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ORIGINAL PAPER

Optimization of Fluoroimmunoassay against C-Reactive Protein Exploiting Immobilized-antigen Glass Slide

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Abstract An optimization experiment for an indirect-competitive (IC) fluoroimmunoassay (FIA) against C-reactive protein (CRP) was conducted exploiting an immobilized-antigen glass slide and an anti-CRP antibody tagged with fluorescent silica nanoparticles (FSNPs). The optimized conditions for the IC FIA were as follows: time and concentration of treatment with glutaraldehyde, 30 min and 1.5 %, respectively; time of reaction with coating antigen and concentration of coating antigen for immobilization, 1 h and 0.1 mg/mL, respectively; concentration of FSNP-anti-CRP antibody conjugate coupled by the biotin-avidin interaction, the bioconjugate, for immune reaction, 0.250 mg/mL; concentration of bovine serum albumin (BSA) for blocking and time of blocking with BSA, 3 % and 30 min, respectively. By using the glass slide, a highly sensitive detection against CRP was possible with the limit of detection below 0.1 ng/mL.

Keywords Optimization · Indirect-competitive fluoroimmunoassay · C-Reactive protein · Antibody tagged with fluorescent silica nanoparticles · Immobilized-antigen glass slide

Introduction

Biomarker proteins in the blood are important indices to carry out diagnosis, prediction of response, and prediction of prognosis and susceptibility, and are normally classified into susceptability biomarkers, early detection biomarkers, prognostic biomarkers, predictive biomarkers and pharmacodynamic biomarkers according to disease process [1]. To conduct detection of serum biomarkers, development of a reliable analytical measure is prerequisite. From available analytical methods that include spectrophotometry, immunoassays and immunosensing, fluoroimmunoassay (FIA) that exploits the complex formation between antigen and antibody, and determines fluorescence emission of a bioconjugate including fluorophore seems to be promising due to its intrinsic high-sensitivity, specificity and rapidity [2–5].

C-Reactive protein (CRP) is a 118-kDa pentameric protein secreted by the liver upon stimulation by interleukin (IL)-6 and IL-1β [6], and is an important biomarker for coronary heart disease, hypertension, and inflammation. A prospective field of application of CRP measurement is homogeneous evaluation of food functionality that is able to claim disease-preventing effects of food in a model animal like rat or human administered with a diet that contains a candidate functional material or functional food [7, 8]. Meanwhile, fluorescent silica nanoparticles (FSNPs) could be key nanomaterials for labeling antibodies or antigens for use in immunosensing. They are bright and photostable, less prone to stochastic blinking with sustained imaging, and compatible with aqueous systems owing to hydrophilic surface [9–12]. In light of these properties, an FIA that exploits FSNP fluorescence could be a good analytical method to detect serum biomarkers sensitively.

As an initial step in developing an assay for biomarker detection to evaluate food functionality, we had developed an immobilized-antigen immunofluorescence glass slide system that measured the fluorescence emission of an FSNP–biomarker antibody conjugate bound to the immobilized CRP on the slide surface [13]. In this study, the assay conditions for an indirect-competitive (IC) FIA against CRP were optimized using the slide.

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Experimental

Reagents and Materials

Recombinant histidine-tagged rat CRP (carrier-free) that had been expressed in a mouse myeloma cell line (NS0) was purchased from R&D Systems (Minneapolis, MN, USA) and used as a target analyte. Its homopentameric structure is composed of three non-covalent and two covalently linked subunits. A monoclonal anti-rat CRP antibody was also obtained from R&D Systems. The antibody was prepared from a hybridoma that resulted from the fusion of a mouse myeloma with B cells acquired from a mouse immunized with purified, NS0-derived, recombinant rat CRP. A water-soluble biotinylation reagent, sulfosuccinimidyl-6-(biotinamido)hexanoate (sulfo-NHS-LC-biotin), streptavidin (SA)-maleimide and phosphate buffered saline (PBS) packs that yielded 500 mL of 0.1 M sodium phosphate buffer (pH7.2, including 0.15 M sodium chloride) were obtained from Pierce Biotechnology (Rockford, IL, USA). 3-Aminopropyltrimethoxysilane (APTMS), glutaraldehyde (GA), dichlorotris(1,10-phenanthroline)ruthenium(II) hydrate (Ruphen) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were guaranteed reagents from various suppliers and double distilled water was used throughout this study. Plain microscopic slides for use as transducers were acquired from Corning (Kennebunk, ME, USA) and a dialysis cassette kit from Pierce Biotechnology was used to remove unreacted reagents after biotinylation of antibody.

Preparation of Immobilized-antigen Glass Slide

CRP was immobilized as a coating antigen using APTMS with referring to a previous report [13]. A glass slide was cleaned by dipping into piranha solution (H_2SO_4: H_2O_2=3:1, v/v) for 10 min, and was rinsed successively with distilled water three times and sonicated in distilled water for 5 min. The slide was hydrated further with hot water at 90 °C for 1 h and dried at room temperature. The cleaned slide was treated with 10 % APTMS in acetone in a Petri dish at room temperature for 1 h, and rinsed with acetone, 50 mM sodium phosphate buffer (pH7.4), the reaction buffer for the system, and distilled water three times. It was then dipped into distilled water for 1 min, dried at room temperature for 30 min, and heat-treated at 120 °C for 5 h. After cooling, the APTMS-treated glass slide was incubated in distilled water for 15 min at room temperature to hydrate the slide surface. The slide was then activated with various concentrations of GA at room temperature for various time, rinsed with the reaction buffer three times, dipped into distilled water for 1 min and dried at room temperature for 30 min. Aliquots of 20 μL of various concentrations of coating antigen were spotted onto the GA-activated glass slide with the aid of a grid-type spotting guide and the slide was incubated at room temperature for various time in a Petri dish. After incubation, the slide immobilized with the coating antigen was rinsed with the reaction buffer three times and dipped into distilled water for 1 min to remove unbound antigen. The part of the slide surface to which no protein was bound was blocked with various concentrations of BSA for various time to prevent non-specific binding of a FSNP–antibody conjugate coupled by the biotin-avidin interaction, the bioconjugate. After rinsing with the reaction buffer three times and dipping into distilled water for 1 min, the BSA-blocked functionalized slide was dried at room temperature for 30 min and stored in a Petri dish inside a desiccator at 4 °C until use.

Preparation of Bioconjugate for Use in Indirect-competitive Fluoroimmunoassay

SA-modified FSNPs that include Ruphen as a fluorescent dye and a biotinylated anti-CRP antibody were prepared in accordance with the methods of Kim et al. [13]. The bioconjugate coupled by the biotin-avidin interaction was manufactured simply by mixing these two components [13].

Fluorescence Measurement

Fluorescence from the immune complex between the antigen and antibody was detected using a fluorescence microscope (Nikon Eclipse 80i, Nikon, Melville, NY, USA), equipped with eye pieces, a monochrome-cooled digital camera head (DS-Qi1, Nikon), a mercury lamp (Osram, München, Germany), a halogen lamp (Osram), a camera control and a PC [14]. Images were collected through a 20× microscope objective using an Epi-fluorescence filter block NB-2A that contained a 450–490-nm band pass excitation filter, a 505 nm dichroic mirror, and a 520 nm barrier filter.

Optimization of Assay Conditions

Assay conditions for the IC FIA against CRP were optimized by measuring binding of the bioconjugate to the immobilized coating antigen with regard to time and concentrations of treatment with GA, time of reaction with coating antigen and concentrations of coating antigen for immobilization, concentrations of BSA for blocking and time of blocking with BSA, and concentrations of the bioconjugate for immune reaction (Fig. 1). For a blank run, BSA was immobilized on the surface of the activated slide and binding of the bioconjugate to the coated BSA was compared with that to the coating antigen. Background interference that was defined as the ratio of fluorescence intensity of the spot immobilized with BSA after the immune reaction against that of the spot coated with CRP after

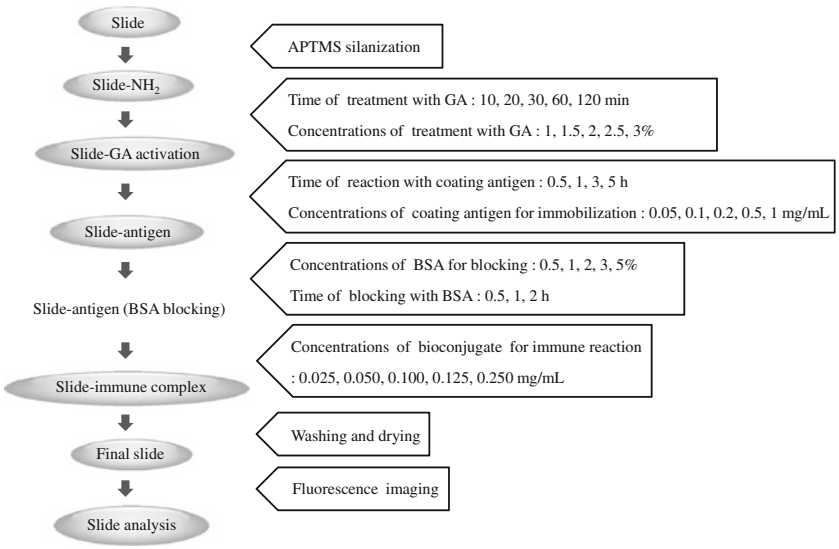

Fig. 1 Conditions for optimization in reaction variables of indirect-competitive fluoroimmunoassay against C-reactive protein

the immune reaction was calculated. The immune reactions were conducted as follows. Aliquots of 20 μL of a diluted bioconjugate solution were spotted onto individual spots of the coating antigen and BSA on a prepared glass slide with the aid of the spotting guide, and the slide was incubated at room temperature for 3 h in a Petri dish. The unbound bioconjugate was removed by rinsing the slide with the reaction buffer three times and dipping it twice into distilled water for 1 h. The slide was then dried with a Kimwipes and examined by fluorescence microscopy.

Detection of C-Reactive Protein

To conduct the IC FIA against CRP, 20 μL of the mixture composed of the optimized bioconjugate solution (0.250 mg/mL) plus each CRP solution at different concentrations of 0.01, 0.05, 0.1, 0.5 and 1 ng/mL were spotted to the individual spots of the immobilized-antigen glass slide as described above, and the resultant slide was incubated at room temperature for 3 h in a Petri dish. The unbound bioconjugate and analyte were removed by rinsing the slide with the reaction buffer three times and dipping it twice into distilled water for 1 h. The slide was then dried with a Kimwipes and examined by fluorescence microscopy. The overall procedure to prepare the immobilized-antigen glass slide and to conduct the IC FIA exploiting the slide and the bioconjugate is schematically depicted stepwise in Fig. 2.

Results and Discussion

In this study, reaction variables during the whole course of the IC FIA against CRP were optimized by comparing fluorescence intensity from sample and blank spots on the surface of the immobilized-antigen glass slide after the immune reaction.

Optimization of Treatment with Glutaraldehyde

Background interference that was defined in the Experimental section was considered as a criterion for optimization in assay procedure. The glass slide treated with 10 % APTMS in acetone was reacted at varying time of treatment with GA in 10–120 min and concentrations of treatment with GA in 1–3 %. At this moment, time of reaction with coating antigen, concentration of coating antigen for immobilization, concentration of BSA for blocking, time of blocking with BSA, concentration of bioconjugate for immune reaction and time of immune reaction with bioconjugate were fixed at 1 h, 0.1 mg/mL, 1 %, 1 h, 0.125 mg/mL and 3 h, respectively. When 2.5 % GA was used for slide activation, background interference was in the lowest values of 0.34 and 0.33 at 30 min of GA treatment on the basis of total fluorescence and number of fluorescent particles, respectively (Fig. 3a). When 30 min of GA treatment was conducted, the optimal concentration of GA for slide activation was 1.5 %, with the background interference of 0.30 on the basis of total fluorescence and number of fluorescent particles (Fig. 3b). It was likely that the degree of GA activation on the surface of the glass slide increased with time lapse. However, slide activation exceeded an optimum level over 30 min of GA treatment, which seemed to indicate that nonspecific binding of the bioconjugate through the antibody component increased at this condition. The same discussion can be given to the effect of GA concentration on background interference. Therefore, further slide activation was conducted with 1.5 % GA for 30 min.

Optimization of Reaction with Coating Antigen

To immobilize the coating antigen to the GA-activated surface of the glass slide, conditions for immobilizing the coating antigen were optimized at varying time of reaction

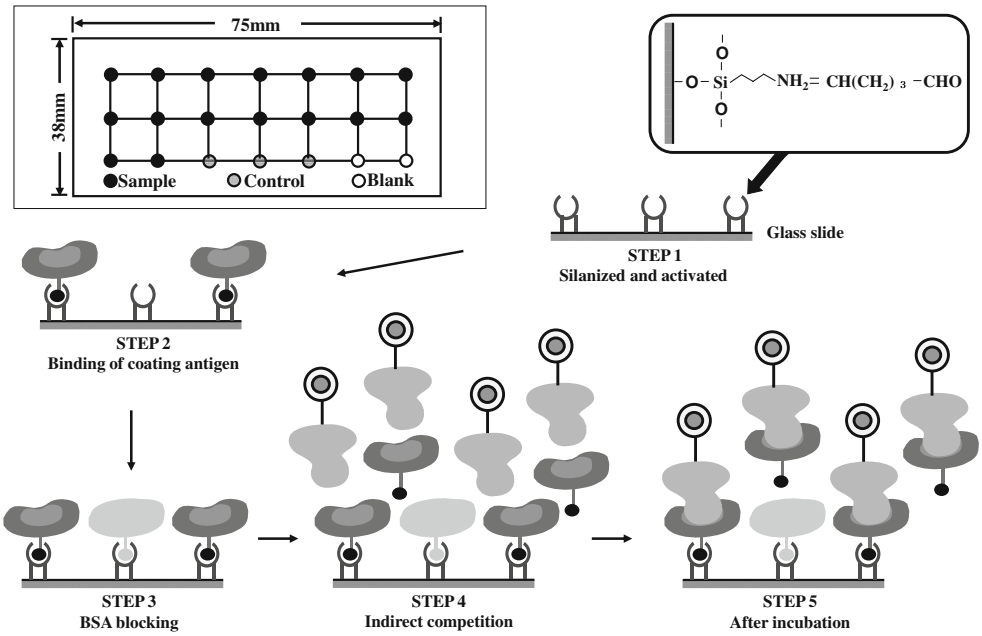

Fig. 2 Slide arrangement and corresponding procedure of indirect-competitive fluoroimmunoassay

with coating antigen in 0.5–5 h and concentrations of coating antigen for immobilization in 0.05–1 mg/mL. At this moment, time of treatment with GA, concentration of treatment with GA, concentration of BSA for blocking, time of blocking with BSA, concentration of bioconjugate for immune reaction and time of immune reaction with bioconjugate were fixed at 30 min, 1.5 %, 1 %, 1 h, 0.125 mg/mL and 3 h, respectively. When measured at 0.1 mg/mL of the coating antigen, background interference decreased sharply from 0.77 and 0.92 to 0.37 and 0.32 according to the increase in time of reaction with coating antigen from 30 min to 1 h on the basis of total fluorescence and number of fluorescent particles, respectively. Background interference increased slightly to 0.50 and 0.46 when time of reaction with coating antigen increased to 5 h (Fig. 4a). From this result, immobilization of the coating antigen was conducted for 1 h for further study. The concentration of the coating antigen that minimized background interference was then determined by using 1 h of immobilization time. Figure 4b indicated that background interference decreased initially up to 0.1 mg/mL of the coating antigen and increased thereafter on the basis of total fluorescence and number of fluorescent particles. Although binding of the coating antigen to the surface of the glass slide seemed to increase time-dependently, the result of Fig. 4a indicated that an optimum binding time of the coating antigen was present for securing adequate spacing to form the immune complex. The concentration effect of the coating antigen in Fig. 4b can be explained similarly. Therefore, 0.1 mg/mL of the coating antigen was used for further study.

Optimization of Immune Reaction with Bioconjugate

It has been reported that concentration of bioconjugate effects on sensitivity of the immobilized-antigen glass slide greatly [13]. In a similar way, a bioconjugate concentration that is able to minimize background interference is likely to be present. In this study, the optimum concentration of the

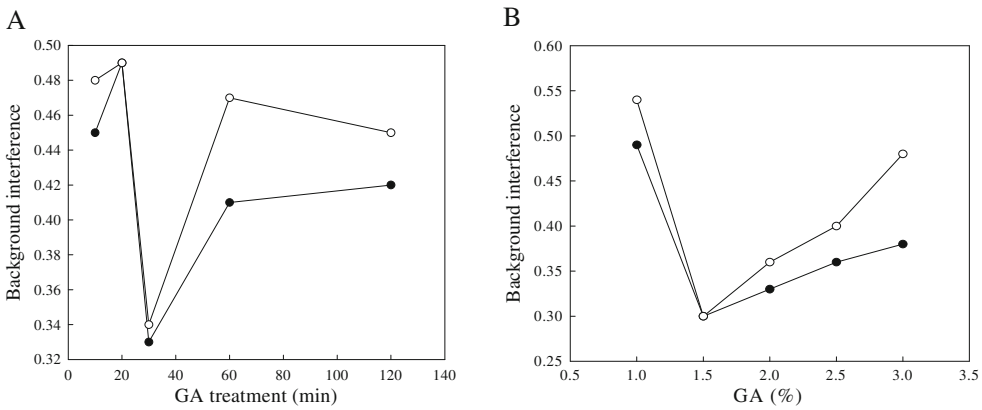

Fig. 3 Effects of treatment with glutaraldehyde on background interference. Panel A and B show background interference according to time of treatment with glutaraldehyde and concentrations of treatment with glutaraldehyde, respectively. Symbols: *white circle*, total fluorescence; *black circle*, number of fluorescent particles

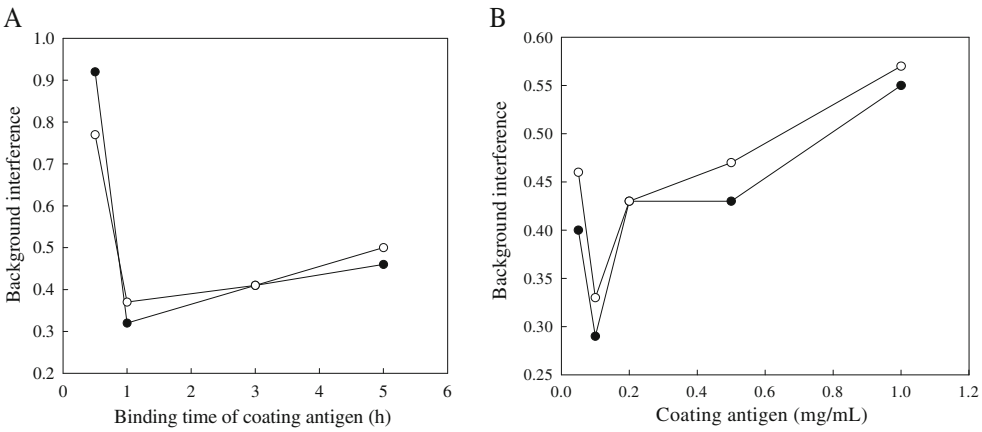

Fig. 4 Effects of reaction with coating antigen on background interference. Panel A and B show background interference according to time of reaction with coating antigen and concentrations of coating antigen for immobilization, respectively. Symbols: *white circle*, total fluorescence; *black circle*, number of fluorescent particles

bioconjugate was determined at varying concentrations of the bioconjugate in 0.025–0.250 mg/mL. At this moment, time of treatment with GA, concentration of treatment with GA, time of reaction with coating antigen, concentration of coating antigen for immobilization, concentration of BSA for blocking, time of blocking with BSA and time of immune reaction with bioconjugate were fixed at 30 min, 1.5 %, 1 h, 0.1 mg/mL, 1 %, 1 h, 3 h, respectively. As shown in Fig. 5, background interference decreased overall although some fluctuation was found. At the bioconjugate concentration of 0.250 mg/mL, it was as low as 0.23 on the basis of number of fluorescent particles. The overall decrease in background interference according to the increase in bioconjugate concentration indicated that bioconjugate concentration was an important factor determining formation of the immune complex and thus a relatively high bioconjugate concentration was desirable to minimize background interference. Also, it was evident that number of fluorescent particles was more effective than total fluorescence with regard to determining the complexation between the antigen and antibody.

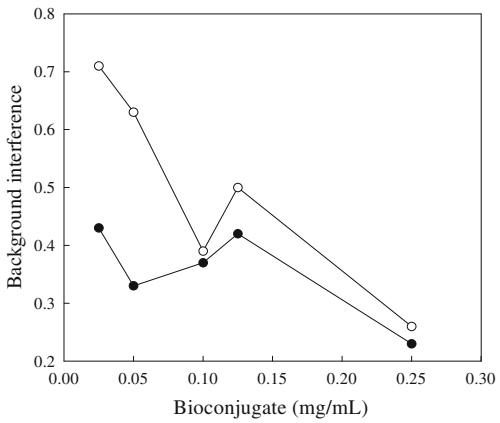

Fig. 5 Effect of immune reaction with bioconjugate on background interference. Symbols: *white circle*, total fluorescence; *black circle*, number of fluorescent particles

Optimization of Blocking with Bovine Serum Albumin

To minimize non-specific binding owing to matrix materials that include proteins and possess amino functionality in a sample like rat serum [15], blocking of the surface of spots on the immobilized-antigen glass slide was practiced with BSA, and the relevant conditions for blocking were optimized at varying concentrations of BSA for blocking in 0.5–5 % and time of blocking with BSA in 0.5–2 h. At this moment, time of treatment with GA, concentration of treatment with GA, time of reaction with coating antigen, concentration of coating antigen for immobilization, concentration of bioconjugate for immune reaction and time of immune reaction with bioconjugate were fixed at 30 min, 1.5 %, 1 h, 0.1 mg/mL, 0.125 mg/mL and 3 h, respectively. When measured at 1 h of blocking time, background interference decreased gradually from the BSA concentration of 0.5 to 3.0 %, however, it increased abruptly at the BSA concentration of 5 % (Fig. 6a). Like Fig. 5, number of fluorescent particles was more effective with respect to obtaining a reduced value in background interference. With fixing the concentration of BSA for blocking at 2 %, the effect of blocking time on background interference was evaluated. As depicted in Fig. 6b, the blocking time over 30 min gave a negative effect. That is, background interference obtained by measuring number of fluorescent particles increased from 0.33 to 0.48 and 0.62 when time of blocking with BSA increased from 30 min to 1 and 2 h, respectively. Thus, 30 min of blocking with BSA was selected. The above result indicated that excessive BSA binding interfered with formation of the immune complex possibly due to insufficient spacing for the immune reaction, which resulted in the increase in background interference.

Indirect-competitive Fluoroimmunoassay against C-Reactive Protein

After optimizing the reaction variables of the IC FIA against CRP, we compared the sensitivity of our assay with that of a commercial sandwich-type enzyme-linked immunosorbent

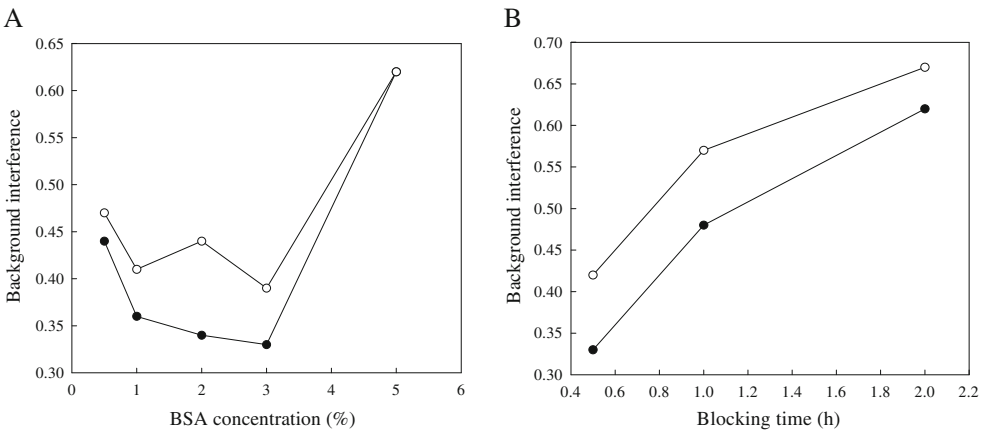

Fig. 6 Effects of blocking with bovine serum albumin on background interference. Panel A and B show background interference according to concentrations of bovine serum albumin for blocking and time of blocking with bovine serum albumin, respectively. Symbols: *white circle*, total fluorescence; *black circle*, number of fluorescent particles

assay (ELISA) kit for a quantitative determination of CRP. In our assay, the coating antigen competes with the analyte for the binding to the bioconjugate, which results in decreased fluorescence signals at increasing analyte concentrations. At all analyte concentrations, fluorescence intensities obtained were considerably lower than that of the control and higher than that of the blank. That is, the number of fluorescent particles of the control and blank were 1032 and 137, respectively, and the resultant background interference was 0.13. Relative fluorescence intensities that were arbitrarily expressed as the percentage response signals of the samples against the control were 59.7, 55.8, 46.4, 34.9 and 28.4 % at the analyte concentrations of 0.01, 0.05, 0.1, 0.5 and 1.0 ng/mL, respectively. The relative fluorescence intensity at 0.05 ng/mL CRP was still lower than that of the control by 44.2 %. The relationship between CRP concentration and fluorescence intensity was expressed as a linear equation by drawing a double-logarithmic plot to show the concentration-dependency needed for analyte quantitation (Fig. 7a). The obtained linear regression equation that encompassed the CRP concentrations of 0.05–1.0 ng/mL was Y ($\log_{10}$ fluorescence intensity)=−0.2144 X ($\log_{10}$ CRP concentration)+2.4764, together with the correlation coefficient (r) of 0.9956. Taking into account the above results, we presumed that the limit of detection (LOD) of the present IC FIA is lower than 0.1 ng/mL. Compared with the double-logarithmic regression of the commercial ELISA kit that showed LOD around 2.5 ng/mL (Fig. 7b), the current assay showed a better sensitivity and significantly lower dynamic range, which seemed to reflect the characteristics of IC immunoassay protocol in which the binding of an antibody, being normally present in a relatively high predetermined concentration, is measured [16]. Also, the IC FIA of this study was more sensitive than a sandwich-type chemiluminescent ELISA against CRP that showed a LOD of 0.3 ng/mL [17]. The sensitivity obtained here was considerably higher than those of various immunosensors that were operated by different transduction mechanisms by more than one decade [18–20].

Conclusion

We optimized the assay conditions for an IC FIA against CRP exploiting an immobilized-antigen glass slide. The assay of this study was very sensitive and thus was responsive to CRP concentrations lower than 0.1 ng/mL. Judging from the obtained results, the IC FIA of this study seems to be applicable to high-sensitivity detection for serum biomarker

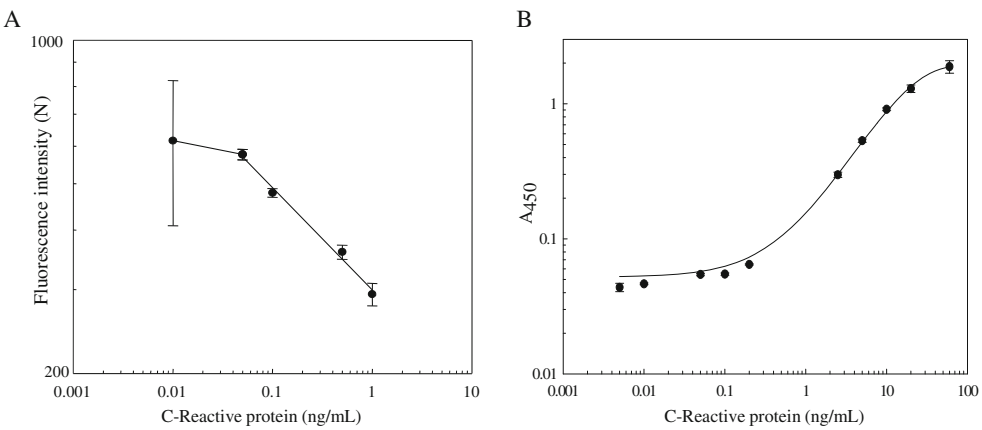

Fig. 7 Calibration curves of indirect-competitive fluoroimmunoassay of this study (**a**) and a commercial sandwich-type ELISA against rat C-reactive protein (**b**) (n=3)

proteins that are present in very low concentrations and are related with food functionality in the near future. Also, an extension of the current assay to microarray format seems to be required for a simultaneous detection of multiple biomarkers in one glass slide.

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