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Journal of Immunological Methods 291 (2004) 165–174

JIM
Journal of Immunological Methods

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Research paper

Chemiluminescent immunosensor for CA19-9 based on antigen immobilization on a cross-linked chitosan membrane

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Received 5 January 2004; received in revised form 14 April 2004; accepted 1 June 2004
Available online 2 July 2004

Abstract

A novel chemiluminescent immunosensor for carbohydrate antigen 19-9 (CA19-9) based on the immobilization of CA19-9 on the cross-linked chitosan membrane was developed. The different membranes were characterized by atomic force microscopy (AFM) and infrared spectrum, respectively. Based on a noncompetitive immunoassay format, this proposed chemiluminescent immunosensor enabled a low-cost, flexible and rapid determination for CA19-9 in combination with flow injection analysis (FIA). After an off-line incubation of the analyte CA19-9 with horseradish peroxidase (HRP)-labeled anti-CA19-9, the mixture was injected into the immunosensor, which led to the trapping of free HRP-labeled anti-CA19-9 by the immobilized antigen in the immunosensor. The trapped HRP-labeled antibody was detected by chemiluminescence due to its catalytic activity following the reaction of luminol and H_2O_2. Under optimal conditions, the decreased chemiluminescent signal of the immunosensor was proportional to the CA19-9 concentration in the range of 2.0–25 U/ml with a detection limit of 1.0 U/ml. The immunosensor showed an acceptable accuracy and good reproducibility. The results of 20 human serum samples detected by this method were in acceptable agreement with those obtained by immunoradiometric assay. The proposed immunosensor provided a new promising tool for practical clinical detection of the serum CA19-9 level.

Keywords: Noncompetitive immunoassay; Immunosensor; Biosensors; Chemiluminescent sensors; Flow injection analysis; Chitosan membrane; Carbohydrate antigen 19-9

1. Introduction

Carbohydrate antigen 19-9 (CA19-9) is one of the most important carbohydrate tumor markers. The determination of serum CA19-9 levels plays an important role in clinical diagnoses of pancreatic, colorectal, gastric and hepatic carcinomas. The levels

Abbreviations: AFM, atomic force microscopy; BSA, bovine serum albumin; CA19-9, carbohydrate antigen 19-9; CV, coefficient of variation; EIA, enzyme immunoassay; FIA, flow injection analysis; T cell receptor Vβ repertoires using real time PCR: a comparison of SYBR green and a dual-labelled HuTrec™ fluorescent probe.

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doi:10.1016/j.jim.2004.06.001

of serum CA19-9 have been determined by enzyme-linked immunosorbent assay (Ohkura et al., 1985), chemiluminescent enzyme immunoassay (EIA) (Nishizono et al., 1991) and electrochemical EIA (Du et al., 2003). Although EIA is a powerful tool for the detection of antigen, and many commercial kits are available for the determination of CA19-9 (Birk et al., 1997; Zhao et al., 1998; Okamura et al., 2002), there are some drawbacks in the conventional assays, including time-consuming, difficult automation and high cost. Thus, it is necessary to improve the traditional immunoassay procedures. Recently, a few commercially automated immunoassay analyzers have been used in clinics characterized by efficient use of labor, minimal sample and reagent manipulation, easy automation and flexibility (Hendriks et al., 2000; Smith et al., 1993). In this study we adopted flow injection immunoassay (FIIA) to develop a novel detection technique for tumor markers due to its simple and flexible operation, rapid assay speed, easy automation and good precision (Liu et al., 1991; Locascio-Brown et al., 1990). As an alternative to robotic systems, FIIA offers a simplified system of automation (Fintschenko and Wilson, 1998).

Since flow injection analysis (FIA), introduced in 1975 (Ruzicka and Hansen, 1975), was combined with immunoassay, FIIA has been proven to be a powerful analytical tool and has been attractively applied in many fields such as environmental, pharmaceutical, food and clinical assays (Bjarnason et al., 1997; Bereczki and Horváth, 1999; Burestedt et al., 2000; Nandakumar et al., 2000). In FIIA heterogeneous immunoassays, a solid support is usually used to immobilize either the antibody or antigen, thus permitting the separation of free fractions from bound immunocomplexes (Botchkareva et al., 2002). A wide variety of matrices, including inorganic surfaces, organic polymers and other commercially available solid supports, have been used for the design of packing materials (Leonard, 1997; Rhemrev-Boom et al., 2001; Butler, 2000). The most commonly used materials are bead supports (silica, agarose, sepharose and polystyrene particles with a magnetic nucleus) and membranes (Morais et al., 1999). Additionally, lower cost is also an important parameter to consider for the future application of the solid phases. Here, we selected chitosan, a naturally abundant material, as the solid phase for the immobilization of CA19-9.

Chitosan is an *N*-deacetylated derivative of natural chitin. It possesses excellent membrane-forming ability, high permeability toward water, good adhesion, biocompatibility, nontoxicity, high mechanical strength and susceptibility to chemical modifications due to the presence of reactive amino groups and hydroxyl functional groups (Cruz et al., 2000). These properties have prompted the extensive applications of chitosan as a matrix for enzyme immobilization (Hikima et al., 1993; Ng and Zhao, 1998; Wang et al., 2003) and affinity purification of biomolecules (Yang and Chen, 2002). To the best of our knowledge, the chitosan membrane has not yet been used as a solid phase matrix in immunoassay. Here, the chitosan was cross-linked by γ-glycidoxypropyltrimethoxy silane (GPS) to immobilize CA19-9 in order to prepare the CA19-9 chemiluminescent immunosensor, which could minimize the leakage of CA19-9 and increase the stability of the coating.

Immunosensors have attracted growing attention with the expectation of obtaining a quick and highly sensitive immunological response (Darain et al., 2003). Various detection methods such as amperometric, electrochemiluminescent, photometric, and chromatographic detection have been combined with immunosensors for antigen analyses (Byfield and Abuknesha, 1994; Kalab and Skladal, 1997; Rishpon and Ivnitski, 1997; Marquette and Blum, 1998; Wang, 1999; Liu et al., 2001; Grogan et al., 2002). Chemiluminescent detection possesses several advantages such as the absence of radioactive waste, the relatively simple instrumentation required, the very low detection limit and a wide dynamic range (Baeyens et al., 1998). It could be versatile and flexible in many different approaches. This work proposes a novel CA19-9/chitosan membrane-based chemiluminescent immunosensor for noncompetitive immunoassay of CA19-9 in combination with FIA detection. This proposed method shortens the analytical time to 35 min including the pre-incubation and detection steps. In comparison with the results obtained by immunoradiometric assay (IRMA), the immunosensor shows an acceptable accuracy. This strategy could be further developed for practical clinical detection of CA19-9 and other important tumor markers.

2. Materials and methods

2.1. Materials and reagents

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 horseradish peroxidase (HRP)-labeled CA19-9 monoclonal antibody from goat. Bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO, USA). Chitosan (MW $1.9-3.1 \times 10^5$; 85–90% deacetylation, Aldrich), GPS (Nanjing Chemical Plant), luminol (Shanxi Normal University, China), *p*-iodophenol (PIP, Weihai Newera Chemical, China), and H_2O_2 (Shanghai Chemical Plant, China) were used as supplied by the manufacturers. All other reagents were of analytical reagent grade and were used without further purification. Luminol (0.01 M) and PIP (0.01 M) stock solutions were kept in the dark and diluted

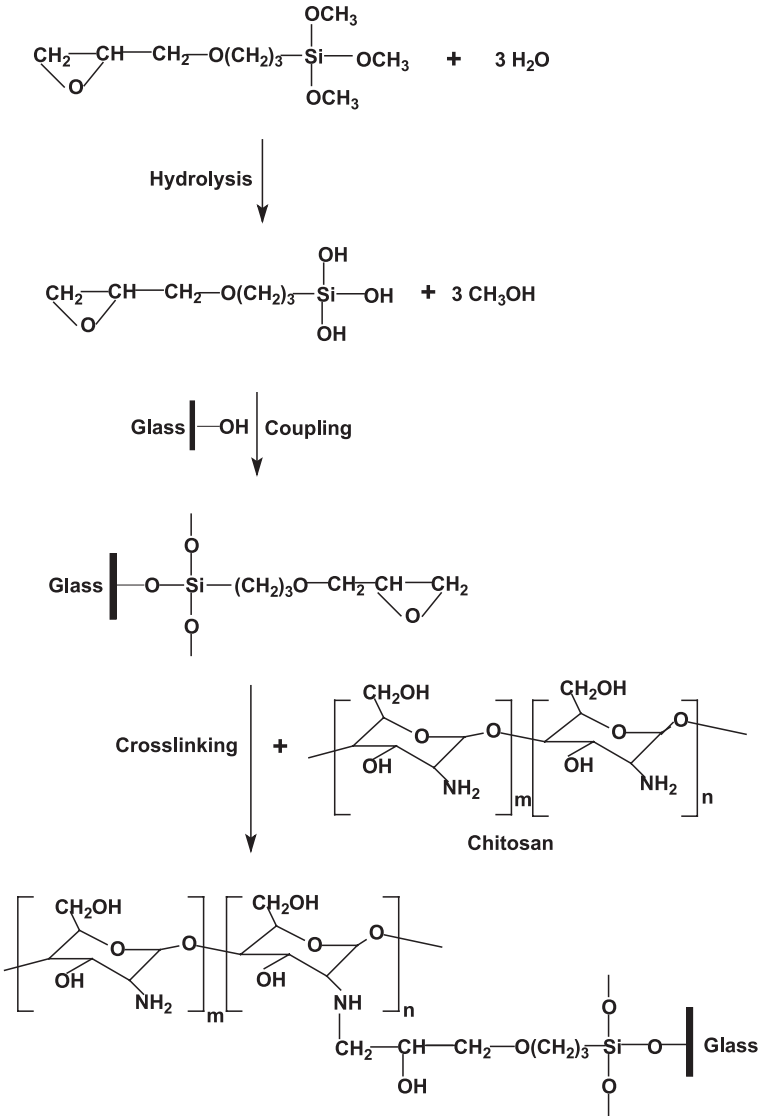

Fig. 1. Reaction mechanism of chitosan cross-linked by GPS.

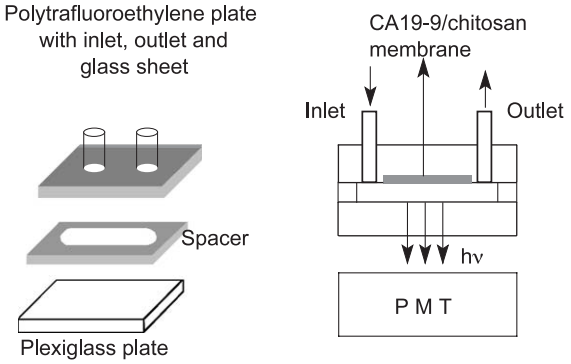

Fig. 2. Configuration of the CA19-9 chemiluminescence immunosensor.

using 0.1 M Tris–HCl buffer solution (pH 8.5) prior to use. H_2O_2 working solution was prepared by appropriate dilution of 30% solution in water daily. Doubly distilled water was used throughout the experiment.

2.2. Instrumentation

The two peristaltic pumps in a luminescence analyzer (IFFM-D, Remex Electronic Instrument, Xi'an, China) were used to deliver flow streams. Polytrafluoroethylene tubing (0.8 mm i.d.) was used to connect all components in the flow system. A multi-way valve was used to shift the directions of the fluids. The CA19-9/chitosan membrane-based immunosensor was linked to the flow system. The chemiluminescent signal was detected by a photo multiplier tube (PMT) under the immunosensor and was recorded with a computer via an A/D conversion card. H_2O was used as the carrier.

Atomic force micrographs (AFM) were obtained with a Seiko Spi3800N atomic force microscope (Seiko, Japan). The IR spectra of the membranes were measured using a Nicolet 400 Fourier transform infrared (FT-IR) (Nicolet, USA).

2.3. Preparations of cross-linked chitosan membrane and CA19-9 immunosensor

A glass sheet (20 × 10 × 1.0 mm) was rinsed with double distilled water and acetone prior to use. According to the method reported previously (Rattana et al., 2002), 1% GPS (in 3:1 ethanol/H_2O) was first set for 60 min for full hydrolysis. Then 50 μl of the solution were dropped onto the sheet and set in an oven at 93 °C for 60 min to coat GPS onto the sheet by silane coupling reaction. After that, 50 μl of 1% chitosan acetate solution were deposited on the GPS coated surfaces and set in the oven at 105 °C for 60 min. The reaction between the primary amine of the

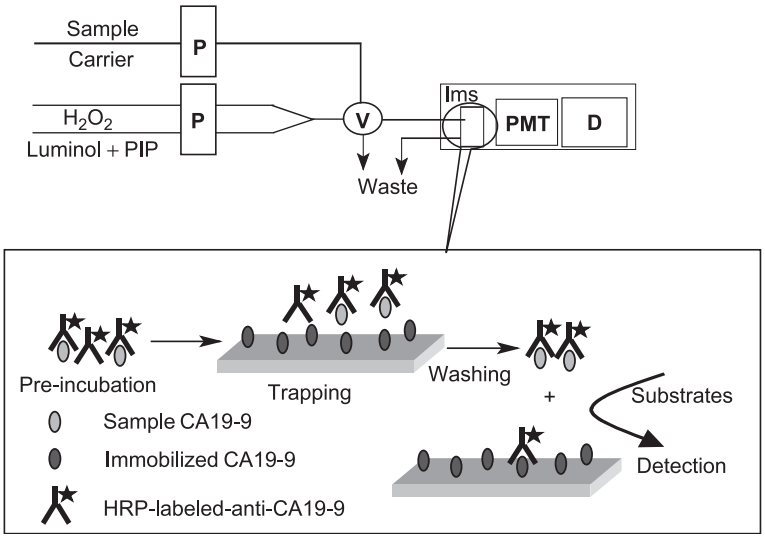

Fig. 3. Schematic diagram of the chemiluminescent noncompetitive FIIA system: P, peristaltic pump; V, eight-way valve; Ims, immunosensor; PMT, photo multiplier; D, detector.

chitosan molecule and GPS coated surfaces occurred via the epoxy ring. The possible reaction mechanism is shown in Fig. 1.

A total of 50 μl of 120 U/ml CA19-9 standard solution were dropped onto the chitosan membrane and kept at 4 °C overnight, and then dipped in BSA solution to block the active sites. The configuration of the immunosensor is shown in Fig. 2. The top polytrafluoroethylene plate was grooved to embed the CA19-9/chitosan membrane coated glass sheet. A spacer was sandwiched between two plates to form a channel for FIIA. After every experiment, the channel was filled with 0.1 M PBS (pH 7.0) and kept at 4 °C.

2.4. Noncompetitive flow injection immunoassay

The detection of CA19-9 was based on a noncompetitive flow injection immunoassay. The schematic diagram of the procedure is shown in Fig. 3. Diluted HRP-labeled anti-CA19-9 solution (50 μl) in 0.1 M PBS (pH 7.0) was mixed with 50 μl CA19-9 standard solution or serum sample. After 30 min at room temperature (25 ± 2 °C), the immunomixture was carried through the immunosensor at a flow rate of 0.1 ml/min. At the same time, luminol, PIP and H_2O_2 were mixed with a Y-shaped element. After the free enzyme conjugate was trapped by the immobilized CA19-9 and retained in the immunosensor, chemiluminescent substrates (100 μl) were injected by turning the valve and carried to the channel to react with the enzyme conjugate trapped in the immunosensor. The chemiluminescent signal was detected and recorded. The immunosensor was regenerated by the flow of 0.1 M glycine–HCl (pH 2.2) for 1 min, and then equilibrated with 0.1 M PBS (pH 7.0) for 1 min. After that, a new assay was ready to begin. The total assay time was 35 min for one sample including 30 min of preincubation and 5 min of detection and regeneration of the immunosensor.

3. Results and discussion

3.1. Characteristic study of the cross-linked chitosan membrane surface and CA19-9/chitosan membrane

The surface morphologies of the membranes were observed by AFM. From the images shown in Fig. 4, it

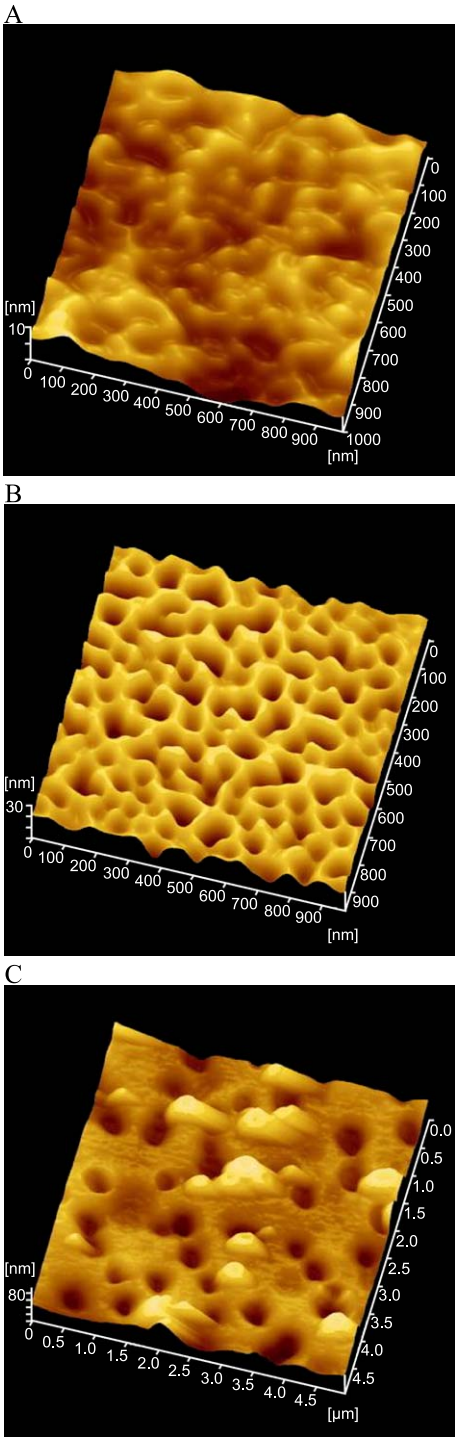

Fig. 4. AFM images of pure chitosan (A), GPS cross-linked chitosan (B) and CA19-9/chitosan (C) membranes.

can be seen that the thickness of the pure chitosan layer (Fig. 4A) was about 10 nm, while the thickness of the cross-linked chitosan layer (Fig. 4B) was about 30 nm. Comparing the images of Fig. 4A and B, it can be concluded that the cross-linked chitosan membrane possessed a more regular and homogeneous surface with a uniform porous network-like structure than the pure chitosan membrane. Fig. 4C shows the absorption of CA19-9 on the cross-linked chitosan membrane.

The shapes of the amide I and amide II infrared absorbance bands of proteins can provide detailed information on the secondary structure of the polypeptide chain (Kauppinen et al., 1981). The amide I band (1700–1600 cm^{-1}) of proteins is caused by C–O stretching vibrations of peptide linkages. The amide II band (1600–1500 cm^{-1}) results from a combination of N–H in-plane bending and C–N stretching of the peptide groups. The FT-IR spectra of CA19-9 showed two bands at 1652 and 1540 cm^{-1}, which were assigned to the C–O stretching vibration of the amide I and a characteristic amide II band of CA19-9, respectively. There were slight changes in the FT-IR spectra of the CA19-9/chitosan membrane. The similarities of the two spectra indicated that CA19-9 retains the essential feature of its native structure in the CA19-9/chitosan membrane.

3.2. Choice of conditions for immunosensor preparation

The preparation of the CA19-9/chitosan membrane was greatly affected by the surface structure of the cross-linked chitosan membrane. The performance of the chitosan membrane mainly depended on the amounts of GPS and chitosan used in its preparation. The optimal concentration of chitosan was 1%. At the same volume of chitosan and GPS solutions, 1% GPS (in 3:1 ethanol/H_2O) was chosen as the coupler concentration. The chitosan membrane cross-linked by GPS not only possessed high adhesive ability, but also high biocompatibility and chemical stability. These properties of the membrane were suitable for its application in a flow injection system.

The chemiluminescent detection partially depended on the binding of HRP-labeled CA19-9 antibody on CA19-9/chitosan membrane, which was related to the immobilization and stability of CA19-9 in the chitosan membrane. An optimal volume of 50 μl for 120 U/ml CA19-9 was selected.

3.3. Optimization of immunoassay procedure

The factors that affected the immunoassay procedure included the working concentration of HRP-labeled anti-CA19-9, the pre-incubation time for the immunoreaction between analyte CA19-9 and HRP-labeled anti-CA19-9, and the flow rate of the immunomixture through the immunosensor after incubation.

The optimal dilution was used as the working concentration of HRP-labeled anti-CA19-9. It was established by injecting different dilutions of HRP-labeled anti-CA19-9 into the immunosensor. The HRP-labeled anti-CA19-9 molecules reacted with the immobilized CA19-9 and were trapped in the CA19-9/chitosan membrane. Thus, the different chemiluminescent signals were obtained due to the catalytic activity of the immobilized enzyme in the chemiluminescent reaction when the substrates flowed through the sensor. Fig. 5A shows the experimental results. The chemiluminescent signal reached a maximum value when the antibody was diluted in the proportions of 1:7.5, which meant that almost all of the specific sites of immobilized CA19-9 were occupied. Therefore, a 1:7.5 dilution of HRP-labeled anti-CA19-9 was chosen as the working solution in this study.

A sample containing 15 U/ml CA19-9 was incubated with a 1:7.5 dilution of enzyme conjugate at room temperature for different time intervals to select an optimal pre-incubation time. With an increasing pre-incubation time, the amount of HRP-labeled anti-CA19-9 available to react with analyte CA19-9 increased, while the free enzyme conjugate in the mixture decreased, which resulted in a decrease in the amount of HRP-labeled anti-CA19-9 trapped in the immunosensors. Thus, the chemiluminescent intensity decreased. Fig. 5B indicates that the signal reached a minimum value at a pre-incubation time of 30 min. Therefore, a pre-incubation time of 30 min was chosen for CA19-9 detection.

Fig. 5C shows the effect of the retention time of the immunomixture in the channel on the trapping efficiency of HRP-labeled anti-CA19-9 by the immunosensor. The retention time was calculated with the flow rate and the volume of the channel. The plot reached a plateau at a retention time of 2 min that corresponded to

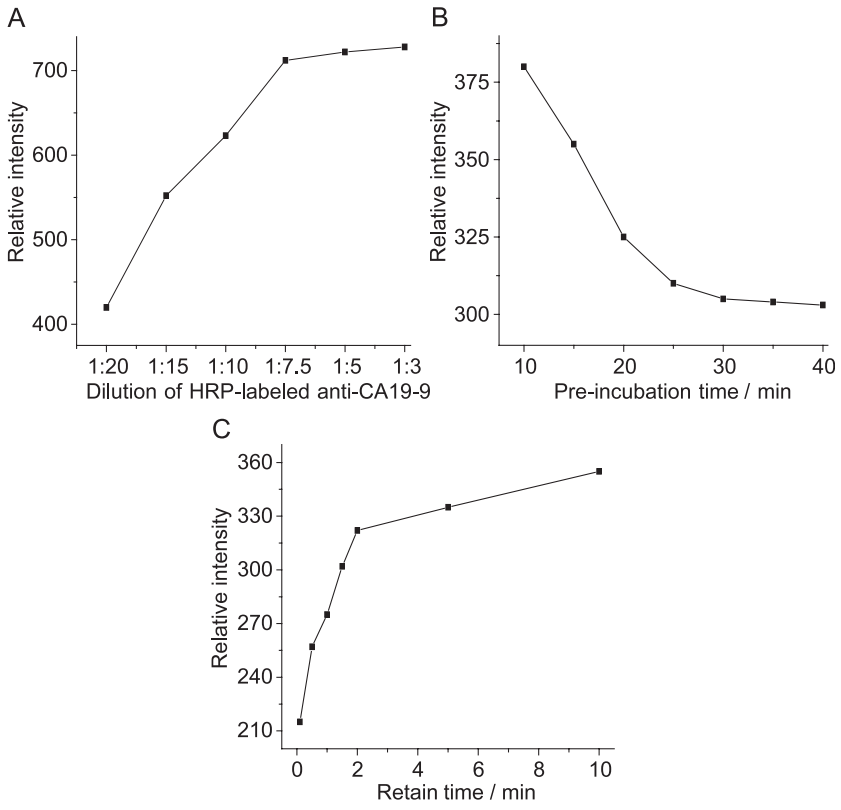

Fig. 5. Effects of dilution of HRP-labeled CA19-9 antibody solution (A), pre-incubation time (B) and retention time of the immunomixture of 15 U/ml CA19-9 and the 1:7.5 dilution of HRP-labeled anti-CA19-9 in the immunosensor (C) on the chemiluminescent intensity of 1.5 mmol/l H_2O_2, 0.5 mmol/l luminol and 0.5 mmol/l PIP.

a flow rate of 0.1 ml/min. The capture of the free enzyme conjugate could reach 90% of that at a static state for 10 min. Considering the total analytical time and the practical operation of this method, a flow rate of 0.1 ml/min was chosen in this experiment.

3.4. Optimal conditions of FIA chemiluminescent detection

The rate of the chemiluminescent substrates flowing through the immunosensor to react with the trapped enzyme conjugate greatly affected the detection of CA19-9. At a high flow rate the recorded signal showed a sharp peak with a high intensity. However, too high a flow rate would affect the chemiluminescent detection. Consequently, a flow rate of 2.0 ml/min was satisfactory for FIIA for CA19-9.

The concentrations of luminol, PIP and H_2O_2, and the pH value of the chemiluminescent detection were also important parameters. In accordance with the experiment reported in Liu et al. (1991), here the optimal concentrations of H_2O_2, luminol and PIP were selected at 1.5, 0.5 and 0.5 mmol/l in pH 8.5 Tris–HCl buffer solution, respectively.

3.5. Noncompetitive FIIA for CA19-9

Typical flow injection chemiluminescent signals obtained under optimal conditions with the noncompetitive immunoassay for CA19-9 are shown in Fig. 6. The blank signal was measured when only the substrates flowed through the sensor. With the increasing CA19-9 concentration, the amount of HRP-labeled anti-CA19-9 trapped in the CA19-9/chitosan membrane decreased, leading to a decrease of the

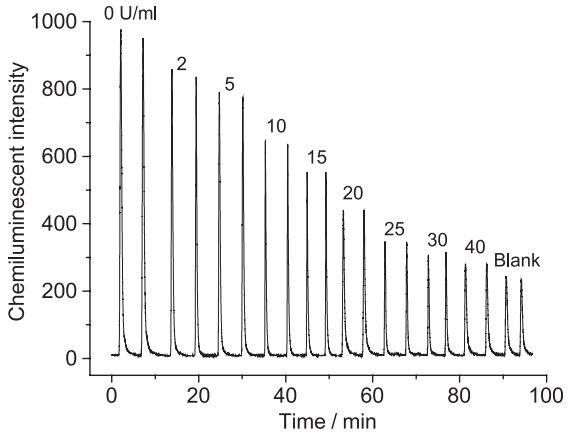

Fig. 6. FIA diagram of noncompetitive immunoassay for CA19-9 at different concentrations at a sampling interval of 5 min. Blank peaks are generated from the reaction between substrates without a sampling step.

chemiluminescent signal. When the CA19-9 concentrations were higher than 40 U/ml, most of the HRP-labeled anti-CA19-9 molecules in the pre-incubation solution bound with analyte CA19-9, so the chemiluminescent signal decreased to the blank value. Fig. 7 shows the plot of the relative chemiluminescent intensity vs. the CA19-9 concentration. The plot shows a linear decrease over the concentration range of CA19-9 from 2.0 to 25 U/ml with a correlation coefficient of 0.998 (inset in Fig. 7). The total assay time including the pre-incubation step was only 35 min for one sample. The detection limit, taken as the concentration equivalent to a 10% decrease in the signal (Gascón et al., 1995; Keay and McNeil, 1998; Du et al., 2003), was calculated to be 1.0 U/ml. When the CA19-9 concentration was higher than 25 U/ml, the detection needed an appropriate dilution of the sample.

3.6. Accuracy and clinical application

The accuracy of CA19-9 detection was examined by comparing the analytical results of 20 serum samples with this method and IRMA. The mean CA19-9 concentrations determined with IRMA were 5.4, 9.5, 16.2, 17.8, 11.6, 16.7, 21.1, 22.5, 17.3, 16.4, 23.2, 14.7, 20.3, 5.8, 15.8, 19.3, 13.8, 14.1, 14.3 and 16.0 U/ml, respectively, while this method resulted in values of 5.05, 10.0, 15.5, 18.5, 10.8, 15.8, 20.1, 20.7, 18.2, 17.8, 22.1, 15.8, 22.1, 5.4, 17.3, 18.2, 15.0, 15.2, 12.9 and 17.2 U/ml, respectively. The relative deviations between the two methods were from −9.79% to 9.49%. Thus, the two methods were in acceptable agreement.

3.7. Stability of the CA19-9 immunosensor

The stability of the immunosensor was assessed from the intra- and inter-assay coefficients of variation (CVs). The intra-assay CV was the difference among three determinations of one sample on the same CA19-9/chitosan membrane after the immunosensor was regenerated. The inter-assay CV was the difference among the measurements of one sample on three different CA19-9/chitosan membranes. The intra- and inter-assay CVs obtained at a CA19-9 concentration of 5.0 U/ml were 1.18% and 9.06%, respectively. Obviously, the preparation of the immunosensor possessed acceptable reproducibility. The low value of intra-assay CV indicated that the immunosensor could be regenerated and used repeatedly. After the immunosensor was used 25 times, the analytical performances did not show an obvious decline. These results demonstrated that the immunosensor possessed good stability and could be prepared in batches.

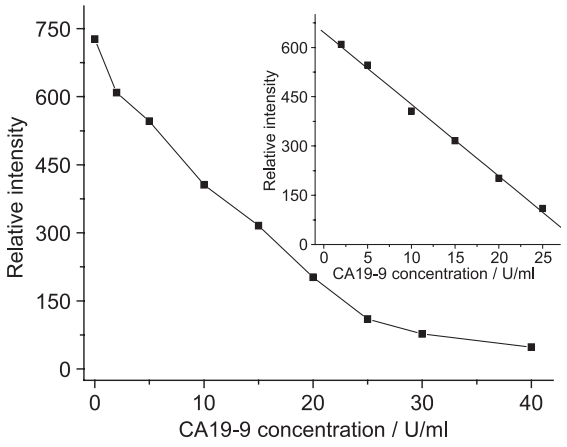

Fig. 7. Calibration curve for CA19-9 determination. Inset: the linear relation between relative chemiluminescent intensity and CA19-9 concentration.

4. Conclusions

A CA19-9/chitosan membrane, which could be replaced freely, was embedded in the flow cell to constitute a novel chemiluminescent immunosensor for the determination of serum CA19-9 levels. This low-cost and flexible chemiluminescent immunosensor, integrating a flow injection system, permits a more simple, sensitive and rapid noncompetitive immunoassay for CA19-9. The cross-linked chitosan membrane shows an efficient immobilization of CA19-9 antigen. The immunosensor shows an acceptable accuracy, good reproducibility and storage stability. In comparison with the conventional EIA approaches, this proposed method is simpler, faster and more flexible, and suitable for automated sample handling. The detection time for each sample is only 35 min including the preincubation step. The cross-linked chitosan membrane could be further used for the preparation of immunosensors for other important tumor markers.

Acknowledgements

This project was supported by the Distinguished Young Scholar Fund awarded to HX Ju (20325518), the National Natural Science Foundation of China (No. 20275017, 90206037), the Specialized Research Funds for the Excellent Young Teachers from the Chinese Ministry of Education, the Science Foundation of Jiangsu (BS2001063) and the Key Project of the Cancer Institute of Jiangsu Province.

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