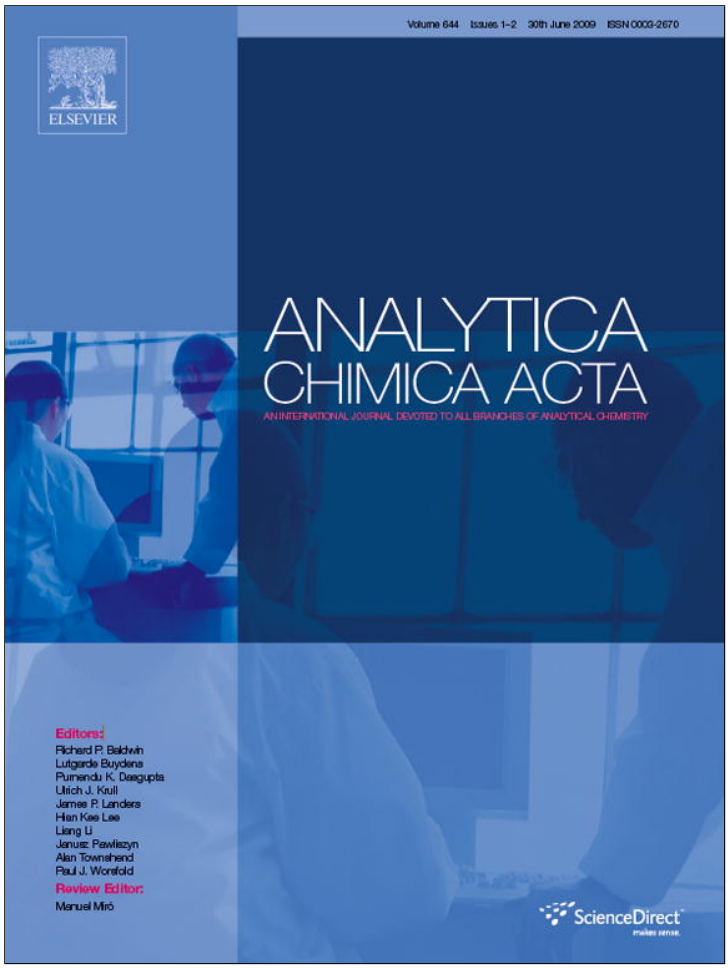

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A rapid and simple method for ultrasensitive electrochemical immunoassay of protein by an electric field-driven strategy

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ABSTRACT

A sensitive, rapid and simple method for analysis of protein was proposed by introducing an electric field-driven technique to the incubation process of immunoassay and immobilizing horseradish peroxidase (HRP) labeled antibody in a newly designed gel matrix to construct an immunosensor. With an electric field-driven technique, the incubation for immuno-recognition could be completed within 2 min. The immobilized HRP showed excellent direct electrochemistry in this new gel matrix, and the detection procedure was greatly simplified by directly monitoring the sensitive electrochemical signal of HRP upon the immunoreaction. Using α-fetoprotein as a model the linear detection range was from 0.02 to 2.0 ng mL^{-1} with a detection limit down to 138 amol mL^{-1}. The immunosensor and the proposed detection method showed good specificity and acceptable stability and accuracy.

1. Introduction

The detection of low-abundant proteins is facing a tremendous challenge in life science. For early diagnosis of disease, finding of biomarkers and proteome research, sensitive detection of proteins with a fast and simple process is essential and has attracted considerable interest [1]. Many strategies have been developed for sensitive measurement of proteins, such as the employment of DNA as amplified signal reporter [2–4], the use of enzyme-bioconjugated nanotube or nanosphere as signal transfer labels [5–7], aptamer-based amplification technology [8–10] and the utility of redox cycling reaction for signal amplification [11,12]. Although these methods can get very low detection limit, most of them focus on the amplification of detection signal, which leads to complex conjugation or detection procedures. In addition, a long analytical time is also inevitable. The biosensing techniques for protein detection are based on the recognition of immobilized biomolecules to target analytes, which generally needs a long incubation time due to the slow diffusion of analyte through an unstirred layer [13]. Both the complex testing process and the long detection time have become general choke points encountered in protein analysis, including immunoassay and other sensitive protein detection techniques.

Enrichment of low-abundant proteins during the analytical process is an attractive strategy for their detection [8,14]. Electric field-driven method, which was early used to control nucleic acid hybridization [15–18], is an excellent enrichment method for low-abundant proteins and has been successfully used to accelerate the transport of proteins in fluorescent [19], UV-absorption [20] and charge-coupled device camera [13,21] immunoassay. In these assays, the charged protein could be concentrated by a local electric field in the vicinity of the membrane surface or capture molecule coated micro-locations, leading to a boosting sensitivity and a rapid recognition process. Obviously, besides the quantitative detection system, these accelerating techniques demand additional potential supply equipment to produce electric field, which results in increased cost and inconvenience. This work combined the electric field-driven method with reagentless electrochemical immunosensing to develop a sensitive, fast and simple immunoassay method. For the intrinsic nature of electrochemistry, the electric field for the enrichment and accelerating transport of charged proteins can be easily produced by the quantitative detection instrument.

The reagentless immunosensors have attracted considerable interest due to their simplicity and low cost for immunoassay in different fields. The electron transfer rate of immobilized redox label and the permeability of the matrix for immobilization of proteins are two key factors for sensitive and fast detection. Several sol–gel matrices have been developed for preparation of reagentless immunosensors, including titania sol–gel [22,23], gold nanoparticles/titania sol–gel composite [24], organically modified silicate

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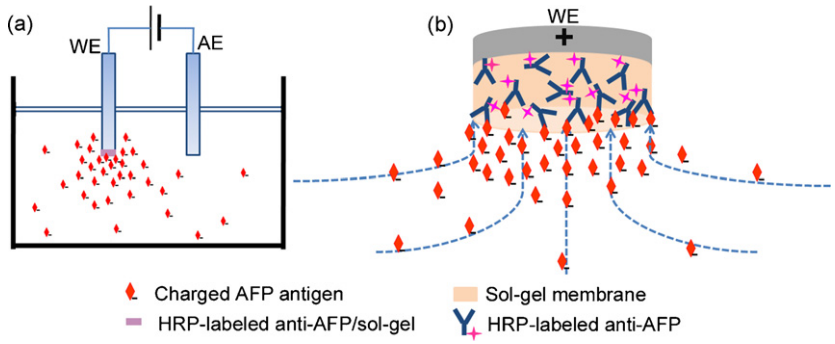

Scheme 1. (a) Schematic representation of the protein concentration in an electric field-driven incubation process, and (b) protein transport for electric field-driven immunoreaction on immunosensor surface.

(ormosil) sol–gel formed in presence of NaOH as catalyst [25], and chitosan/silica sol–gel matrix formed in presence of HCl as catalyst [26]. In these matrices the label, horseradish peroxidase (HRP), on proteins shows the direct electrochemical signal, which decreases upon the formation of immunocomplexes. To improve the electron transfer, gold nanoparticles have been mixed in the sol–gel [24,25]. However, both the sensitivity and the mass transport rate of analytes in these matrices need to be further improved for practical application in fast protein analysis. In this work the conditions for synthesis of a stable and biocompatible ormosil matrix was further optimized. Based on the monitoring of sensitive direct electrochemical response of HRP in the optimal ormosil matrix and the effective concentration of the protein to immunosensor surface in a constringent electric field, a greatly simplified strategy was proposed for sensitive and rapid immunoassay of proteins (Scheme 1). The constringent electric field created by a low driving potential, greatly accelerated the molecular recognition and amplified the detection signal. This proposed method provided a powerful tool for simple, rapid and sensitive detection of low-abundant proteins.

2. Experimental

2.1. Materials and reagents

α-Fetoprotein (AFP) enzyme-linked immunosorbent assay (ELISA) kits, containing a series of AFP standard solutions from 0 to 1000 ng mL^{-1}, and a stock solution of HRP labeled AFP monoclonal antibody (HRP-anti-AFP) from mouse, were purchased from BioCheck, Inc. (Foster City, USA). Human serum chorionic gonadotrophin (hCG), carcinoma antigen 125 (CA 125) and carcinoembryonic antigen (CEA) were obtained from their assay kits purchased from BioCheck, Inc. (USA, for hCG) and CanAg Diagnostics AB (Sweden, for CA 125 and CEA). Aminopropyl triethoxysilane (APTES) was from Sigma Chemical Company (St. Louis, MO, USA). All other reagents including tetraethyoxysilane (TEOS) were of analytical grade. Phosphate buffer solution (PBS) was prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4. Doubly distilled water was used in all experiments.

2.2. Preparation of the AFP immunosensor

The homemade graphite electrode (GE, 4 mm in diameter) was polished to a mirror-like phase using 0.3 and 0.05 μm alumina slurry (Buehler) followed by thoroughly rinse with doubly distilled water. After sonicated successively in 1:1 nitric acid, acetone and twice distilled water, the electrode was rinsed with doubly distilled water and allowed to dry at room temperature. The ormosil sol was prepared by mixing 100 μL of APTES, TEOS and 10 mM NaOH as catalyst and 1000 μL H_2O in a small test tube under stirring for 5 min at room temperature. To immobilize HRP-anti-AFP in the gel matrix, the HRP-anti-AFP stock solution offered in ELISA kits was mixed completely with as-obtained ormosil sol in a volume ratio of 1:1, and 4 μL of the mixture was dropped on the pretreated GE surface and allowed to dry under ambient condition for 12 h. After twice rinsing the modified electrode with doubly distilled water, the immunosensor, HRP-anti-AFP/ormosil gel/GE, was obtained. When not in use, it was stored in desiccator at room temperature.

2.3. Apparatus

Electrochemical measurements were performed on a BAS Epsilon-EC electrochemical workstation using a conventional three-electrode system (Bioanalytical Systems, Inc., USA). The electric field-driven incubation process was carried out in a two-electrode system on this workstation. Scanning electron microscope (SEM, HITACHI S-3400, Japan) was used for morphological analysis of different films at 20 kV.

2.4. Analytical procedure

The direct electrochemical signal of the immobilized HRP labeled to antibodies was firstly measured in 1 mL 0.1 M pH 7.0 PBS with differential pulse voltammetry (DPV) from 200 to −250 mV (*vs.* $Hg/HgCl_2$) at a pulse amplitude of 50 mV, a pulse width of 50 ms and a scan rate of 50 mV s^{-1}. Afterwards, 4 μL certain concentration of AFP or sample was dropped in this PBS and mixed completely, subsequently applying a potential of +0.5 V on the immunosensor for 2 min to perform the proposed electric field-driven incubation. After the active incubation process, the direct electrochemical signal of HRP at the resulting immunosensor was read again in this solution with the same detection process. The decrease of the peak current was proportional to the concentration of antigen in the incubation mixture.

3. Results and discussion

3.1. Fast electrochemical immunoassay

The detection signal was derived from the reduction of immobilized HRP from its resting state (Fe(III)) to (Fe(II)). The one-step immunoassay including incubation and detection was accomplished in the same electrochemical cell containing 0.1 M pH 7.0 PBS and the analyte. Because the isolated point of most proteins are between 4 and 6, the proteins will be negatively charged in pH 7.0 PBS. During incubation, applying a positive potential on the immunosensor, an electric field will be generated in its vicinity, and the negative charged antigen molecules could be quickly travelled to the electrode surface under the electrophoretic force, which also increased the antigen concentration near the surface. In this work, AFP, whose isolated point is around 5, was used

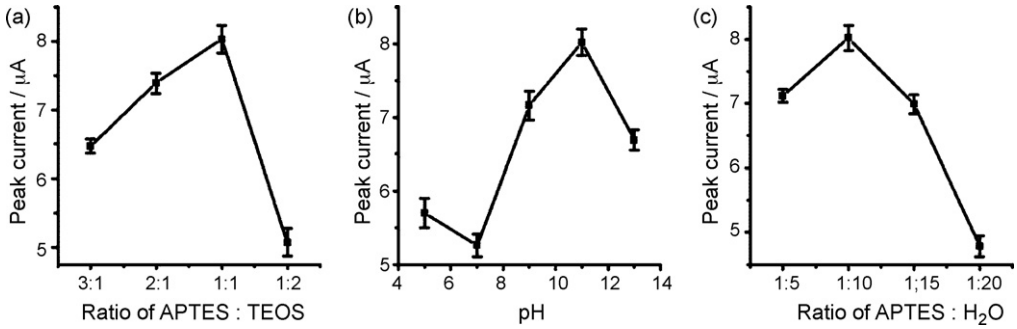

Fig. 1. Dependence of DPV peak current for direct electron transfer of the HRP on volume ratio of APTES:TEOS (a), pH of the solution before reaction (b) and volume ratio of APTES:H_2O (c) for fabrication of the ormosil sol.

as a model analyte. As shown in Scheme 1b, stronger electric field and electrophoretic force could be produced on the interface closer to immunosensor. The constringent electric field and transport layer concentrated and brought more antigen molecules from bulk solution to the gel matrix, which was proved in the electrophoresis-assisted immunoassay [20], leading to extremely high concentration efficiency and detection sensitivity. In addition, the external force could shorten the transport time of antigen to immunosensor surface, leading to an accelerated specific combination and a very rapid detection procedure. Upon the formation of the HRP-Ab/Ag immunocomplex, the electron transfer between HRP and the electrode was blocked, leading to a decreased direct electrochemical signal. The signal decrease was proportional to the amount of formed immunocomplex and original analyte concentration. Therefore, the concentration of the analyte could be determined by monitoring the signal change resulted from the blocking to direct electrochemistry of HRP.

3.2. Optimal conditions for AFP immunosensor preparation

The ratio of hydrophilic to hydrophobic monomers in ormosil gel is crucial in preparing biocompatible matrices and designing biosensors. The structure, permeability and properties of doped sol–gels also depends on the operational factors such as water/silica molar ratio, solvent, catalyst, and pH, which greatly influence the hydrolysis and condensation. In this system, the condition optimization for preparation of ormosil gel was carried out by monitoring the direct electrochemical signal of HRP immobilized in the resulting immunosensor. When the as-prepared immunosensor showed the maximum response, it could provide the best sensitivity for specific recognition of the given analyte. As shown in Fig. 1, the optimal conditions for ormosil sol preparation were at the volume ratios of 1:1:1:10 of APTES:TEOS:10 mM NaOH:H_2O, at which the pH of the mixture before reaction was 11, and the formed ormosil gel membrane was uniform and showed a porous structure with a very narrow particle size distribution (Fig. 2a). The biocompatible porous matrix with the excellent morphology was favour of the immobilization and immunorecognition of HRP-anti-AFP and provided a necessary conduction pathway to assist the direct electron transfer between the enzyme and the electrode. After immobilization step, bright particles of HRP-anti-AFP were evenly distributed in the porous gel membrane (Fig. 2b). After the electric field-driven incubation in 2.0 ng mL^{-1} AFP solution for 2 min, the surface became rough and uneven due to the formation of immunocomplex (Fig. 2c), which blocked the electron transfer between immobilized HRP and sensing surface.

3.3. Optimization of electric field-driven incubation

The concentration and travelling speed of the antigen depended on the driving potential, and pH and salt concentration of incubation solution. These conditions could be optimized by monitoring the decrease of DPV peak current of the HRP upon an incubation step of 2 min. In 0.1 M pH 7.0 PBS, at which the antigen molecules are negatively charged, with the positive increase of driving potential, the peak current decreased sharply, indicating that the formation of immunocomplex on immunosensor surface was faster at more positive driving potential (Fig. 3a). However, at the driving potentials higher than +0.5 V the decrease of peak current decreased slightly and trended to a steady value, indicating a little effect of the more positive driving potential on immunoreaction efficiency. Also, for avoiding the interference from the oxidation of reductive species at a higher positive potential, +0.5 V was chosen as the optimal driving potential. At this driving potential the pH and salt concentration of the incubation solution were optimized.

As shown in Fig. 3b, the peak current decreased and reached relatively stable values in the pH range of 7.0 to 8.2, which indicated that the negative charges on the AFP reached saturation at the solution pHs higher than 7.0. So, pH 7.0 PBS was used for the electric field-driven incubation process. Again, at this pH the maximum peak current decrease occurred at the PBS concentration of 0.1 M (Fig. 3c). The smaller change of the peak current at salt concentrations lower than 0.1 M was due to the slow immunoreaction rate, which resulted from the unstable pH and the low dielectric constant in the vicinity of immunosensor. The latter decreased the electric field intensity. At high salt concentration, more electrolyte anions

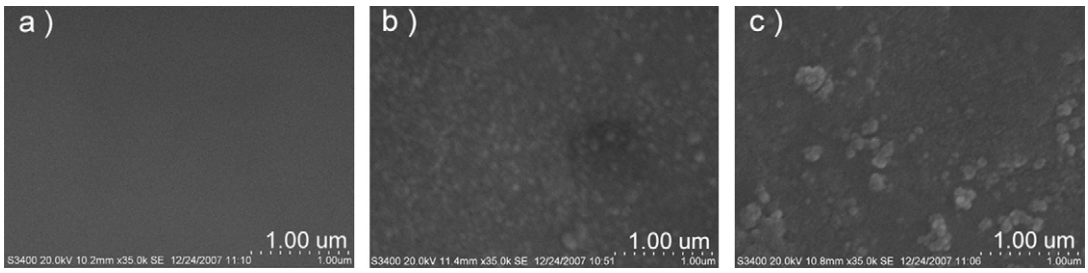

Fig. 2. Scanning electron micrographs of graphitic electrodes coated with ormosil gel (a), HRP-anti-AFP/ormosil gel (b) and AFP-HRP-anti-AFP/ormosil gel (c) at ×35.0 K.

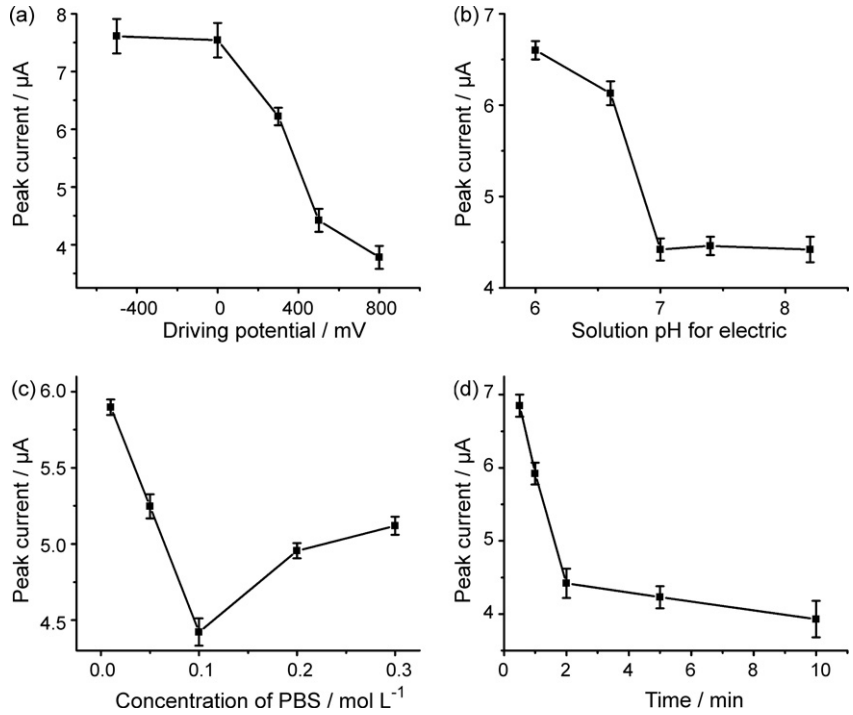

Fig. 3. Effects of driving potential (a), solution pH (b), concentration of PBS (c) and incubation time (d) on the immunoreaction in PBS containing 2.0 ng mL^{-1} AFP at room temperature. When changing one parameter others are in their optimal values.

could be transported to immunosensor surface, which decreased the concentration efficiency of negatively charged antigen.

The electric field-driven incubation time was also a crucial factor. Under optimal conditions, with the increasing incubation time, the DPV peak current of the resulting immunosensor decreased quickly, followed by a slow decrease after 2 min (Fig. 3d), indicating saturated formation of immunocomplex on immunosensor surface. So 2 min was used for electric field-driven incubation. In addition, most enzymes display good activity only in a limited range of pH. Thus the acidity of detection solution could affect the direct electrochemical response of the HRP. Coincidentally, the maximum response of the immunosensor in 0.1 M PBS occurred at pH 7.0 (Fig. 4a). Thus the detection could be performed in the same solution as that for incubation process, leading to the one-step immunoassay.

3.4. Specificity and stability of the immunosensor

Under an electric field-driven mode the specificity of the proposed AFP immunosensor was examined by exposing it to different incubation solutions (Fig. 4b). Only slight difference of the reserved percentage was observed after the immunosensor was incubated in the solution containing other antigens. Thus the cross-reactivity, nonspecific adsorption of other proteins in the optimized gel material or false positive result was negligible, indicating excellent specificity of the immunosensor.

After the immunosensors were refrigerated at +4 °C for a variable time up to 12 weeks, the DPV response remained more than 90% of initial response. Thus, the storage stability of the immunosensor was acceptable.

3.5. Immunoassay of AFP

Under the optimal conditions, the DPV peak current of the immunosensor after electric field-driven incubation decreased linearly with the increasing AFP concentration from 0.02 to 2.0 ng mL^{-1} with an equation of I_p (μA) = 8.04–1.76c (ng mL^{-1}) and a correlation coefficient of 0.997 (Fig. 5). The detection limit corresponding to the signal of 3SD was 9.67 pg mL^{-1} (138 amol mL^{-1}), which was the lowest detectable AFP concentration (Table 1) except

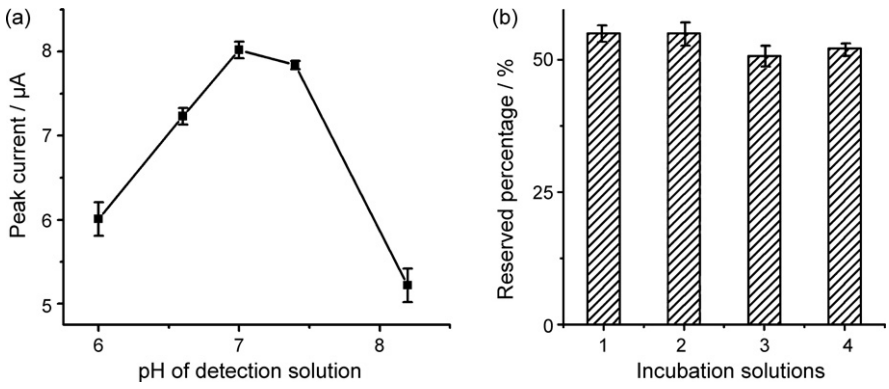

Fig. 4. Effect of pH of detection solution on the direct electrochemical response of HRP-antibody at immunosensor (a), and specific response of the immunosensor to 0.5 M pH 7.0 PBS containing 2.0 ng mL^{-1} AFP (1) and 2.0 ng mL^{-1} AFP + 1.0 mIU mL^{-1} hCG (2), 1.5 ng mL^{-1} CA 125 (3) and 2.0 ng mL^{-1} CEA (4) (b).

Table 1
Analytical performance of AFP immunoassays reported previously.

Ref.	Immunoassay format	Detection method	Time	Linear range	LOD
[28]	Sandwich immunoassay using a microfluidic device	Fluoroimmunoassay	50 min		0.01 ng mL^{-1}
[29]	ArcDia TPX2 binding assay	Fluoroimmunoassay	>2 h		0.41 ng mL^{-1}
[30]	Anion-exchange column chromatography	Fluoroimmunoassay	3 min	0–10 μg mL^{-1}	0.5 ng mL^{-1}
[31]	Capillary electrophoresis on a microchip	Fluoroimmunoassay	>30 min	0–20 nM	300 pM
[32]	Sandwich immunoassay	Fluoroimmunoassay	3 h		44 pg mL^{-1}
[33]	Sandwich immunoassay	Fluoroimmunoassay	2.5 h	15–400 ng mL^{-1}	0.17 nM
[27]	Sandwich immunoassay	Fluoroimmunoassay	>2 h	0–100 pg mL^{-1}	0.04 pg mL^{-1}
[34]	Sandwich non-competitive immunoassay	ICP-MS	>2 h	4.6–500 ng mL^{-1}	1.2 ng mL^{-1}
[35]	Sandwich immunoassay	ICP-MS	>4 h		47.1 ng mL^{-1}
[36]	Direct immunoassay method	QCM immunoassay	>30 min	0.1–100 ng mL^{-1}	
[37]	Sandwich heterogeneous immunoassay	Electrochemical immunoassay	2 h	0.3–100 ng mL^{-1}	0.16 ng mL^{-1}

one sandwich time-resolved fluorometric immunoassay with two one-hour incubation steps and a series of rinsing steps using a core-shell-type fluorescent nanosphere as a label [27]. The sensitivity was also close to that for PSA with a 75-min incubation step by combining carbon nanotube amplification and enzymatic cycle [6]. In this work, because the incubation process could be completed in 2 min with the benefit of electric field-driven strategy, the whole assay time for protein could be shortened to 3 min. It could be seen from Table 1 that the proposed system was the fastest one for sensitive detection of protein at low concentration.

The inter-assay precision of the immunosensor preparation was evaluated using three immunosensors prepared at the same electrode. The coefficients of variation for inter-assay were 8.7% and 10.6% for 0.08 and 0.6 ng mL^{-1} AFP (4 μL of 20 and 150 ng mL^{-1} AFP solution was mixed with 1.0 mL PBS), respectively, indicating acceptable fabrication reproducibility. In this work new immunosensor was always used for another measurement due to the formation of immunocomplex.

3.6. Application in detection of serum tumor marker

When using this method for clinic test, a dilution of serum sample with 250 times 0.1 M pH 7.0 PBS (v/v) was needed, at which the detectable linear range could be extended to 5.0–500 ng mL^{-1} for practical clinic application. 4 μL of serum samples were mixed with 1 mL 0.1 M pH 7.0 PBS, and the mixtures were then used as the incubation solutions for the electric field-driven incubation. The accuracy of AFP detection with the proposed immunosensor was examined by comparing the results obtained from five sera with this method and the commercial electrochemiluminescent analysis performed with Elecsys 2010 analyzer (Roche). The proposed method showed that the AFP levels in the five patients were 7.2, 67, 131, 227 and 409 ng mL^{-1}, respectively, while the results gained from the commercial test were 8.25, 58, 144, 202 and 398 ng mL^{-1}, respectively. The standard deviations of both data were between 2.7% and 15.6%, which indicated an acceptable accuracy of the proposed method.

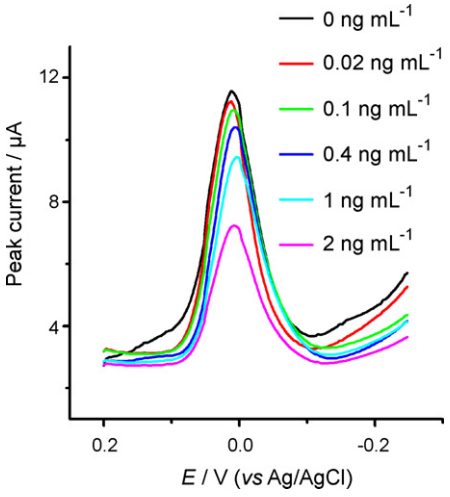

Fig. 5. DPV responses for immunoassay of AFP at different concentrations with an electric field-driven incubation at +0.5 V for 2 min.

4. Conclusions

A very simple, extremely fast and sensitive electrochemical immunoassay for low-abundant protein is proposed by combining electric field-driven strategy with electrochemical immunosensing. This method can detect AFP concentration down to 138 amol mL^{-1} in one step with a whole analytical time of less than 3 min. The designed biocompatible ormosil gel provides a porous structure and pleasant surroundings for fast immuno-recognition and a necessary conduction pathway for direct electron transfer between the HRP labeled to antibody and the electrode, producing a reagentless immunoassay procedure. The immunosensor for protein shows good stability and acceptable accuracy. The proposed method shows excellent practicability and provides a new promising platform for rapid detection of low-abundant proteins and biomarkers for clinic early screening.

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