Anal. Chem. **2008,** *80,* 6072–6077

Electric Field-Driven Strategy for Multiplexed Detection of Protein Biomarkers Using a Disposable Reagentless Electrochemical Immunosensor Array

Jie Wu,[†] Yuetian Yan,[†] Feng Yan,*[,‡] and Huangxian Ju*[,†]

Key Laboratory of Analytical Chemistry for Life Science (Ministry of Education of China), Department of Chemistry, Nanjing University, Nanjing 210093, and Jiangsu Institute of Cancer Research, Nanjing 210009, P.R. China

A fast, simple, sensitive, and low-cost method for electrochemical multianalyte immunoassay was developed by combining newly designed electric field-driven incubation with a screen-printed reagentless immunosensor array. The disposable array was prepared by immobilizing respectively horseradish peroxidase (HRP)-labeled antibodies modified gold nanoparticles in biopolymer/sol−gel modified electrodes to obtain direct electrochemical responses of HRP. Upon the formation of immunocomplexes, the responses decreased due to increasing spatial blocking and impedance. At a driving potential of 0.5 V, the incubation process could be accomplished within 2 min. Under optimal conditions, this method could simultaneously detect carbohydrate antigens 153, 125, and199 and carcinoembryonic antigens ranging from 0.084 to 16, 0.11 to 13, and 0.16 to 15 U mL^{-1} and 0.16 to 9.2 ng mL^{-1} with a detection time of less than 5 min, and the detection limits corresponding to the signals of 3SD were 0.06, 0.03, and 0.10 U mL^{-1} and 0.04 ng mL^{-1}, respectively. The disposable immunosensor array and simple detection system for fast measurement of panels of tumor markers show significant clinical value for application in cancer screening and provide great potential for convenient point-of-care testing and commercial application.

Immunoassay is one of the most effective approaches for specific detection of proteins and has been used extensively for disease diagnoses.[1–3] Although many immunoassay methods that can realize ultrasensitive,[4,5] label-free,[6,7] simple,[8] or high-throughput[9] detection of a single protein have been well developed, their applications in disease diagnosis are facing great challenge because most protein biomarkers are not specific to a particular disease, particularly tumor, and most cancers have more than one biomarker associated with their incidence. Thus, the development of a multiplexed immunoassay for panels of biomarkers possesses important significance for diagnostic cancer screening.[10–14]

Multiplexed immunoassay is a promising analytical method in protein analysis with the advantages of shortened analysis time, simplified analytical procedure, decreased sampling volume, improved test efficiency, and reduced cost as compared to parallel single-analyte assays. In recent years, many immunosensor arrays, particularly optical immunosensor arrays,[15–18] have been developed for accurate, simple, and sensitive multiplexed detection of protein biomarkers; however, expensive array detectors, for example, the charge-coupled device camera, are needed for optical detection. For practical clinical application, now tremendous efforts have been focused on developing low-cost and portable immunosensor arrays. Electrochemical immunosensor array (ECIA), which distinguishes itself by convenient miniaturization and low cost of the entire assay, can be an ideal alternative.

Up to now, different ECIAs have been prepared for multiplexed electrochemical immunoassay.[19–24] For example, Kojima et al.[19]

* To whom correspondence should be addressed. Tel./Fax: +86-25-83593593. E-mail: yanfeng2007@sohu.com; hxju@nju.edu.cn.

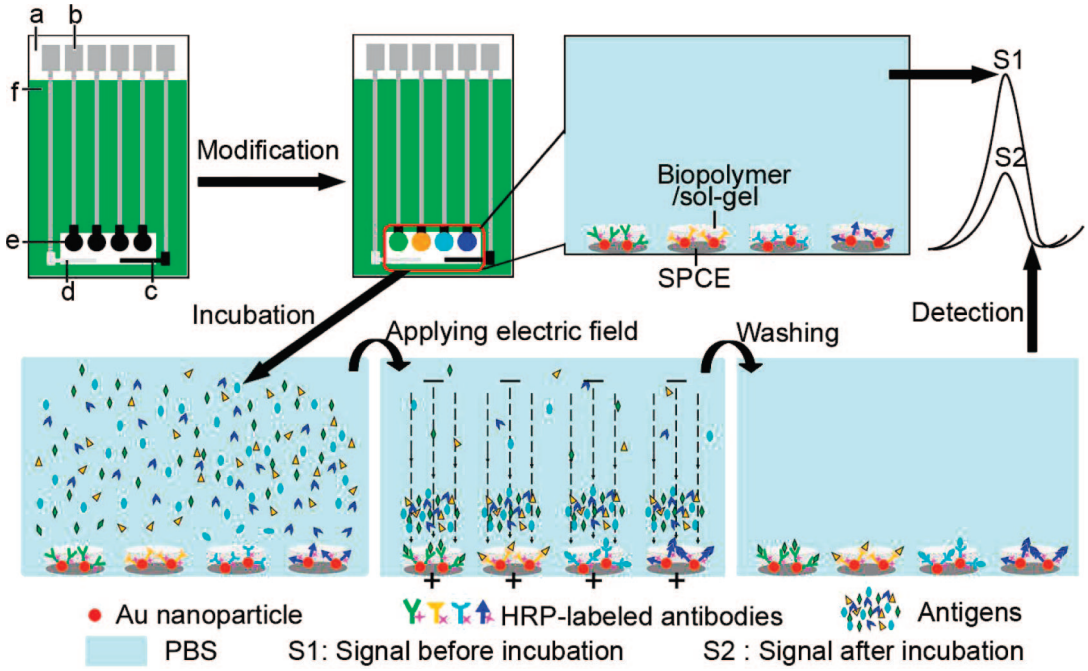
[†] Nanjing University.

[‡] Jiangsu Institute of Cancer Research.

(1) Andreescu, S.; Sadik, O. A. *Pure Appl. Chem.* **2004**, *76*, 861–878.
(2) Lin, J. H.; Ju, H. X. *Biosens. Bioelectron.* **2005**, *20*, 1461–1470.
(3) Rasooly, A.; Jacobson, J *Biosens. Bioelectron* **2006**, *21*, 1851–1858.
(4) Yu, X.; Munge, B.; Patel, V.; Jensen, G.; Bhirde, A.; Gong, J. D.; Kim, S. N.; Gillespie, J.; Gutkind, J. S.; Papadimitrakopoulos, F.; Rusling, J. F. *J. Am. Chem. Soc.* **2006**, *128*, 11199–11205.
(5) Tang, D. P.; Yuan, R.; Chai, Y. Q. *Anal. Chem.* **2008**, *80*, 1582–1589.
(6) Kerman, K.; Nagatani, N.; Chikae, M.; Yuhi, T.; Takamura, Y.; Tamiya, E. *Anal. Chem.* **2006**, *78*, 5612–5616.
(7) Okuno, J.; Maehashi, K.; Kerman, K.; Takamura, Y.; Matsumoto, K.; Tamiya, E. *Biosens. Bioelectron.* **2007**, *22*, 2377–2381.
(8) Liu, X.; Dai, Q.; Austin, L.; Coutts, J.; Knowles, G.; Zou, J. H.; Chen, H.; Huo, Q. *J. Am. Chem. Soc.* **2008**, *130*, 2780–2782.
(9) Wang, J.; Liu, G. D.; Wu, H.; Lin, Y. H. *Small* **2008**, *4*, 82–86.
(10) Wilson, D. S.; Nock, S. *Angew. Chem., Int. Ed.* **2003**, *42*, 494–500.
(11) Seidel, M.; Gauglitz, G. *TrAC-Trends Anal. Chem.* **2003**, *22*, 385–394.
(12) Haab, B. B. *Mol. Cell. Proteomics* **2005**, *4*, 377–383.
(13) Wang, J *Biosens. Bioelectron* **2006**, *21*, 1887–1892.
(14) Wu, J.; Fu, Z. F.; Yan, F.; Ju, H. X. *TrAC-Trends Anal. Chem.* **2007**, *26*, 679–688.
(15) Cho, E. J.; Tao, Z.; Tehan, E. C.; Bright, F. V. *Anal. Chem.* **2002**, *74*, 6177–6184.
(16) Knecht, B. G.; Strasser, A.; Dietrich, R.; Märtlbauer, E.; Niessner, R.; Weller, M. G. *Anal. Chem.* **2004**, *76*, 646–654.
(17) Barry, R.; Soloviev, M. *Proteomics* **2004**, *4*, 3717–3726.
(18) Stoeva, S. I.; Lee, J. S.; Smith, J. E.; Rosen, S. T.; Mirkin, C. A. *J. Am. Chem. Soc.* **2006**, *128*, 8378–8379.
(19) Kojima, K.; Hiratsuka, A.; Suzuki, H.; Yano, K.; Ikebukuro, K.; Karube, I. *Anal. Chem.* **2003**, *75*, 1116–1122.
(20) Wilson, M. S. *Anal. Chem.* **2005**, *77*, 1496–1502.
(21) Wilson, M. S.; Nie, W. Y. *Anal. Chem.* **2006**, *78*, 2507–2513.
(22) Wilson, M. S.; Nie, W. Y. *Anal. Chem.* **2006**, *78*, 6476–6483.
(23) Wu, J.; Tang, J. H.; Dai, Z.; Yan, F.; Ju, H. X.; Murrc, N. E. *Biosens. Bioelectron.* **2006**, *22*, 102–108.

10.1021/ac800905k CCC: $40.75
Published on Web 07/02/2008

Scheme 1. Schematic Representation of ECIA and the Electrochemical Multiplexed Immunoassay with an Electric Field-Driven Incubation Process. (a) Nylon Sheet, (b) Silver Ink, (c) Graphite Auxiliary Electrode, (d) Ag/AgCl Reference Electrode, (e) Graphite Working Electrode, and (f) Insulating Dielectric



fabricated a pioneer ECIA with 6 × 6 immunosensors for detection of two tumor markers, and Wilson[20–22] reported a series of ECIAs and realized the simultaneous quantitative detection of seven tumor markers.[22] These ECIAs were prepared by immobilizing immunoreagents on photolithographic and sputter-deposited meal layers adhered on a glass substrate. Recently, our group immobilized the electron-transfer mediators and antigens on a screen-printed carbon electrode array to develop two types of disposable ECIAs for low-cost multiplexed immunoassay of proteins.[23,24] These ECIAs could completely avoid electrochemical cross talk due to the settled electrochemical mediator−enzymatic cycle. However, the detection process needed the help of electron-transfer mediators and the participation of additional substrate, and more importantly, the multiplexed immunoassay needed long incubation time due to the slow diffusion of analyte through an unstirred layer to form the immunocomplex. These disadvantages limited greatly their application in disease diagnosis.

Many technologies have been explored to accelerate the immunoreaction on the immunosensor surface, such as magnetic stirring,[25] low-power microwave radiation,[26,27] and magnetic field combined with superparamagnetic labels.[28] An electrophoresis-assisted optical immunoassay has also been successfully developed for accelerating the transport of protein.[29–32] All these incubation processes need additional equipment, leading to the increased cost and inconvenience. Here, we made use of the electrochemical analyzer to integrate the newly designed electric field-driven strategy with a novel reagentless ECIA and developed a fast, sensitive, and simple electrochemical multiplexed immunoassay method. Using carbohydrate antigens 153, 125, and 199 (CA 153, CA 125, CA 199) and carcinoembryonic antigen (CEA) as model analytes, their antibodies labeled with horseradish peroxidase (HRP) were immobilized in biopolymer/sol−gel modified electrodes of a screen-printed carbon array, respectively. In the presence of gold nanoparticles, the HRP could show enhanced direct electrochemical responses; and the formation of immunocomplexes led to a decrease in the electrochemical signals due to the increasing spatial blocking and impedance caused by the nonconductive immunocomplexes, which blocked the electron transfer between the electrode and HRP-labeled antibodies.[33–36] The ECIA was disposable and reagentless, and benefiting from the electric field-driven incubation strategy, the whole detection of four protein biomarkers could be completed in less than 5 min. Thus, this method showed a great potential for convenient clinical and commercial applications.

EXPERIMENTAL SECTION

Reagents. CA 153, CA 125, CA 199, and CEA ELISA kits were purchased from CanAg Diagnostics AB (Gothenburg, Sweden). They consisted of a series of CA 153, CA 125, CA 199, and CEA standard solutions with concentrations from 0 to 250, 0 to 500,

(24) Wu, J.; Yan, F.; Tang, J. H.; Zhai, C.; Ju, H. X. *Clin. Chem.* **2007**, *53*, 1495–1502.
(25) Tanaka, K.; Imagawa, H. *Talanta* **2005**, *68*, 437–441.
(26) Aslan, K.; Geddes, C. D. *Anal. Chem.* **2005**, *77*, 8057–8067.
(27) Previte, M. J. R.; Aslan, K.; Malyn, S. N.; Geddes, C. D. *Anal. Chem.* **2006**, *78*, 8020–8027.
(28) Driskell, J. D.; Uhlenkamp, J. M.; Lipert, R. J.; Porter, M. D. *Anal. Chem.* **2007**, *79*, 4141–4148.
(29) Ewalt, K. L.; Haigis, R. W.; Rooney, R.; Ackley, D.; Krihak, M. *Anal. Biochem.* **2001**, *289*, 162–172.
(30) Morozov, V. N.; Morozova, T. Y. *Anal. Chem.* **2003**, *75*, 6813–6819.
(31) Morozov, V. N.; Morozova, T. Y. *Anal. Chim. Acta* **2006**, *564*, 40–52.
(32) Morozov, V. N.; Groves, S.; Turell, M. J.; Bailey, C. *J. Am. Chem. Soc.* **2007**, *129*, 12628–12629.
(33) Dai, Z.; Yan, F.; Chen, J.; Ju, H. X. *Anal. Chem.* **2003**, *75*, 5429–5434.
(34) Chen, J.; Yan, F.; Dai, Z.; Ju, H. X. *Biosens. Bioelectron.* **2005**, *21*, 330–336.
(35) Tan, F.; Yan, F.; Ju, H. X. *Electrochem. Commun.* **2006**, *8*, 1835–1839.
(36) Tan, F.; Yan, F.; Ju, H. X. *Biosens. Bioelectron.* **2007**, *22*, 2945–2951.

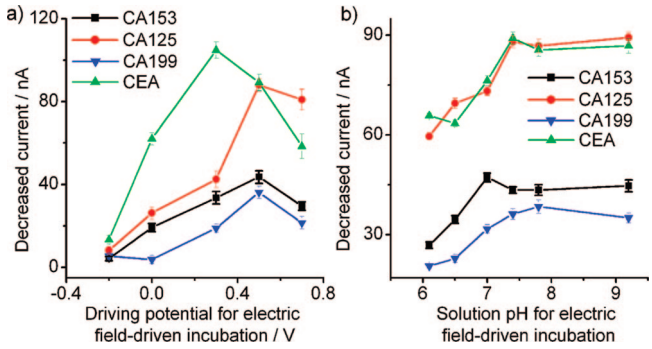


Figure 1. Effects of driving potential (a) at pH 7.4 and solution pH (b) at a driving potential of +0.5 V for electric field-driven incubation of 2 min at room temperature on immunosensing responses in 0.2 M pH 7.4 PBS. The incubation solution is 0.2 M PBS containing 80 U mL^{-1} CA 153, 67 U mL^{-1} CA 125, 77 U mL^{-1} CA 199, and 46 ng mL^{-1} CEA.

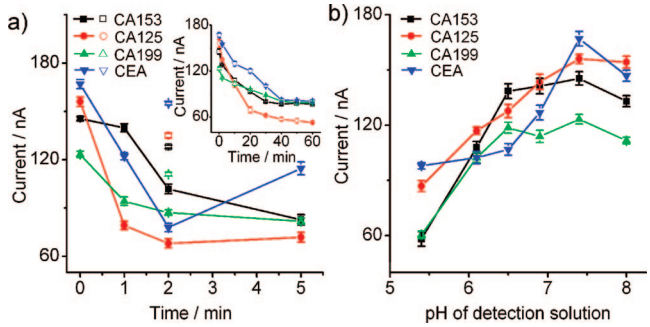


Figure 2. (a) Dependence of DPV peak currents in 0.2 M pH 7.4 PBS on incubation time with (solid symbols) and without (hollow symbols) electric field driving at +0.5 V in 0.2 M pH 7.4 PBS containing 80 U mL^{-1} CA 153, 67 U mL^{-1} CA 125, 77 U mL^{-1} CA 199, and 46 ng mL^{-1} CEA, and (b) effect of pH of detection solution on the direct electrochemical responses of HRP--antibodies at immunosensors for CA 153, CA 125, CA 199, and CEA. Inset in (a): dependence of peak currents on incubation time at the same conditions as (a) without electric field driving.

and 0 to 240 U mL^{-1} and 0 to 75 ng mL^{-1}, and the stock solutions of HRP-labeled CA 153, CA 125, CA 199, and CEA mouse monoclonal antibodies, respectively. Bovine serum albumin, (3-aminopropyl)triethoxysilane, and chitosan (MW ~1×10^6; ~85% deacetylation) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Tetraethyoxysilane (analytical reagent grade) was from Shanghai Chemical Co. All other reagents were of analytical reagent grade and used without further purification. The 0.1 M Tris-HCl (pH 7.2) containing 1% bovine serum albumin was used as blocking buffer. The 0.2 M phosphate-buffered saline (PBS) with various pHs was prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4 and then adjusting the pH with 0.2 M NaOH and H_3PO_4, respectively. Doubly distilled water was used throughout the experiments. Serum specimens from Jiangsu Institute of Cancer Prevention and Cure were stored at 4 °C.

Apparatus. Electrochemical measurements and the electric field-driven incubation process were performed on an eDAQ four-channel potentiostat (eDAQ Co.) equipped with an e-corder system. The control levels of the tumor markers in sera were detected with an automation electrochemiluminescent analyzer (Elecsys 2010, Roche).

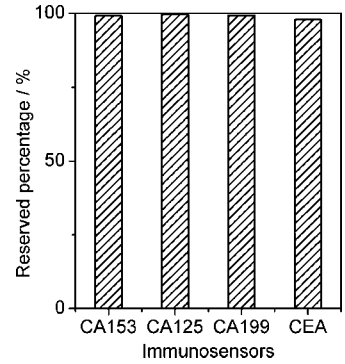


Figure 3. Reserved percentage of the direct electrochemical responses of HRP on four immunosensors after applying a potential of +0.5 V for 10 min in 50 μL of 0.2 M pH 7.4 PBS without the presence of antigen.

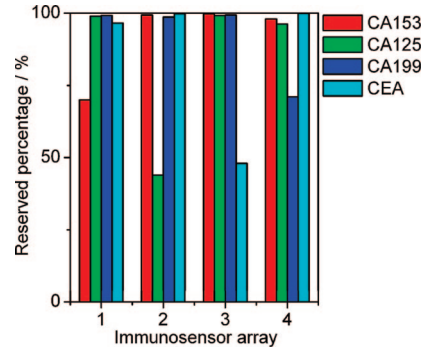


Figure 4. Reserved percentage of the direct electrochemical responses of HRP on four immunosensors after immunosensor arrays were incubated in 0.2 M pH 7.4 PBS containing 80 U mL^{-1} CA 153, 67 U mL^{-1} CA 125, 77 U mL^{-1} CA 199, or 46 ng mL^{-1} CEA for 2 min at a driving potential of +0.5 V, respectively.

Preparation of Immunosensor Array. The multiplexed immunosensor array was fabricated on a carbon electrode array containing four graphite working electrodes (2 mm in diameter, 0.5 mm edge-to-edge separation) (Scheme 1), which was prepared with screen-printed technology.[37] All working electrodes shared the same Ag/AgCl reference and graphite auxiliary electrodes. The insulating layer printed around the working area constituted an electrochemical microcell. Chitosan solution (0.3 wt %) was prepared by ultrasonically dissolving chitosan powder in 1% acetic acid. The biopolymer/sol−gel was prepared by mixing 50 μL of (3-aminopropyl)triethoxysilane, 25 μL of tetraethyoxysilane, and 10 μL of 10 mM HCl as catalyst and 700 μL of 0.3 wt % chitosan solution in a small test tube under stirring for 5−6 min at room temperature. The ratios of (3-aminopropyl)triethoxysilane, tetraethyoxysilane to chitosan were optimized according to the direct electrochemical response of the HRP. The 24-nm-diameter colloidal gold nanoparticles were prepared according to the literature.[38]

HRP-labeled CA 153, CA 125, CA 199, and CEA monoclonal antibodies were mixed with the gold colloidal solution in the volume ratio of 1:1, and the mixtures were placed at 4 °C for 12 h to obtain HRP-labeled antibody modified gold nanoparticles,

(37) Wu, J.; Tang, J. H.; Dai, Z.; Yan, F.; Ju, H. X.; Murr, N. E. *Biosens. Bioelectron.* **2006**, *22*, 102–108.
(38) Liu, S. Q.; Ju, H. X. *Anal. Biochem.* **2002**, *307*, 110–116.

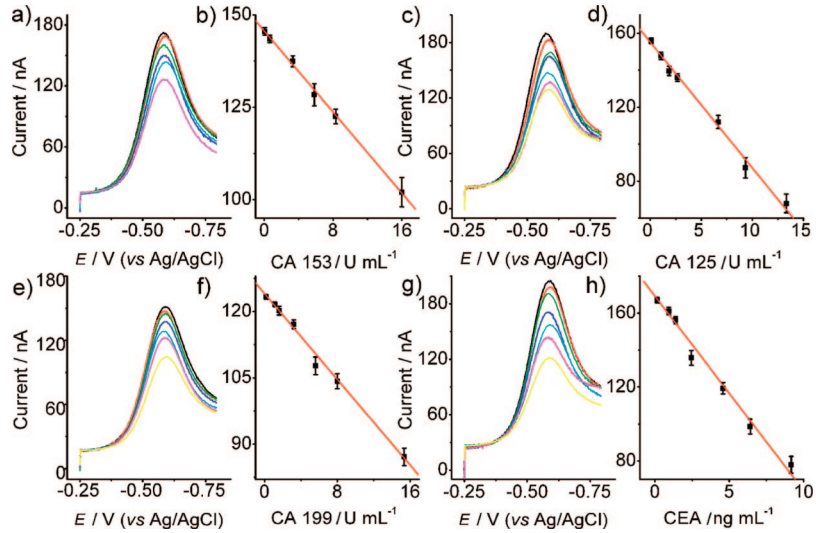


Figure 5. DPV responses (a, c, e, f) and calibration curves (b, d, f, h) for simultaneous multiplexed immunoassay of CA 153 (a, b), CA 125 (c, d), CA 199 (e, f), and CEA (g, h) with electric field-driven incubation at +0.5 V for 2 min.

respectively. These mixtures were then mixed with biopolymer/sol−gel at the volume ratio of 2:1, respectively, and 0.5 μL of each was individually dropped on the working electrodes and dried under ambient conditions for 2−3 h to form an immunosensor array. The formed immunosensor arrays were incubated with 0.1 M Tris-HCl (pH 7.2) containing 1% bovine serum albumin for 20 min to block the sites against nonspecific adsorption. After being thoroughly rinsed with doubly distilled water, the resulting ECIAs were stored in air prior to use.

Analytical Procedure. As shown in Scheme 1, the direct electrochemical signals of the immobilized HRP labeled to antibodies were first measured as S1 using 40 μL of anaerobic 0.2 M pH 7.4 PBS as detection solution with differential pulse voltammetry (DPV) from −250 to −800 mV (vs Ag/AgCl) at a pulse amplitude of 50 mV, a pulse width of 50 ms, and a scan rate of 20 mV s^{-1}. The mixture of 40 μL of 0.2 M pH 7.4 PBS and 10 μL of antigen solution containing known amounts of CA 153, CA 125, CA 199, and CEA or sample was then dropped on the entire ECIA to perform the proposed electric field-driven incubation step by applying a potential of +0.5 V on all the immunosensors for 2 min. After the active incubation process, the residual was removed with doubly distilled water, and the direct electrochemical signals of HRP at the resulting immunosensors were detected as S2 with the same detection process. The obtained S1 could be used for all samples and standard solutions due to the acceptable fabrication reproducibility of the arrays; thus, only one single run was needed to obtain the decreases of the peak currents for each practical sample, which were proportional to the concentrations of antigens in the incubation mixture.

RESULTS AND DISCUSSION

Fast Electrochemical Multianalyte Immunoassay. The multiplexed immunoassay was carried out with an assay format of direct capture coupled with electrochemical measurement in the supporting electrolyte, in which the HRP-labeled antibodies immobilized on working electrodes could recognize the corresponding antigens in sample solution, respectively, and the electrochemical responses were derived from the direct reduction of immobilized HRP from its resting state (Fe(III)) (S1 in Scheme 1). The recognition process was driven by applying a driving potential to the ECIA, at which an electric field could be produced nearby the electrode interfaces, respectively, leading to an external electrophoretic force to accelerate the transport of charged antigen molecules to electrodes and thus accelerate the immunoreactions and formation of immunocomplexes (Scheme 1). The saturated binding of immobilized HRP-labeled antibodies to corresponding antigens could be achieved within 2 min. The formation of HRP−Ab/Ag immunocomplex blocked the direct electron transfer between HRP and electrode due to the increasing spatial blocking and impedance, which resulted from the entrapment of more insulating protein into the membrane on the electrode surface, thus produced a lowered direct electrochemical signal (S2 in Scheme 1). The decrease of reduction current of HRP was proportional to the amount of formed immunocomplex on immunosensor and analyte in sample solution, respectively. Therefore, the concentrations of multiplexed analytes could be simultaneously determined by monitoring the corresponding signal changes of the HRP on ECIA without help of any electron-transfer mediator or enzymatic substrate.

Optimal Conditions for Electric Field-Driven Incubation. In the incubation process, the immunoreaction rates were related to the transport rates of the corresponding antigens to immunosensor interfaces. After an electric field was produced near the electrode interfaces, the transport rates of these charged proteins could be accelerated. Thus, the transport rates depended on solution pH and the strength of electric field or driving potential. The former decided the charge of protein. Both the solution pH and driving potential could be optimized by monitoring the decrease of DPV peak currents upon the incubation step.

In pH 7.4 PBS, at which these antigen molecules are negatively charged because of their isoelectric points between 3 and 5, with the positive increase of driving potential, all decreased values of the peak currents at ECIA containing four immunosensors for CA 153, CA 125, CA 199, and CEA with a fixed incubation time of 2 min increased and then decreased at different critical driving potentials (Figure 1a). The quick increase of peak current decrease indicated that the formation of immunocomplex was

Table 1. Assay Results of Clinical Sera Using the Proposed and Reference Methods

	CA 153 (U mL^{-1})		CA 125 (U mL^{-1})	
sample	proposed method	reference method	proposed method	reference method
1	43.9	43.4	23.6	24.5
2	11.6	13.3	25.7	26.2
3	22.0	21.0	31.4	28.7
4	31.7	35.3	48.0	45.8
5	54.0	75.2	63.1	69.3

	CA 199 (U mL^{-1})		CEA (ng mL^{-1})	
sample	proposed method	reference method	proposed method	reference method
1	21.9	20.1	2.86	3.17
2	54.3	62.3	16.8	17.0
3	81.1	77.8	7.07	6.51
4	57.4	62.6	13.3	12.1
5	65.3	74.3	25.8	26.6

accelerated and more immunocomplex was formed on the ECIA surface during the limited time, producing a greater block to the direct electron transfer of HRP. At high driving potential, the decrease of peak current decrease was attributed to the affection of the electrostatic repulsion among concentrated antigen molecules due to the relatively lower immunoreaction rate for binding the antigen to the immobilized HRP-labeled antibody than the driven transport rate. The critical driving potentials for CA 153, CA 125, and CA 199 were +0.5 V, while it was +0.3 V for CEA (Figure 1a). Considering convenient manipulation, +0.5 V was chosen as the driving potential for the proposed electrochemical multiplexed immunoassay, at which the peak current decrease for CEA was also sensitive enough for its immunoassay.

At the driving potential of +0.5 V, upon the incubation process of 2 min, the peak current decreases increased and reached relatively stable values in the pH range of 7.4−9.2 (Figure 1b), which indicated that all the four tumor markers became negatively charged at solution pHs higher than 7.4. So, pH 7.4 PBS was used for the electric field-driven incubation process.

The time for the electric field-driven incubation process was a crucial factor for a fast multiplexed immunoassay. At the driving potential of +0.5 V, with increasing incubation time, the DPV responses of the HRP at all four immunosensors quickly decreased and trended to the lowest values at 2 min (Figure 2a), indicating saturated formation of immunocomplexes on the ECIA surface. At the incubation time of 2 min, the peak decreases for CA 153, CA 125, CA 199, and CEA were 30, 56, 29, and 52%, respectively. Compared with the peak decreases of the HRP at the ECIA upon a 2-min incubation process without electric field driving, which were 12, 13.5, 10, and 7.2%, respectively, electric field-driven incubation greatly enhanced the incubation efficiency. In addition, the profile of current change against incubation time without electric field-driven incubation (inset, Figure 2a) showed an incubation time of 40 min for the saturated formation of immunocomplexes, at which the peak decreases for CA 153, CA 125, CA 199, and CEA were 47.1, 63.3, 34.5, and 50.7%, respectively. The decrease percentage for CA 153 was similar to that of 44% obtained with electric field-driven incubation for 5 min, and the others were similar to those obtained with 2-min electric field-driven incubation. These results further affirmed the saturated formation of immunocomplexes. So 2 min was used for electric field-driven incubation.

Optimization of Detection Condition. The proposed method was based on the measurement of direct electrochemical signal of immobilized HRP. When HRP-labeled antibody was immobilized in the biopolymer/sol−gel, the DPV curve could show the direct electrochemical response with regard to Fe(III)−Fe(II) conversion. In the presence of gold nanoparticles in the biopolymer/sol−gel, the reduction peak of the equal amount of HRP−antibody conjugate increased for 2.1 times. Thus, the Au nanoparticles could accelerate the direct electrochemistry of HRP to further amplify the detectable signal. The direct electrochemical signal was related to the acidity of the detection solution. As shown in Figure 2b, all of the maximum DPV peak currents of HRP-labeled antibodies at four immunosensors occurred at pH 7.4. Therefore, pH 7.4 PBS was used for signal detection.

Stability of Immunosensor Array for the Electric Field-Driven Process. The stability of ECIA during the electric field-driven process was of vital importance. It should be sure that the decreased signal resulted from the formation of the immunocomplexes on immunosensor surfaces rather than the leakage or deactivation of the HRP. Thus, the ECIA was examined by incubation in 0.2 M pH 7.4 PBS without the presence of antigens. After applying a potential of +0.5 V for 10 min, these immunosensors could reserve more than 98% of their initial responses (Figure 3), showing acceptable stability for electric field-driven incubation.

Evaluation of Cross-Reactivity or Specificity. The proposed electrochemical multiplexed immunoassay method did not need any substrate or mediator in the detection solution; thus, no electrochemical cross talk occurred among neighboring electrodes. The cross-reactivity among the antibodies and nonspecific antigens was studied by incubating the ECIA in a series of solutions containing only one protein biomarker. The experimental results indicated that only the immunosensor for the corresponding antigen in the incubation solution showed a sharp decrease of direct electrochemical signal of the HRP, while other nonspecific immunosensors could retain at least 96% of their original responses (Figure 4). Therefore, cross-reactivity at the array was negligible, making it possible for multiplexed immunoassay of the four protein biomarkers in a single run without interfering with each other. These results also indicated the ECIA and proposed method were of good specificity.

Multiplexed Immunoassay of Four Tumor Markers. After immunocomplexes were formed on the immunosensor array with an electric field-driven incubation step in a direct capture immunoassay format, the multiplexed immunoassay was performed in 0.2 M pH 7.4 PBS. The DPV peak currents of the immunosensors after the electric field-driven incubation decreased linearly with increasing concentrations of tumor markers in the incubation mixture, respectively (Figure 5). The linear calibration ranges were 0.084−16 (CA 153, $R = 0.9985$), 0.11−13 (CA 125, $R = 0.9957$), and 0.16–15 U mL^{-1} (CA 199, $R = 0.9962$) and 0.16−9.2 ng mL^{-1} (CEA, $R = 0.9947$). The detection limits corresponding to the signals of 3SD for the four analytes were 0.06, 0.03, and 0.10 U mL^{-1} and 0.04 ng mL^{-1}, respectively. Thus, the ECIA and

proposed method with an electric field-driven incubation step showed high sensitivity.

According to the analytical process, the detectable linear ranges in the sample with a dilute factor of 5 were 0.42−80, 0.55−65, and 0.8−75 U mL^{-1} and 0.8−46 ng mL^{-1} for CA 153, CA 125, CA 199, and CEA, respectively. The proposed detection ranges were sufficient for simultaneous detection of CA 153, CA 125, CA 199, and CEA levels in clinical samples with the cutoff values of 25, 35, and 37 U mL^{-1} and 3 ng mL^{-1}, respectively.

Application in Detection of Serum Tumor Markers. Ten microliters of serum samples were mixed with 40 μL of 0.2 M pH 7.4 PBS, and the mixtures were then used as the incubation solutions for the electric field-driven incubation, respectively. The accuracy of multiplexed measurement of CA 153, CA 125, CA 199, and CEA by the proposed immunosensor arrays was examined by comparing the results obtained from five sera with this method, and the commercial electrochemiluminescent single-analyte test was performed in Jiangsu Institute of Cancer Prevention and Cure. In comparison with the results obtained with commercial method, the results obtained with the proposed method for the five serum samples showed acceptable accuracy (Table 1). Thus, the proposed four-analyte immunosensor array and the immunoassay system could be anticipated to rapidly detect CA 153, CA 125, CA 199, and CEA levels in clinical diagnosis.

Reproducibility and Stability of the Immunosensor Arrays. The interassay precision of the immunosensor arrays was examined with two panels of tumor markers at various concentrations. Each panel was measured for five times using five arrays. The coefficients of variation were 3.7, 8.9, 4.1, and 2.3% for 16.7 U mL^{-1} CA 153, 13.3 U mL^{-1} CA 125, 16.0 U mL^{-1} CA 199, and 8.2 ng mL^{-1} CEA, and 2.5, 2.3, 6.5, and 7.8% for 41.7 U mL^{-1} CA 153, 46.7 U mL^{-1} CA 125, 40.0 U mL^{-1} CA 199, and 32.1 ng mL^{-1} CEA. These results indicated acceptable precision and fabrication reproducibility of the immunosensor arrays.

The ECIA could be stored in dry air at room temperature. The DPV responses were all >90% of initial responses after a storage period of 30 days. Thus, the storage stability of the ECIA was acceptable, and the proposed ECIA was suitable for clinical diagnostics.

CONCLUSIONS

In this paper, a simple, sensitive, and fast electrochemical multiplexed immunoassay for protein biomarkers is proposed. By integrating an electric field-driven strategy with a newly designed ECIA, the time for multiplexed immunoassay is greatly shortened. The incubation time of 2 min leads to a fast electrochemical multiplexed immunoassay method. The reagentless detection process based on the changes of direct electrochemical signals of HRP-labeled antibodies upon incubation greatly simplifies the manipulation. The results show acceptable linear ranges and accuracy for detection of panels of tumor markers. The disposable ECIA and portable detection system used for the multiplexed immunoassay can satisfactorily meet the requirements of clinical diagnostics and commercial application with simplicity and low cost.

ACKNOWLEDGMENT

This work was financially supported by the National Science Funds for Creative Research Groups (20521503), the Key Program (20535010) and Major Research Plan (90713015) from National Natural Science Foundation of China, the Outstanding Medical Talents Program (RC2007069) from Department of Health of Jiangsu Province, and the Social Development Projects of Jiangsu Province (BS2006006, BS2006074).

Received for review May 2, 2008. Accepted June 3, 2008.

AC800905K