ELSEVIER

Journal of Immunological Methods 283 (2003) 67–75

JIM
Journal of Immunological Methods

www.elsevier.com/locate/jim

Immunological assay for carbohydrate antigen 19-9 using an electrochemical immunosensor and antigen immobilization in titania sol–gel matrix

Dan Du[a], Feng Yan[b], Shengli Liu[c], Huangxian Ju[a,*]

[a] *State Key Laboratory of Coordination Chemistry, Department of Chemistry, Institute of Analytical Science, Nanjing University, Nanjing 210093, PR China*

[b] *Jiangsu Institute of Cancer Prevention and Cure, Nanjing 210009, PR China*

[c] *The Affiliated Zhongda Hospital, Southeast University, Nanjing 210009, PR China*

Received 28 April 2003; received in revised form 2 July 2003; accepted 11 August 2003

Abstract

We describe a novel electrochemical immunosensor for carbohydrate antigen 19-9 (CA19-9) based on the immobilization of CA19-9 with titania sol–gel on a graphite electrode (GE) by vapor deposition. The CA19-9 membrane was characterized using scanning electron microscopy and proved to be chemically clean, porous and homogeneous. The incubation of the immunosensor in a solution containing horseradish peroxidase (HRP)-labeled CA19-9 antibody led to the binding of HRP-labeled antibody with the immobilized antigen. The immobilized HRP catalyzed the oxidation of catechol by H_2O_2 and this provided a competitive method for the measurement of serum CA19-9. The response current decreased with increasing CA19-9 concentration in the incubation solution. The effects of pH, amount of HRP-labeled antibody, incubation time and temperature were explored to provide optimum analytical performance. Under optimal conditions, the current decrease of the immunosensor was proportional to CA19-9 concentrations in the range of 3–20 U/ml with a detection limit of 2.68 U/ml at a current decrease of 10%. The detection of CA19-9 in two serum samples obtained from clinically diagnosed patients with pancreatic carcinoma showed acceptable accuracy. The proposed immunosensor provides a new promising tool for the clinical immunoassay of CA19-9.

Keywords: Immunosensor; Electrochemical sensors; Biosensors; Enzyme-labeled antibody; Carbohydrate antigen 19-9; CA19-9; Titania sol–gel; Pancreatic carcinoma

1. Introduction

In recent years, immunosensors developed for the diagnostic assay of biomolecules have attracted considerable interest (Wang, 1999; Liu et al., 2001; Grogan et al., 2002). Electrochemical immunosensors combine simple, portable, low-cost electrochemical measurement systems with specific and sensitive

Abbreviations: BSA, bovine serum albumin; CA19-9, carbohydrate antigen 19-9; DPV, differential pulse voltammetry; EDTA, ethylenediamine tetraacetic acid; ELISA, enzyme-linked immunoabsorbent assay; GE, graphite electrode; HRP, horseradish peroxidase; IRMA, immunoradiometric assay; PBS, phosphate buffered saline; SCE, saturated calomel electrode.

* Corresponding author. Tel.: +86-25-3593593; fax: +86-25-3593593.

E-mail address: hxju@nju.edu.cn (H. Ju).

0022-1759/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.jim.2003.08.014

immunoassay procedures and thus represent a promising approach in clinical (Clerico et al., 2000; Worwood, 2002), biochemical (Gervay and McReynolds, 1999; Sellrie et al., 2002) and environmental (Van Emon et al., 1998; Fahnrich et al., 2002) analyses.

Electrochemical immunosensors are usually prepared by immobilizing various kinds of antibody or antigen on the electrode surface, and the analytes are measured through the immunoreaction between the immobilized ligand and the analytes or labeled conjugate species. Enzymes have been extensively used as markers to improve the sensitivity of immunoassays by electrochemical amplification of the signals (McNeil et al., 1995; Keay and McNeil, 1998; Wang et al., 1998; Crowley et al., 1999; Santandreu et al., 1999; Kuznetsov et al., 2001). Many enzymes such as horseradish peroxidase (HRP) (McNeil et al., 1995; Santandreu et al., 1999), alkaline phosphatase (Wang et al., 1998; Crowley et al., 1999), laccase (Kuznetsov et al., 2001) and glucose oxidase (Keay and McNeil, 1998; Campanella et al., 1999) have been used to label the antibody or antigen and to produce the electrochemically active species for amperometric immunosensor preparation. Correspondingly, some substrates such as H_2O_2 or O_2, naphthyl phosphate or *p*-aminophenol phosphate, O_2 or ferrocene and glucose or O_2 and immobilized HRP are needed in the test solutions for HRP, alkaline phosphatase, laccase and glucose oxidase, respectively. Sometimes, when HRP is used as the marker an additional substrate such as hydroquinone (Santandreu et al., 1999) or *o*-aminophenol (Liu et al., 2001) is also added to the detection solution as a mediator to transfer electrons between H_2O_2 and the enzyme. These amperometric immunosensors have been used for the measurement of progesterone in cow's milk (Pemberton et al., 1999), mouse IgG (Toda et al., 2002), creatinine (Benkert et al., 2000) and bacteria such as *E. coli* and *Salmonella* (Abdel-Hamid et al., 1999). Here, we report a novel immunosensor for carbohydrate antigen 19-9 (CA19-9) by immobilizing CA19-9 on a graphite electrode (GE) with titania sol–gel membrane. The titania sol–gel thin film has been shown to be clean, porous and homogeneous and to have a very narrow particle size distribution for protein immobilization (Yu and Ju, 2002). The immobilized CA19-9 then competes with free CA19-9 in the sample solution to conjugate the HRP-labeled CA19-9 antibody. This procedure produces an HRP modified surface, which is used for amperometric immunoassay of CA19-9.

CA19-9 is one of the most important carbohydrate tumor markers. The determination of serum CA19-9 levels is of great importance for clinical diagnoses of pancreatic, colorectal, gastric and hepatic carcinomas. In an earlier study, Ohkura et al. (1985) reported a method for determining serum CA19-9 levels ranging from 0 to 240 U/ml by enzyme-linked immunoabsorbent assay (ELISA). In 1991 a chemiluminescent enzyme immunoassay was developed using alkaline phosphatase as a labeling enzyme to quantify several tumor markers including CA19-9 (Nishizono et al., 1991). Zucchelli et al. (1992, 1994) then provided an external quality assessment of the analytical performance of CA19-9, CA125 and CA153 immunoassays. Recently, a number of immunoassay kits and new methods for CA19-9 determinations have become available (Birk et al., 1997; Zhao et al., 1998; Okamura et al., 2002). Although enzyme immunoassay is a powerful tool for the detection of antigen and many commercial kits are available for the determination of CA19-9, there involve several incubation and washing steps followed by spectrophotometric detection using a chromogenic substrate (Van Emon and Lobez-Avila, 1992). Immunosensors avoid these disadvantages and the CA19-9 immunosensor described here provides a separation-free electrochemical immunoasssy for the determination of serum CA19-9. This method is not subject to interference from many absorbing and fluorescent compounds in typical serum samples. In comparison with the results obtained by immunoradiometric assay (IRMA), the immunosensor shows an acceptable accuracy and appears to be practical, convenient and reliable.

2. Materials and methods

2.1. Materials

CA19-9 ELISA kits were purchased from Diagnostic Products (DPC, USA). The ELISA kits consisted of a series of CA19-9 standard solutions with different concentrations from 0 to 240 U/ml and a stock solution of HRP-labeled CA19-9 specific antibody from goat. Bovine serum albumin (BSA) was obtained from

Sigma (St. Louis, MO, USA). The dilution solution for the enzyme conjugate contained 0.04% BSA and 1.0 mM ethylenediamine tetraacetic acid (EDTA) and 0.1 M phosphate buffered saline (PBS). Titanium isopropoxide ($Ti[OCH(CH_3)_2]_4$) was obtained from Aldrich. All other reagents were of analytical grade. All solutions were made up with deionized water of 18 MΩ purified from a Milli-Q purification system. The serum samples were separated by centrifugation from blood, which was obtained from clinically diagnosed patients with pancreatic cancer.

2.2. Instruments

Electrochemical measurements were performed on a CHI 730 electrochemical analyzer (CHI, USA) with a conventional three-electrode system comprising platinum wire as auxiliary electrode, saturated calomel electrode (SCE) as reference and HRP/CA19-9 modified graphite electrode as working electrode. The IRMA procedure was carried out with a FMJ-182 Immunoradiometric Gramma Counter (China) according to the instructions in the operator's manual. Scanning electron micrographs of titania sol–gel and CA19-9 sol–gel membranes were obtained with a Hitachi X-650 scanning electron microscope (Hitachi, Tokyo, Japan) at an acceleration voltage of 20 kV.

2.3. Preparation of CA19-9 immunosensor

The GE (6-mm diameter) was polished to a mirror finish using 0.3- and 0.05-μm alumina slurry (Beuhler) followed by thorough rinsing with deionized water. After sonication successively in 1:1 nitric acid, acetone and double distilled water, the electrode was rinsed with distilled water and allowed to dry at room temperature. Before immobilization of CA19-9, the electrode was pretreated electrochemically by applying a potential of +1.75 V in 0.1 M PBS pH 5.0 for 300 s, and cyclical scanning between +0.3 and +1.2 V for 30 cycles and then +0.3 and −1.3 V until a steady-state current–voltage curve was observed (Wang et al., 2001). A 10-μl aliquot of a 240 U/ml CA19-9 standard solution was dropped onto the treated electrode surface. The electrode was then suspended vertically above titanium isopropoxide in a sealed flask and kept at a constant temperature of 25 °C for 6 h. This resulted in adsorption of saturated titanium isopropoxide vapor by the CA19-9 solution and slow formation of a titania sol–gel membrane through hydrolysis of titanium isopropoxide on the surface, which trapped the CA19-9 molecules in the membrane (Yu and Ju, 2002). After rinsing thoroughly with deionized water, an immunosensor for CA19-9 was obtained, which was kept in PBS pH 6.6 at 4 °C prior to use.

2.4. Measurement procedure

The immunosensor for CA19-9 was incubated in an incubation solution of 1.0 ml at 30 °C for 2 h and then washed carefully with deionized water. The incubation solutions were prepared by diluting different volumes of the enzyme conjugate solution with BSA/EDTA/PBS dilution solution to 1.0 ml. For the measurement of CA19-9, one competitive assay configuration was used and 10-μl CA19-9 standard solution or serum sample was added to 0.99-ml incubation solution. The schematic diagram of the procedure is shown in Fig. 1. All electrochemical measurements

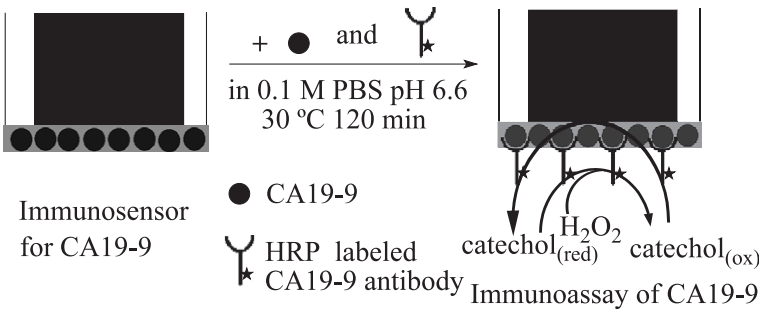

Fig. 1. Principle of the CA19-9 immunosensor based on immobilized antigen, competitive immunoreaction and electrochemical detection.

were done in an unstirred electrochemical cell containing 0.1-ml 5.0 mM catechol, 0.1-ml 5.0 mM H_2O_2 and 0.8-ml PBS at 20 ± 0.5 °C. After the incubation step, the HRP/CA19-9 modified electrode was immersed in the electrochemical cell to study the electrochemical response of H_2O_2 and catechol solution using cyclic voltammetry and differential pulse voltammetry (DPV). The DPV measurements were performed from $+0.7$ to -0.2 V with the pulse amplitude of 50 mV and the pulse width of 50 ms. Although the dissolved oxygen in the detection solution showed little effect on the electrochemical measurements, in order to obtain more accurate results all solutions for electrochemical detections were deaerated by high-purity nitrogen for 5 min and a nitrogen atmosphere was maintained over the solutions during the measurements.

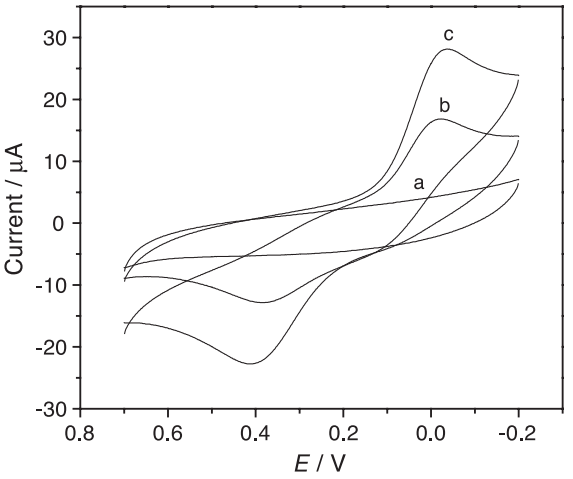

Fig. 2. Cyclic voltammograms of HRP/CA19-9 modified electrode in (a) 0.1 M PBS pH 6.6, (b) (a) +0.5 mM catechol and (c) (b) +0.5 mM H_2O_2. Scan rate: 50 mV/s.

3. Results and discussion

3.1. Cyclic voltammetric behavior of HRP/CA19-9 modified electrode

After the HRP/CA19-9 modified electrode was immersed in 0.1 M PBS pH 6.6 containing 0.5 mM catechol and 0.5 mM H_2O_2 for 40 s, both the cyclic voltammogram and DPV gave steady responses. The HRP/CA19-9 modified electrode showed a low background current in 0.1 M PBS pH 6.6 (curve a in Fig. 2). When 0.5 mM catechol was added in PBS, the cyclic voltammogram showed a couple of oxidation and reduction peaks of catechol at 50 mV/s (curve b in Fig. 2). Upon addition of H_2O_2 to the solution, the reduction peak current increased dramatically, the oxidation peak current decreased (curve c in Fig. 2), and the reduction peak potential of catechol shifted slightly in a negative direction, indicating an obvious electrocatalytic characteristic of the immobilized HRP to the reduction of catechol. The electrocatalytic process was the same as that described by Xiao et al. (1999).

3.2. Scanning electron microscopy of titania sol–gel and CA19-9/titania sol–gel films

The surface morphology of the titania sol–gel matrix is an important factor affecting the immobilization of CA19-9. Fig. 3 shows the surface features of titania sol–gel and CA19-9/titania sol–gel films. The micrograph of titania sol–gel film displayed a chemically clean three-dimensional uniform porous structure. The aggregates of the titania sol–gel matrix on the electrode surface showed a very narrow particle size distribution (Fig. 3a). This uniform open structure provided a significant increase of effective electrode surface for CA19-9 loading and a good preparation reproducibility for the immobilized CA19-9 electrode. When CA19-9 was immobilized on the titania sol–gel matrix, the uniform open structure was retained and bright particles of CA19-9 were observed (Fig. 3b). This structure resulted in free binding of the enzyme conjugate with immobilized CA19-9 and a good amperometric response.

3.3. Optimization of immunosensor preparation

The electrocatalytic activity achieved mainly depended on the surface binding of HRP-labeled CA19-9 antibody, which was related to the immobilization and stability of CA19-9 on the titania sol–gel membrane. The amount of CA19-9 deposited on the electrode surface greatly affected its immobilization and satisfactory preparation of the immunosensor. An optimal volume of 10 μl for 240 U/ml CA19-9 standard solution was selected. CA19-9 molecules were diffi-

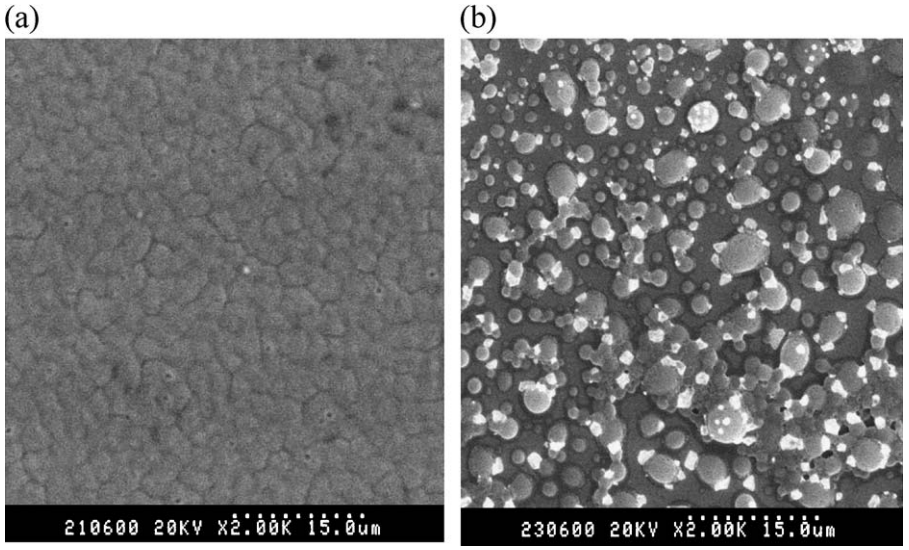

Fig. 3. Scanning electron micrographs of graphite electrodes coated with (a) titania sol–gel and (b) CA19-9/titania sol–gel films.

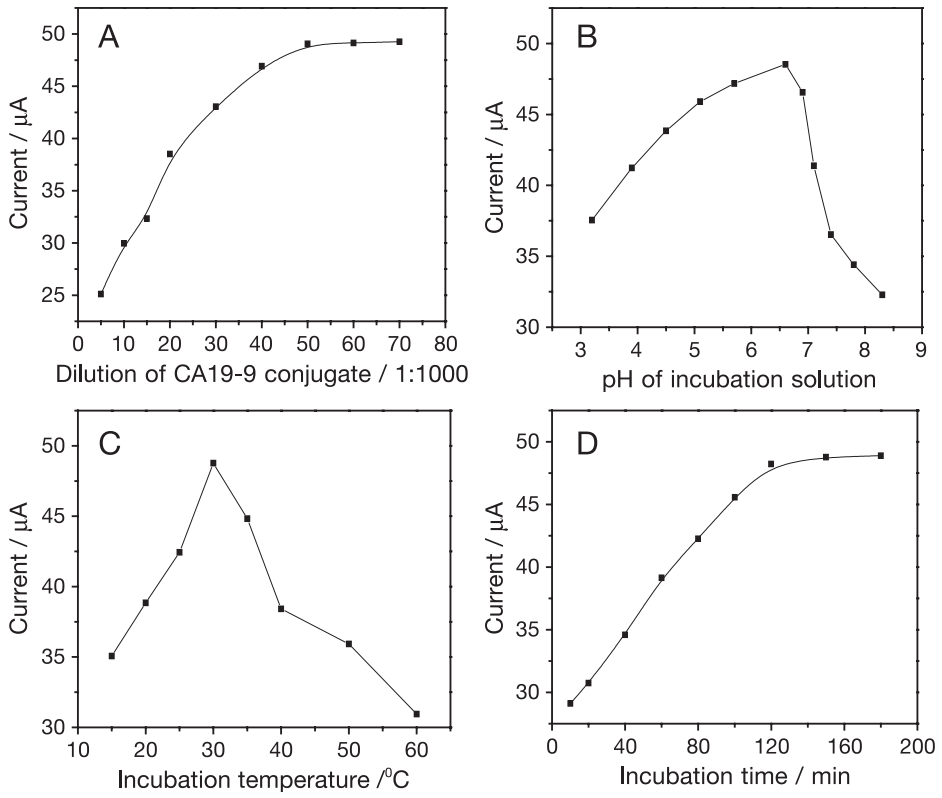

Fig. 4. Effects of dilution of HRP-labeled CA19-9 antibody solution (A), pH of incubation solution (B), incubation temperature (C) and incubation time (D) on DPV peak current of the immunosensor in 0.1 M PBS pH 6.6 containing 0.5 mM catechol and 0.5 mM H_2O_2.

cult to disperse on a hydrophobic electrode surface and discrete foci of CA19-9 molecules were obtained on the surface. In contrast, a hydrophilic surface was beneficial to the immobilization of CA19-9. Thus the electrode was firstly scanned between +0.3 and +1.2 V for 30 cycles and then +0.3 and −1.3 V to increase its surface hydrophilicity (Kepley and Bard, 1988; Wang et al., 2001). The stability of the titania sol–gel membrane depended on the deposition temperature and membrane thickness. The temperature affected directly the vapor pressure of titanium isopropoxide and thus controlled the hydrolysis rate. The experimental results showed that the optimal conditions were at 25 °C for 6 h (Yu and Ju, 2002).

3.4. Optimization of immunoassay conditions

The concentration of HRP-labeled CA19-9 antibody in the incubation solution was another important parameter. The effect of dilution of HRP-labeled CA19-9 antibody solution on the DPV peak current is shown in Fig. 4A. With increasing volume of HRP-labeled CA19-9 antibody solution added in the incubation solution, the catalytic peak current increased and then tended to stabilise at a constant value. At a dilution of 50:950, the current reached a maximum value, indicating that the amount of the HRP-labeled CA19-9 antibody in the incubation solution was enough to match the amount of the immobilized CA19-9 in the titania sol–gel membrane. Thus, 1.0 ml of incubation solution containing 50-μl HRP conjugate was used for the immunoreaction.

Other factors which influenced the immunoreaction between the immobilized CA19-9 and HRP-labeled CA19-9 antibody included the pH of the incubation solution, incubation temperature and incubation time. The relationship between the catalytic peak current of the HRP/CA19-9 modified electrode to the H_2O_2 and catechol system and the pH of the incubation solution indicated that the optimal pH range was between 5.1 and 6.9, with the maximum response at pH 6.6 (Fig. 4B), which was close to the pH value usually used for the binding between antigen and its enzyme conjugate. Accordingly, pH 6.6 PBS was selected for immunoassay.

The effect of incubation temperature on the DPV peak current was studied over a temperature range from 15 to 60 °C. The maximum response occurred at an incubation temperature of 30 °C (Fig. 4C). With increasing incubation time the catalytic response of the immobilized HRP to the H_2O_2 and catechol system increased and reached a maximum at 120 min. Longer incubation times did not improve the response. Therefore, the optimal incubation conditions were PBS pH 6.6 containing 1/20 HRP conjugate solution at 30 °C for 120 min.

3.5. Electrochemical response of the immunosensor to CA19-9 concentration

A competitive assay configuration was used for the determination of CA19-9. The standard solution of CA19-9 at a known concentration or one serum sample was added to the incubation solution containing 1/20 HRP conjugate solution. The CA19-9 in the solution competed with the immobilized CA19-9 on the membrane to bind to the limited binding sites of the HRP-labeled CA19-9 specific antibody. As expected for a competitive mechanism, the DPV catalytic peak current of the immobilized HRP to the H_2O_2 and catechol system showed a decrease with increasing CA19-9 concentrations in the incubation solution (Fig. 5). The decrease in peak current was

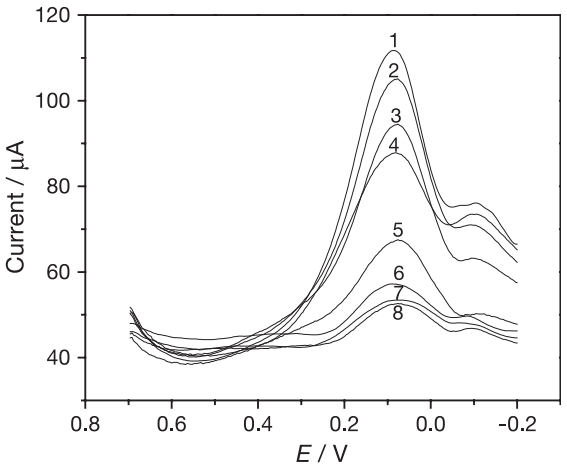

Fig. 5. Differential pulse voltammograms of the immunosensor in 0.1 M PBS pH 6.6 containing 0.5 mM catechol and 0.5 mM H_2O_2 after incubation in 1.0-ml pH 6.6 incubation solutions containing 50-μl HRP-labeled CA19-9 antibody and (1) 0, (2) 3.0, (3) 6.0, (4) 10.0, (5) 16.0, (6) 20.0, (7) 25.0 and (8) 30.0 U/ml CA19-9 at 30 °C for 120 min.

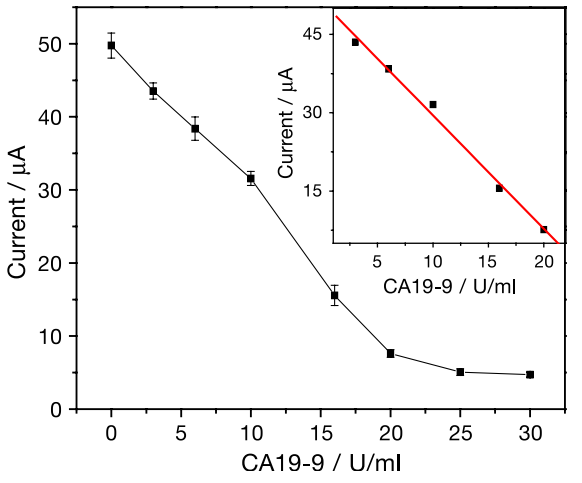

Fig. 6. Calibration curve for CA19-9 determination. Inset: linear relationship between DPV peak current and CA19-9 concentration.

proportional to the CA19-9 concentration over the range of 3–20 U/ml with a correlation coefficient of 0.9965 and a linear slope of 2.171 (± 0.1053) μA/U/ml (Fig. 6). The detection limit was calculated to be 2.68 U/ml taken as the concentration equivalent to a 10% decrease in signal (Keay and McNeil, 1998). Higher serum CA19-9 levels could be detected with an appropriate dilution.

3.6. Selectivity and application of CA19-9 immunosensor

Because of the expression of other antigens such as CA125 in many gastrointestinal malignancies including pancreatic, colorectal, gastric, and hepatic carcinomas, it was important to examine the selectivity of CA19-9 immunosensor. When the incubation solution contained 10 U/ml CA125 and 10 U/ml CA19-9, no difference in current was observable in comparison with the result obtained in the presence of CA19-9 alone. An increase in CA125 concentration did not lead to a significant change in current. Thus, the immunosensor had a good selectivity to CA19-9.

After nine serum samples were diluted with the appropriate volumes of dilution solution, serum CA19-9 concentrations were detected from DPV peak currents of the CA19-9 immunosensor. In parallel, the CA19-9 concentrations were also detected with IRMA. The results are shown in Table 1. The relative deviations between the two methods were in the range of 9.2–4.8%, indicating an acceptable agreement. Thus, the immunosensor could be satisfactorily applied to the clinical determination of CA19-9 levels in pancreatic cancer samples.

3.7. Reproducibility of the CA19-9 immunosensor

The intra-assay precision of the immunosensors was evaluated by assaying the CA19-9 levels of two sera for five replicate measurements in the same run. The intra-assay coefficients of variation with this method were 6.9% and 4.2% at CA19-9 concentrations of 3.8 and 12 U/ml, respectively. The inter-assay precision, or fabrication reproducibility, was estimated by determining in duplicate the CA19-9 level in one serum sample using five immunosensors made independently at the same electrode. The inter-assay precision was 10.4% at a CA19-9 concentration of 18 U/ml, indicating an acceptable reproducibility.

3.8. Stability of the CA19-9 immunosensor

The immunosensor rapidly lost its sensitivity when stored in air. However, the immunosensor could retain its current response after a storage period of 3 weeks in PBS pH 6.6 at 4 °C. This indicated that titania sol–gel prepared by the vapor deposition method provided a biocompatible microenvironment around the antigen molecule and essentially stabilized its biological activity. The large quantities of hydroxyl groups in the sol–gel hybrid material were able to form strong

Table 1
Comparison of serum CA19-9 levels determined using two methods

Serum samples	1	2	3	4	5	6	7	8	9
Immunosensor (U/ml)	9.5	17.1	19.8	30.6	37.6	42.8	54.3	61.2	965
IRMA (U/ml)	8.7	16.6	20.8	28.7	38.2	44.5	57.5	59.5	979
Relative deviation (%)	9.2	3.0	−4.8	6.6	−1.6	−3.8	−5.6	2.9	−1.4

hydrogen bonds (Yu and Ju, 2002). These hydrogen bonds and the intermolecular interactions between antigen molecules and specific sites of the titania sol–gel prevented the immobilized antigen from leaking out of the thin film. On the other hand, the titania sol–gel did retain its porous structure on the storage so that the immobilized antigen was able to bind freely to the HRP-labeled antibody.

4. Conclusions

We have developed a novel immunosensor and immunoassay method for CA19-9 in serum samples by immobilizing CA19-9 in a titania sol–gel thin film. A competitive immunoreaction results in binding of HRP-labeled CA19-9-specific antibody to the immobilized CA19-9. The electrocatalytic response of the immobilized HRP to the H_2O_2 and catechol system shows a decrease proportional to the CA19-9 concentration and this can be used for the determination of serum CA-19-9 levels without the requirement for separation or washing steps. The immunosensor demonstrated good accuracy, acceptable selectivity, sensitivity, reproducibility, storage stability and precision. The sol–gel film is very efficient for the immobilization of antigen and could be extended to the preparation of other amperometric immunosensors for the detection of important antigens.

Acknowledgements

This project was supported by the National Natural Science Foundation of China (No.20275017, 90206037), the Specialized Research Funds for Excellent Young Teachers from the Chinese Ministry of Education, the Science Foundation of Jiangsu (no. BS2001063) and the Key Project of Cancer Institute of Jiangsu Province.

References

Abdel-Hamid, I., Ivnitski, D., Atanasov, P., Wilkins, E., 1999. Highly sensitive flow-injection immunoassay system for rapid detection of bacteria. Anal. Chim. Acta 399, 99–108.

Benkert, A., Scheller, F., Schossler, W., Hentschel, C., Micheel, B., Behrsing, O., Scharte, G., Stocklein, W., Warsinke, A., 2000. Development of a creatinine ELISA and an amperometric antibody-based creatinine sensor with a detection limit in the nanomolar range. Anal. Chem. 72, 916–921.

Birk, B., Henne, V., Hipp, B., Meyer, A., 1997. Core CA 19-9 II EIA: new CA 19-9 enzyme immunoassay with high correlation to radioimmunoassays. Anticancer Res. 17, 2911–2914.

Campanella, L., Attioli, R., Colapicchioni, C., Tomassetti, M., 1999. New amperometric and potentiometric immunosensors for anti-human immunoglobulin G determinations. Sens. Actuators, B, Chem. 55, 23–32.

Clerico, A., Del Ry, S., Giannessi, D., 2000. Measurement of cardiac natriuretic hormones (atrial natriuretic peptide, brain natriuretic peptide, and related peptides) in clinical practice: the need for a new generation of immunoassay methods. Clin. Chem. 46, 1529–1534.

Crowley, E., O'Sullivan, C., Guilbault, G.G., 1999. Amperometric immunosensor for granulocyte-macrophage colony-stimulating factor using screen-printed electrodes. Anal. Chim. Acta 389, 171–178.

Fahnrich, K.A., Pravda, M., Guilbault, G.G., 2002. Immunochemical detection of polycyclic aromatic hydrocarbons (PAHs). Anal. Lett. 35, 1269–1300.

Gervay, J., McReynolds, K.D., 1999. Utilization of ELISA technology to measure biological activities of carbohydrates relevant in disease states. Curr. Med. Chem. 6, 129–153.

Grogan, C., Raiteri, R., O'Connor, G.M., Glynn, T.J., Cunningham, V., Kane, M., Charlton, M., Leech, D., 2002. Characterisation of an antibody coated microcantilever as a potential immuno-based biosensor. Biosens. Bioelectron. 17, 201–207.

Keay, R.W., McNeil, C.J., 1998. Separation-free electrochemical immunosensor for rapid determination of atrazine. Biosens. Bioelectron. 13, 963–970.

Kepley, L.J., Bard, A.J., 1988. Ellipsometric, electrochemical and elememtal characterization of the surface phase produced on glassy carbon electrodes by electrochemical activation. Anal. Chem. 60, 1459–1467.

Kuznetsov, B.A., Shumakovich, G.P., Koroleva, O.V., Yaropolov, A.I., 2001. On applicability of laccase as label in the mediated and mediatorless electroimmunoassay: effect of distance on the direct electron transfer between laccase and electrode. Biosens. Bioelectron. 16, 73–84.

Liu, G.D., Wu, Z.Y., Wang, S.P., Shen, G.L., Yu, R.Q., 2001. Renewable amperometric immunosensor for *Schistosoma japonium* antibody assay. Anal. Chem. 73, 3219–3226.

McNeil, C.J., Athey, D., Ho, W.O., 1995. Direct electron transfer bioelectronic interfaces: application to clinical analysis. Biosens. Bioelectron. 1, 75–83.

Nishizono, I., Üda, S., Suzuki, N., Kawada, H., Murakami, H., Ashihara, Y., Okada, M., 1991. Rapid and sensitive chemiluminescent enzyme immunoassay for measuring tumor markers. Clin. Chem. 37, 1639–1644.

Ohkura, H., Sakawaki, O., Ozaki, H., 1985. Enzyme immunoassay of CA19-9. Enzyme immunoassay and its clinical application. Kan, Tan, Sui, Japan 11, 21–28.

Okamura, K., Kiyoshima, T., Shima, K., Kobayashi, I., Matsuo, K., Ishibashi, H., Komatsu, S., Rasul, A.M.E., Sakai, H., 2002.

Immunohistochemical expression of CA19-9 and CA125 in mucoepidermoid and adenoid cystic carcinomas of the salivary gland. Oral Oncol. 38, 244–250.

Pemberton, R.M., Hart, J.P., Stoddard, P., Foulkes, J.A., 1999. Comparison of 1-naphthyl phosphate and 4 aminophenyl phosphate as enzyme substrates for use with a screen-printed amperometric immunosensor for progesterone in cows' milk. Biosens. Bioelectron. 14, 495–503.

Santandreu, M., Alegret, S., Fàbregas, E., 1999. Determination of beta-HCG using amperometric immunosensors based on a conducting immunocomposite. Anal. Chim. Acta 396, 181–188.

Sellrie, F., Schenk, J.A., Behrsing, O., Böttger, V., Micheel, B., 2002. A competitive immunoassay to detect a hapten using an enzyme-labelled peptide mimotope as tracer. J. Immunol. Methods 261, 141–144.

Toda, K., Tsuboi, M., Sekiya, N., Ikeda, M., Yoshioka, K.I., 2002. Electrochemical enzyme immunoassay using immobilized antibody on gold film with monitoring of surface plasmon resonance signal. Anal. Chim. Acta 463, 219–227.

Van Emon, J.M., Lobez-Avila, V., 1992. Immunochemical methods for environmental analysis. Anal. Chem. 64, 79A–88A.

Van Emon, J.M., Gerlach, C.L., Bowman, K., 1998. Bioseparation and bioanalytical techniques in environmental monitoring. J. Chromatogr., B, Biomed. Sci. Appl. 715, 211–228.

Wang, J., 1999. Electroanalysis and biosensors. Anal. Chem. 71, 328R–332R.

Wang, J., Pamidi, P.V.A., Rogers, K.R., 1998. Sol–Gel derived thick-film amperometric immunosensors. Anal. Chem. 70, 1171–1175.

Wang, H.S., Ju, H.X., Chen, H.Y., 2001. Voltammetric behavior and detection of DNA at electrochemically pretreated glassy carbon electrode. Electroanalysis 13, 1105–1109.

Worwood, M., 2002. Serum transferrin receptor assays and their application. Ann. Clin. Biochem. 39, 221–230.

Xiao, Y., Ju, H.X., Chen, H.Y., 1999. Hydrogen peroxide sensor based on horseradish peroxidase-labeled Au colloids immobilized on gold electrode surface by cysteamine monolayer. Anal. Chim. Acta 391, 73–82.

Yu, J.H., Ju, H.X., 2002. Preparation of porous titania sol–gel matrix for immobilization of horseradish peroxidase by a vapor deposition method. Anal. Chem. 74, 3579–3583.

Zhao, X.Y., Yu, S.Y., Da, S.P., Bai, L., Guo, X.Z., Dai, X.J., Wang, Y.M., 1998. A clinical evaluation of serological diagnosis for pancreatic cancer. World J. Gastroenterol. 4, 147–149.

Zucchelli, G.C., Pilo, A., Jaworek, D., Masini, S., Chiesa, M.R., 1992. Immunoassay of CEA, CA19-9, CA125, and CA15-3 on the automated systems ES 300 and ES 600: methodological evaluation from a multicenter collaborative study. Eur. J. Clin. Chem. Clin. Biochem. 30, 875–879.

Zucchelli, G.C., Pilo, A., Chiesa, M.R., Masini, S., Clerico, A., 1994. Analytical performance of CA19-9, CA125 and CA153 assays as observed through an external quality assessment program. Int. J. Biol. Markers 9, 43–47.