Available online at www.sciencedirect.com
ScienceDirect
ELSEVIER
Electrochemistry Communications 8 (2006) 1835–1839
electrochemistry communications
www.elsevier.com/locate/elecom

A designer ormosil gel for preparation of sensitive immunosensor for carcinoembryonic antigen based on simple direct electron transfer

Fang Tan [a], Feng Yan [b], Huangxian Ju [a,*]

[a] *Key Laboratory of Analytical Chemistry for Life Science (Education Ministry of China), Department of Chemistry, Nanjing University, Nanjing 210093, China*
[b] *Jiangsu Institute of Cancer Prevention and Cure, Nanjing 210009, PR China*

Received 26 July 2006; received in revised form 12 August 2006; accepted 15 August 2006
Available online 11 September 2006

Abstract

The excellent direct electron transfer (DET) of enzyme labeled to antibody immobilized in designer organically modified silicate (ormosil) sol–gel was achieved at an electrode, which was used to construct a novel reagentless immunosensor for antigen determination. The synthesized ormosil architecture provided a hydrophilic interface for retaining the activity of immobilized enzyme labeled immunocomponent. The proposed immunosensor for carcinoembryonic antigen (CEA) prepared by immobilizing horseradish peroxidase-labeled CEA antibody (HRP-*anti*-CEA) in the architecture showed a surface-controlled electrode process attributed to the DET between electrode and HRP with a rate constant of $5.94 \pm 0.40\ s^{-1}$. The formation of immunocomplex upon incubation in CEA or sample solution led to block of DET and linearly decrease in voltammetric response over CEA concentration ranging from 0.5 to 3.0 and 3.0 to 120 ng ml^{-1}. The limit of detection for CEA was 0.4 ng ml^{-1}. The immunosensor showed good accuracy and acceptable storage stability, precision and reproducibility. The proposed method was simple, low-cost and potentially attractive for clinical immunoassays.
© 2006 Elsevier B.V. All rights reserved.

Keywords: Ormosil; Sol–gel; Carcinoembryonic antigen; Immunosensor; Horseradish peroxidase; Graphite electrode

1. Introduction

The development of simple, sensitive and specific immunoassays for quantitation of analytes is attracting great interest in biological and food analysis and clinical diagnostics. Various conceptual approaches have been reported in this field [1–3]. In conjunction the specificity of immunoreaction with the amplification feature of an enzyme label, electrochemical immunoassay is being developed to provide a highly selective, sensitive and low-cost detection system [4,5]. The key procedure in this goal is the immobilization of biorecognition substance on electrode surface and preserving sufficient bioactivity of the immobilized immunocomponent.

Several methods such as avidin–biotin interaction [6], covalent coupling [7] and entrapment in sol–gel [8] have been proposed for immobilization of immunocomponents. Of these methods, sol–gel processes offer a relatively mild route for the immobilization of biomolecules, which are entrapped in the growing covalent gel network rather than being chemically attached to an inorganic material [9]. This interest derives mainly from the design flexibility through the easy control of chemical composition. The gel network can be prepared under ambient conditions and exhibits tunable porosity, high thermal stability, chemical inertness and negligible swelling, thus biomolecules entrapped in sol–gel-derived matrix can exhibit improved performances with respect to thermal and chemical denaturation, long-term storage, and operational stability. In this work, a novel designer organically modified silicate (ormosil) sol–gel architecture containing electroactive enzyme labeled antibody was formed on electrode surface for immunosensing.

Direct electron transfer (DET) of enzyme labeled on antibody is difficult because of the low concentration of

* Corresponding author. Tel./fax: +86 25 83593593.
E-mail address: hxju@nju.edu.cn (H. Ju).

1388-2481/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.elecom.2006.08.026

labeled enzyme and the high molecular weight of antibody, which blocks the electron transfer of labeled enzyme. Although our previous works have observed the DET of HRP labeled to carcinoma antigen-125 (CA125) [10] and human serum chorionic gonadotrophin (hCG) [11] antibodies encapsulated in a titania sol–gel film by vapor deposition method and competitive immunoreaction, the electrochemical signal from the DET is weak due to the relatively slow electron transfer rate. In this work the intrinsic nanoporous property of the designed ormosil architecture and the small sol–gel particles acted as electron-conduction pathways in the architecture promote the electrical communication between redox sites of enzyme and sensing surface. Thus a more sensitive immunosensor could be obtained for direct electrochemical immunoassay of proteins, using carcinoembryonic antigen (CEA) as a model.

CEA is one of the most extensively used clinical tumor markers. A number of immunoassay methods for CEA, including radioimmunoassay [12], chemiluminescence immunoassay [13], enzyme-linked immunosorbent assay [14], fluoroimmunoassay [15] and electrochemical immunoassay [16], have been reported. These methods are usually time and sample consuming due to several incubation and washing steps needed in detection procedure. This proposed strategy avoids the trouble of washing and adding a mediator to the sample solution, and appears to be simple, practical, convenient and significant in clinic diagnoses.

2. Experimental

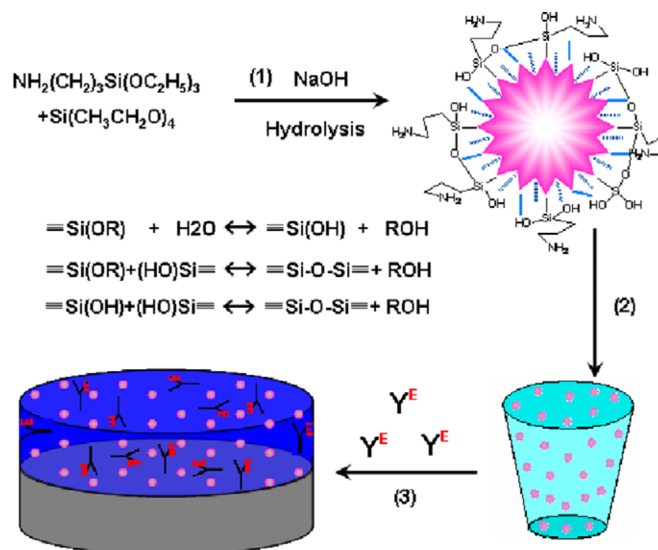
2.1. Materials

CEA enzyme-linked immunosorbent assay (ELISA) kits were purchased from BioCheck, Inc. (Foster city, USA). The kits consisted of a series of CEA standard solutions with different concentrations from 0 to 120 ng ml^{-1}, a stock solution of HRP labeled CEA monoclonal antibody from goat. Bovine serum albumin and (3-aminopropyl) triethoxysilane (APTES) were the products of Sigma Chemical Company (St. Louis, MO USA). The dilute solution of enzyme conjugate contained 0.04% bovine serum albumin, 1.0 mM ethylenediamine tetraacetic acid and 0.1 M phosphate buffer (PBS). All other reagents were of analytical grade and made in Chinese chemical company. All solutions were made up with deionized water of 18 MΩ purified from a Milli-Q purification system.

2.2. Preparation of HRP-*anti*-CEA/sol–gel immunosensor

Graphite electrode (GE) (4 mm diameter) was polished to a mirror finish using 0.3 and 0.05 μm alumina slurry (Beuhler) followed by rinsing thoroughly with deionized water. After sonicated successively in 1:1 nitric acid, acetone and deionized water, the electrode was allowed to dry at room temperature.

As shown in Scheme 1, a homogeneous stock ormosil sol was prepared by mixing 150 μl of APTES, 50 μl of tetraethoxysilane, 100 μl of 10 mM NaOH as catalyst and 700 μl of H_2O in a small test tube under stirring for 4–5 min at room temperature. To immobilize HRP-*anti*-CEA in the gel network, 2 μl HRP-*anti*-CEA mixed with 2 μl ormosil sol was dropped on the pretreated GE surface and allowed to dry for the formation of gel under ambient condition for 3 h. After the modified electrode was rinsed with deionized twice or thrice, the immunosensor, HRP-*anti*-CEA/sol–gel/GE, was obtained. When not in use, the sensor was stored in 0.1 M pH 7.0 phosphate buffer (PBS) at 4 °C.



Scheme 1. Fabrication procedure of ormosil sol–gel film: (1) hydrolysis of two precursors under weak basic condition, (2) formation of ormosil sol–gel, and (3) preparation of bioactive surface.

For morphological analysis the film was prepared in the same way on a glass slide dealt with nitric acid and the mixture H_2SO_4:H_2O_2 (1:1). After coated with Au film to improve the conductivity, the film was examined under a scanning electron microscope (SEM, LEO 1530 VP, Germany) at 5.00 kV.

2.3. Measurement procedure

Electrochemical measurements were performed on a CHI 660B electrochemical workstation (Shanghai Chenhua Apparatus Corporation, China). All experiments were carried out using a conventional three-electrode system with the GE as working, a platinum wire as auxiliary, and saturated calomel electrode as reference electrodes.

The one-step immunoreaction was accomplished on the immunosensor surface. Ten microliters CEA standard or sample solution was firstly diluted with 40 μl dilute solution to a total volume of 50 μl in an incubation cup. The immunosensor was then incubated in the solution at 30 °C for 35 min and washed carefully with deionized water to obtain an immobilized immunoconjugate layer, named as CEA/HRP-*anti*-CEA/sol–gel/GE. The amount of the immobilized immunocomplex depended on the concentra-

tion of CEA in standard or sample solution. The detection solution was 0.1 M pH 7.0 PBS. The differential pulse voltammetric (DPV) measurements were performed from +200 to −200 mV with a pulse amplitude of 50 mV and a pulse width of 50 ms.

3. Results and discussion

3.1. Characterization of ormosil sol–gel/GE

Fig. 1 shows the morphology of ormosil sol–gel film observed with SEM. The film showed a uniform and continuous surface without macroscopic granularity or crack. The aggregate of the ormosil matrix on the electrode surface produced a nanoporous structure of nanoparticles with a very narrow size distribution. The diameter of these nanoparticles was about 15–20 nm. Such a platform provided a necessary conduction pathway and assisted the electron transfer between the immobilized labeled enzyme and the electrode.

3.2. Cyclic voltammetric behavior of HRP-anti-CEA/sol–gel/GE

The cyclic voltammograms of different electrodes immersed in 0.1 M pH 7.0 PBS are shown in Fig. 2. No response was observed at unpolished, polished and ormosil sol–gel modified GEs (curves a–c). The polished GE showed a lower charging current than the unpolished GE. The coating of ormosil sol–gel led to a less background current. After mixing HRP-*anti*-CEA with the sol–gel, a pair of stable and well defined redox peaks with regard to the DET of Fe(III) to Fe(II) conversion of the immobilized HRP were observed (curve d). The anodic and cathodic peak potentials were −36 and −60 mV (*vs.* SCE) at 50 mV s^{-1}, respectively. After HRP-*anti*-CEA/sol–gel/GE was incubated in 50 µl incubation solution containing 10 µl of 30.0 ng ml^{-1} CEA for 35 min, the DET response of the immobilized HRP decreased due to the increased barrier resulted from the formation of immunocomplex (curve e).

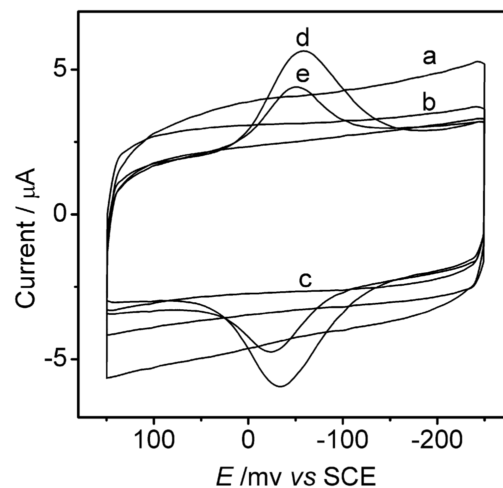


Fig. 2. Cyclic voltammograms of different electrodes in pH 7.0 PBS at 50 mV s^{-1}: (a) unpolished bare GE, (b) polished bare GE, (c) sol–gel/GE and (d) HRP-*anti*-CEA/sol–gel/GE, (e) CEA/HRP-*anti*-CEA/sol–gel/GE obtained after incubating (d) in 50 µl incubation solution containing 10 µl of 30.0 ng ml^{-1} CEA at 30 °C for 35 min.

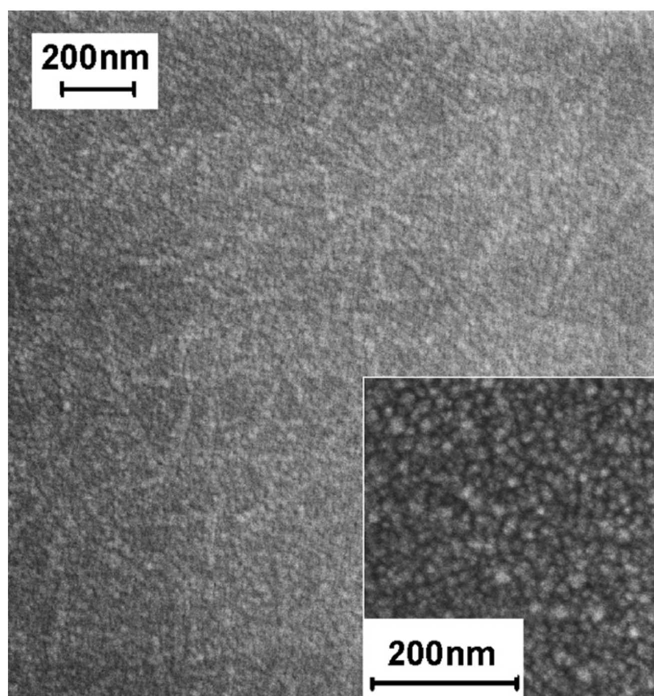


Fig. 1. Scanning electron micrograph of graphitic electrode coated with ormosil sol–gel at ×40.0 K. Inset: ×80.0 K.

The cyclic voltammograms of HRP-*anti*-CEA/sol–gel modified GE showed a thin-layer electrochemical behavior (not shown). From the peak-to-peak separations at different scan rates, an average electron transfer rate constant of $5.94 \pm 0.40\ s^{-1}$ was obtained [17]. The DET rate was faster than those of 3.04 ± 1.21 and $1.35 \pm 0.40\ s^{-1}$ for the labeled HRP immobilized in titania sol–gel [10,11].

3.3. Optimization conditions of immunoassay

The effect of volume fraction of HRP-*anti*-CEA of given concentration to ormosil on DPV peak current at a fixed total volume was shown in Fig. 3A. The peak current reached a maximum value at the volume fraction of 0.5 (1:1, V/V). As shown in Fig. 3B, the response reached a platform at an incubation temperature of 30 °C, at which the best appropriate incubation time was 35 min (Fig. 3C).

3.4. DPV response of immunosensor to CEA concentration

Under optimal incubation conditions, the CEA molecules in incubation solution could bind to the encapsulated HRP-*anti*-CEA to form immunocomplex, which decreased

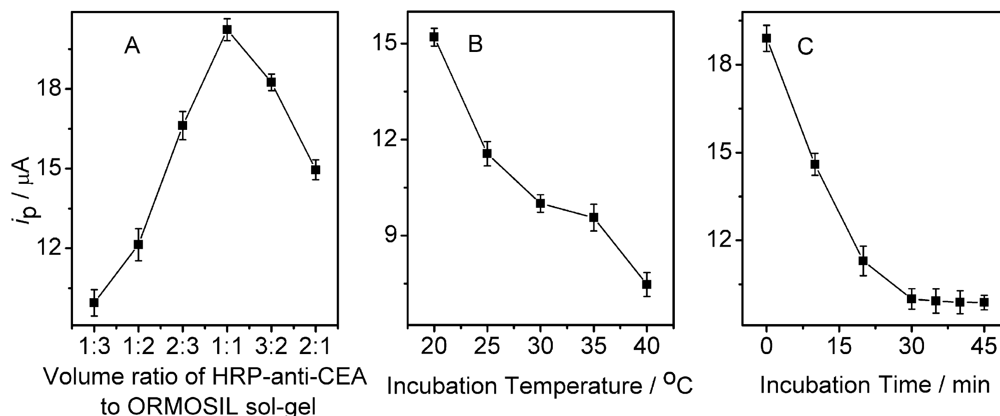


Fig. 3. Effect of volume ratio of HRP-*anti*-CEA to ormosil sol for immunosensor preparation, on DPV peak current (A) and dependence of DPV peak current of CEA/HRP-*anti*-CEA/sol–gel/GE in 0.1 M pH 7.0 PBS on incubation temperature (B) and time (C) in 50 µl incubation solution containing 10 µl of 30.0 ng ml^{-1} CEA.

the rate of DET and the DPV peak current. With an increasing CEA concentration the DPV response decreased (Fig. 4). The plot of the average peak current ($n = 5$) *vs.* CEA concentration in standard solution or original sample for preparation of incubation solution showed two linear ranges from 0.5 to 3.0 and 3.0 to 120 ng ml^{-1} with the slopes of 1.12 and 0.088 µA (ng ml^{-1})$^{-1}$ and the correlation coefficients of 0.993 and 0.996 (inset of Fig. 4). From the slope of 1.12 µA (ng ml^{-1})$^{-1}$ the limit of detection was calculated to be 0.4 ng ml^{-1} at 3σ. When the dilution fraction of 10/50 (V/V) for preparation of incubation solution was taken into account, the practical linear ranges were from 0.1 to 24.0 ng ml^{-1}, and the detection limit in the incubation solution was 0.08 ng ml^{-1}. The proposed limit of detection was comparable to a number of reported heterogeneous CEA assays using electrochemical analytical principle [18].

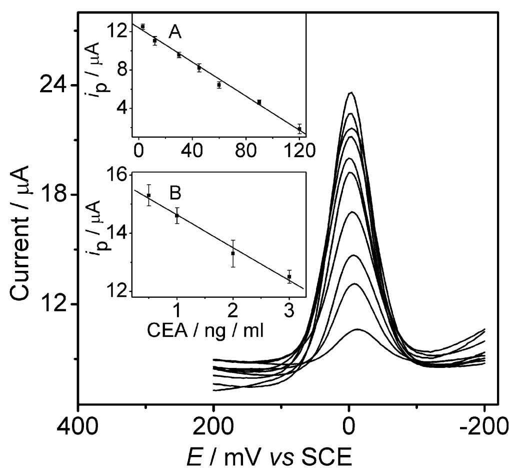


Fig. 4. DPV of HRP-*anti*-CEA/sol–gel/GE after incubated in 50 µl solutions containing 10 µl of 0.5, 1.0, 2.0 3.0, 12, 30, 45, 60, 90 and 120 ng ml^{-1} CEA (from highest to lowest peak current) at 30 °C for 35 min. Inset: (A) plot of DPV peak current *vs.* CEA concentration; (B) linear calibration for 0.5–3.0 ng ml^{-1} CEA.

3.5. Detection of CEA in a clinical serum sample

After five serum samples from different persons were diluted with the appropriate volumes of dilution solution, the serum CEA concentrations were detected with the calibration curve of the CEA immunosensors to be 1.4, 4.8, 15.7, 25.7 and 71.4 ng ml^{-1}. As control, the concentrations were detected with Elecsys 2010 analyzer (Roche), a commonly used analyzer in clinic laboratory. The results were 1.3, 4.6, 16.0, 24.6 and 79.8 ng ml^{-1}, respectively. The relative deviations between two methods were in the range of −10.3% to 7.8%.

3.6. Reproducibility and stability of the CEA immunosensor

The reproducibility of the immunoassay system was assessed by intra- and inter-assay coefficients of variation (CVs). The intra-assay CV was the difference between three determinations of one sample on the same electrode. The variation coefficients of the results with this method were 6.8% and 5.4% at the CEA concentrations of 3.0 and 30.0 ng ml^{-1} in serum samples, respectively. The inter-assay precision or the fabrication reproducibility was estimated by determining in duplicate the CEA level in one serum sample with three immunosensors made at the same electrode independently. The inter-assay precision of this method was 7.0% at the CEA concentration of 30 ng ml^{-1}, showing acceptable fabrication reproducibility.

When the modified electrode was not in use, it was stored in PBS (pH 7.0) at 4 °C. No obvious change was observed after storing for 10 days. The performance stability of the biosensor was examined by storage in air at 4 °C, it retained 90% of its initial current after a storage period of 30 days.

4. Conclusions

This work introduces a simple and efficient method for fabrication of an immunosensor by immobilization of

HRP-*anti*-CEA in one novel ormosil sol–gel. The intrinsic nanoporous property of the ormosil architecture and the small sol–gel particles acted as electron-conduction pathways promote the electrical communication between redox sites of immobilized enzyme and sensing surface, lead to the direct electron transfer of the enzyme. The fast electron transfer produces a sensitive immunosensing due to the barrier resulted from the formation of immunocomplex upon incubation in testing solution. The proposed CEA immunosensor shows good accuracy and acceptable storage stability, precision and reproducibility. The method avoids the addition of electron transfer mediator and does not require separation and washing steps, thus is much simpler and cheaper than traditional assays.

Acknowledgements

We gratefully acknowledge to the National Science Funds for Distinguished Young Scholars (20325518) and Creative Research Groups (20521503), the Key Program (20535010) from the National Natural Science Foundation of China and the Science Foundation of Jiangsu (BS2006006, BS2006074) for financial support of this research.

References

[1] F. Yu, W. Knoll, Anal. Chem. 76 (2004) 1971.
[2] J. Wang, A. Ibanez, M.P. Chatrathi, A. Escarpa, Anal. Chem. 73 (2001) 5323.
[3] T.K. Lim, T. Matsunaga, Biosens. Bioelectron. 16 (2001) 1063.
[4] Z.P. Chen, J.H. Jiang, G.L. Shen, R.Q. Yu, Anal. Chim. Acta 553 (2005) 190.
[5] W.C. Mak, K.Y. Cheung, D. Trau, A. Warsinke, F. Scheller, R. Renneberg, Anal. Chem. 77 (2005) 2835.
[6] M. Tudorache, M. Co, H. Lifgren, J. Emneus, Anal. Chem. 77 (2005) 7156.
[7] N. Sanvicens, V. Pichon, M.C. Hennion, M.P. Marco, J. Agr. Food Chem. 51 (2003) 156.
[8] T. Peng, Q. Cheng, R.C. Stevens, Anal. Chem. 72 (2000) 1611.
[9] Y.D. Kim, C.B. Park, D.S. Clark, Biotechnol. Bioeng. 73 (2001) 331.
[10] Z. Dai, F. Yan, J. Chen, H.X. Ju, Anal. Chem. 75 (2003) 5429.
[11] J. Chen, F. Yan, Z. Dai, H.X. Ju, Biosens. Bioelectron. 21 (2005) 330.
[12] M. Cioffi, M.T. Vietri, P. Gazzerro, R. Magneta, A. D'Auria, A. Durante, E. Nola, G.A. Puca, A.M. Molinari, Lung Cancer 33 (2001) 163.
[13] J.C. Forest, J. Masse, A. Lane, Clin. Biochem. 31 (1998) 81.
[14] L.S. Zhao, S.Y. Xu, G. Fjaertoft, K. Pauksen, L. Hakansson, P. Venge, J. Immunol. Methods 293 (2004) 207.
[15] F. Yan, J.N. Zhou, J.H. Lin, H.X. Ju, X.Y. Hu, J. Immunol. Methods 305 (2005) 120.
[16] Z. Dai, F. Yan, H. Yu, X.Y. Hu, H.X. Ju, J. Immunol. Methods 287 (2004) 13.
[17] E. Laviron, J. Electroanal. Chem. 101 (1979) 19.
[18] Y. Liu, H. Jiang, Electroanalysis 18 (2006) 1007.