount of 40 μg was, thus, chosen as a compromise for the subsequent experiment.

Cyclic voltammograms (CVs) analysis using [Fe(CN)₆]³⁻/⁴⁻ redox probe was used to characterize this immunoassay. As shown in the Supplementary Fig. S3, strong declines in electric conductivity were observed when biomolecules (4D1 IgG and immunocomplex) attached on the surface of GMBs. To address this problem, MCNTs that possess high roughness and area, unique electronic were selected to improve the assay. After the immunoreaction, MCNTs were mixed with IGMBs, and then the poorly wrapped MCNTs were removed by washing. Transmission Electron Microscope (TEM) was used to describe the morphology of the IGMBs with and without MCNTs, respectively. The structure of IGMBs in the presence of MCNTs (Supplementary Fig. S4(b)) is more incompact than that of IGMBs (Supplementary Fig. S4(a)), owing to the fact that MCNTs can nonspecifically absorb onto the protein surface (Baldrich et al., 2011). The CVs in Fig. 2a shows that ΔEₚ became smaller and the redox peak currents increased dramatically when MCNTs were added. This demonstrates that MCNTs can obviously enhance the electron transfer between the redox probe and the electrode (Yang et al., 2010). The influence of the amounts of MCNTs (0–15 μg) on sensitivity was examined by differential pulse voltammograms (DPVs) in H₂O₂-hydroquinone-HRP system. As shown in Fig. 2b, the cathodic currents enhanced with an increase of the amount of MCNTs from 0–12 μg. The best signal-to-noise ratio could be attained when the amount of MCNTs was 12 μg (Fig. 2c). Further increasing the amount of MCNTs leads to IGMBs partially confined on the detection surfaces, and results in the declines of cathodic currents and the signal-to-noise ratio (Fig. 2 b and c). MCNTs at 12 μg were therefore, selected for the subsequent assay.

The concentration of mediator, H₂O₂ and PBS solution, as well as the pH of PBS solution were further investigated. Optimization of electrochemical detection was achieved in the 10 mmol/L PBS solution (pH = 7.0) containing 3 mmol/L hydroquinone and 0.8 mmol/L H₂O₂.

3.3. Performances of the immunoassay

Under optimal conditions, the increase of the cathodic peak current in the DPV response was proportional to the logarithm of the concentration of E protein in the range from 0.84 to 11200 ng/mL (Supplementary Fig. S5(a)). The linear regression equation is \( i = 5.4387 \log_{C_{\text{protein}}} + 6.2031 \) (\( R^2 = 0.9934 \)), where \( i \) is the increase of cathodic current and \( C_{\text{protein}} \) is the concentration of E protein in ng/mL. The detection limit is 0.56 ng/mL (S/N = 3). The reproducibility and repeatability of the developed assay were also examined. A series of repetitive measurements of 56 ng/mL E protein gave sensor-to-sensor reproducible results with a relative standard deviation (RSD) of 7.64% (n = 11) for this biosensor.
This suggested a good reliability of the MBs-based electrochemical immunoassay. The repeatability of the electrochemical detections for 56 ng/mL E protein was checked by taking the reduction peaks of the immunoassay. The repeatability of the electrochemical detections for clinical samples. Under the optimization conditions, JEV at 1.0×10^6 PFU/mL was serially diluted and tested. The results show that the increase of the cathodic current was proportional to the logarithm of the titer of JEV in the range of 2×10^3 to 5×10^5 PFU/mL (Supplementary Fig. S5(b)). The linear regression equation is \( i = 7.1232 \times 10^{-4} \times C_{JEV} – 20.423 \) (\( R^2 = 0.9816 \)), where \( i \) is the increase of cathodic current and \( C_{JEV} \) is the titer of JEV in PFU/mL. The relative standard deviation of this approach was 9.38% (\( n = 11 \)) with the detection of 5×10^3 PFU/mL JEV. The minimal amount that could be detected was 2.0×10^3 PFU/mL which is 2 orders of magnitude lower than that of immunochromatographic strip (ICS) and similar to that obtained from RT-PCR (Li et al., 2010).

### 3.4. Application of the immunoassay in clinical diagnosis

The magnetic electrochemical immunoassay strategy was applied in JEV diagnostic test of clinical samples. Under the optimization conditions, JEV at 1.0×10^6 PFU/mL was serially diluted and tested. The results show that the increase of the cathodic current was proportional to the logarithm of the titer of JEV in the range of 2×10^3 to 5×10^5 PFU/mL (Supplementary Fig. S5(b)). The linear regression equation is \( i = 7.1232 \times 10^{-4} \times C_{JEV} – 20.423 \) (\( R^2 = 0.9816 \)), where \( i \) is the increase of cathodic current and \( C_{JEV} \) is the titer of JEV in PFU/mL. The relative standard deviation of this approach was 9.38% (\( n = 11 \)) with the detection of 5×10^3 PFU/mL JEV. The minimal amount that could be detected was 2.0×10^3 PFU/mL which is 2 orders of magnitude lower than that of immunochromatographic strip (ICS) and similar to that obtained from RT-PCR (Li et al., 2010).

As a potential preliminary screening tool for JEV, ICS has the characteristics of rapid, inexpensive and easy of handling. The major limitation of ICS is its low sensitivity which prevents the use of ICS for confirmatory diagnosis of JEV. At present, RT-PCR with remarkable sensitivity is generally recognized as gold standard in JEV confirmatory diagnosis. Nevertheless, its inherent drawbacks such as the high cost and the requirement of sophisticate instrument limit the prevalence. The method developed in this study is rapid, sensitive, convenient, compact and cost-effective, indicating that it can be employed as a promising fast screening and confirmatory diagnostic method for the detection of JEV.

Several viruses associated with epizootic viral diseases in swine production were used as nonspecific virus samples to investigate the specificity for the diagnosis of JEV (Supplementary Fig. S6). As expected, the negative results were obtained from pseudorabies virus (PRV), porcine circovirus (PCV), porcine parvovirus (PPV), porcine respiratory syndrome virus (PPRSV), classical swine fever virus (CSFV) and swine influenza virus (SIV), suggesting that this assay has high specificity for JEV.

The clinical specimens (n = 60), collected from mosquitoes, swine (brain tissue), and the human patients (the cerebrospinal fluid), were detected by this method and RT-PCR, respectively. As shown in Table 1, the proposed method had an efficiency of 95% compared to the results of RT-PCR. This is telling that the proposed method can be satisfactorily used to detect JEV in clinical diagnostic test.

### 4. Conclusions

In summary, we have reported a novel MBs-based electrochemical immunoassay strategy for the diagnosis of JEV based on the homemade magnetic gold electrode for the capture and direct sensing. It effectively reduces the complexity and time required for sensing applications. MCNTs were successfully introduced to improve the electric conductivity and enhance the sensitivity. The presented strategy was applied in the clinical diagnosis of JEV, and had a good diagnostic agreement with the results from RT-PCR. This impressive, 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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### Supplementary data


### References


| Table 1 | Comparative results of the proposed method and RT-PCR of 60 clinical samples. |
|---------|--------------------------------------|------------------|------------------|
| RT-PCR  | Positive | Negative | Total |
| The magnetic electrochemical assay | 17 | 0 | 17 |
| Negative | 3 | 40 | 43 |
| Total | 20 | 40 | 60 |

The efficiency value was calculated using following equations (Shukla et al., 2009): Efficiency = (TP + TN) × 100/Total = 95.0 (TP = 17, TN = 40, Total = 60). TP: true positive; TN: true negative.