Food Engineering Progress
Vol. 14, No. 1. pp. 21~26 (2010.2)

산업식품공학
Food Engineering Progress

Glass Slide-based Immunosensing for C-Reactive Protein Using Quantum Dot-Antibody Conjugate

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Abstract

C-Reactive protein (CRP), which is an 118 kDa pentameric protein, was secreted by the liver is an important biomarker for coronary disease, hypertension and inflammation. In this study, a method for CRP detection exploiting quantum dot (Qdot)-antibody conjugate was developed according to an indirect-competitive immunosensing protocol. For this purpose, a streptavidin-bound $Qdot_{605}$ was linked with a separately prepared biotinylated monoclonal anti-rat CRP antibody to produce a Qdot-antibody conjugate. The immunosensing was performed at 0.1 and 20 nM of the coating antigen and conjugate, respectively. The current method was found very sensitive in CRP detection, judging from the concentration-dependent fluorescence emission.

Key words: glass slide, immunosensing, C-reactive protein, quantum dot-antibody conjugate

Introduction

Functional food which is often named as nutraceutical, pharmafood or designer food contains biologically active substances effective for physiological functions of the human body. Recently, the total market of functional food in Japan, US, EU, China, Korea, and Brazil are increasing rapidly with the share approximating to 35.2 billion dollars (Bech-Larsen & Scholderer, 2007). To be recognized as a functional food, a targeted functionality such as cardio-protective property must be proved scientifically, which greatly increases the importance of functionality evaluation on food (Kim et al., 2007). Until now, functionality assessment on food has been undertaken by *in vitro* assay, *in vivo* assay using a model animal and clinical demonstration for major functional properties. As a previous step preceding clinical demonstration, *in vivo* assay using a model animal conventionally measures the changes in physical parameters such as body weight, blood pressure and organ morphology. However, it is heterogeneous in itself and suffers from diversification in assay protocol, requirement for well-trained personnel and necessity for expensive analytical instruments (Malone et al., 2006). Therefore, the importance to develop a new version of *in vivo* assay using a model animal has strongly been urged to meet the recent demand for the assessment on food functionality.

One homogeneous assessment method on *in vivo* food functionality is to determine the rise and fall of specific biomarker proteins related with a disease or metabolic syndrome in the blood of a model animal like rat. In this case, the model animal is administered with a diet containing a candidate functional food material or functional food (Noone et al., 2002; Anil, 2007).

To undertake *in vivo* biomarker assay efficiently, the development of a novel analytical tool for serum biomarkers is essential. Out of the possible analytical methods, including spectrophotometry and enzyme-linked immunosorbent assay (ELISA), biosensing based on the formation of antibody-antigen complex is powerful and efficient for homogeneous *in vivo* functionality evaluation on food owing to its intrinsic high-sensitivity, specificity and rapidity. Moreover, it can reuse expensive reagents like antibody and present the sensor response in a real-time scale on computer screen (Park & Kim, 2006; Lee & Yoon, 2007).

An optoelectronic immonosensor based on quantum dot (Qdot) fluorescence seems to be a promising analytical tool for biomarker detection, possibly due to extraordinary signal intensity and photochemical stability (Clarke et al., 2008; Ma et al., 2008). Also, it can be easily expanded to an arrayed simultaneous detection (Cui et al., 2007), suitable for the detection of multiple biomarkers in the blood of a model animal (Du et al., 2008). Therefore, we have tried to develop a

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Received October 7, 2009; revised 1st November 19, 2009, revised 2nd February 3, 2010, accepted February 4, 2010

disposable Qdot-based glass slide immunosensor for biomarker detection. In this case, C-reactive protein (CRP), which is a 118 kDa pentameric protein secreted by the liver upon stimulation by interleukin (IL)-6 and IL-1β (Weinhold & Rüther, 1997) and is an important biomarker for coronary heart disease, hypertension and inflammation, was used as the model biomarker for assay development. Until now, CRP was mainly determined by ELISA (Parra et al., 2005) and its plasma level is normally lower than 3 μg/mL (Gabay & Kushner, 1999; Rifai & Ridker, 2001).

As an initial step for serum biomarker detection, we developed an indirect-competitive (IC) immunosensor which measures the fluorescence emission of a Qdot-anti-CRP antibody conjugate bound over a glass slide platform immobilized with CRP and reported on its basal sensor properties in CRP detection.

Materials and Methods

Materials and reagents

A recombinant histidine tagged rat CRP (carrier-free) which was expressed in a mouse myeloma cell line (NS0) was obtained from R&D Systems, Inc. (Minneapolis, MN, USA) and used throughout this study. Its homopentameric structure consists of three non-covalent and two covalently linked subunits. A monoclonal anti-rat CRP antibody which was produced from a hybridoma resulting from the fusion of a mouse myeloma with B cells acquired from a mouse immunized with purified, NS0-derived, recombinant rat CRP was also obtained from R&D Systems, Inc. Glass slide (25×76 mm) and streptavidin (SA)-coated $Qdot_{605}$ were the products of Corning Inc. (Kennebunk, ME, USA) and Invitrogen Co. (Carlsbad, CA, USA), respectively. A water-soluble biotinylation reagent, sulfosuccinimidyl-6-(biotinamido) hexanoate (sulfo-NHS-LC-biotin), was purchased from Pierce Biotechnology, Inc. (Rockford, IL, USA). 3-Aminopropy ltrimethoxysilane (APTMS), glutaraldehyde and bovine serum albumin (BSA) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals were guaranteed reagents from various suppliers and double distilled water was used throughout this study.

Biotinylation of antibody and successive bioconjugation

A biotinylated anti-CRP antibody was prepared in phosphate buffered saline (PBS, pH 7.2) using sulfo-NHS-LC-biotin according to a previous procedure (Kim & Cho, 2008). In the next step, 0.500 mg/mL of the biotinylated antibody was added to 80 nM of SA-coated $Qdot_{605}$ in an Eppendorf tube in equal volume. After swirling gently, the resulting mixture was reacted for 2 hr inside ice flakes to prepare a nanoprobe, antibody-Qdot conjugate, linked by biotin-SA linkage.

Procedure of IC immunosensing for CRP

The detection principle of IC immunosensing is shown in Fig. 1. The formation of complex between the nanoprobe and the coating antigen (CRP itself) immobilized on the glass slide or the analyte in sample during immune reaction occurred in an IC way. After rinsing the glass slide with distilled water three times and dipping it in distilled water for 2 min to remove unbound components of immune reaction, the nanoprobe-bound slide was examined for fluorescence emission.

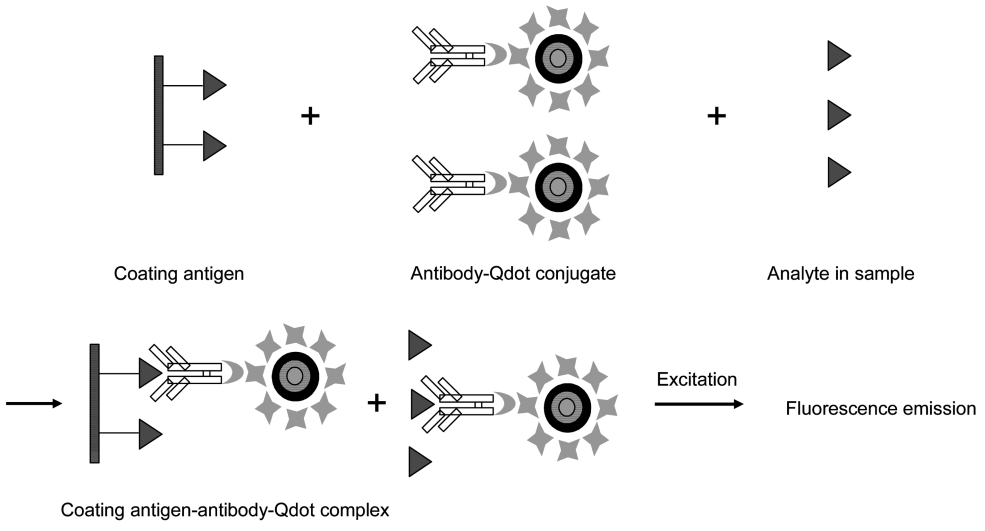

Fig. 1. Operating principle of the IC immunosensing based on Qdot fluorescence.

The experimental procedure is summarized as follow. The glass slide was cleaned by dipping into a piranha solution (H_2SO_4 : H_2O_2 = 3 : 1, v/v) for 10 min, followed by a successive rinsing with distilled water three times and sonication in distilled water for 5 min. It was further hydrated with 90°C hot water for 1 hr and dried at room temperature. The cleaned slide was treated with 10% acetonic APTMS for 1 hr at room temperature and was successively rinsed with acetone, 50 mM sodium phosphate buffer (pH 7.4)- the reaction buffer, and distilled water three times. It was then dipped into distilled water for 1 min and was heat-treated at 120°C for 5 hr after drying at room temperature. After cooling, the APTMS-treated glass slide was added into distilled water for 15 min for hydration. Then, it was activated with 2.5% glutaraldehyde at room temperature for 1 hr, followed by rinsing with the reaction buffer three times and dipping into distilled water for 1 min, and drying at room temperature. To the glutaraldehyde-activated glass slide, 20 μL each of 0.1 mg/mL of the coating antigen and the reaction buffer as a blank was spotted and incubated at room temperature for 1 hr. The resulting slide was rinsed with the reaction buffer three times and dipped into distilled water for 1 min. It was further treated with 1% BSA to exclude the possible non-specific interaction between the nanoprobe and glass slide. 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. The nanoprobe solution as described above and each CRP solution at varying concentration range of 0.0005-5 ng/mL were mixed in an equal volume and pre-incubated for 1 hr. To the bio-transducer, the pre-incubation mixture was spotted by 20 μL and then was incubated at room temperature for 1 hr. The resulting slide was rinsed with distilled water three times and dipped into distilled water twice for 1 min each. The finally obtained slide was dried over soft Kimwipes and examined by fluorescence microscopy.

Fluorescence detection

The detection system (Nikon Eclipse 80i, Nikon Inc., Melville, NY, USA) for nanoprobe fluorescence is depicted in Fig. 2. Fluorescence images were collected through a 20× microscope objective using a Epi-fluorescence filter block N B-2A containing 450-490 nm band pass excitation filter, a 505 nm dichroic mirror and a 520 nm barrier filter. A monochrome cooled digital camera head DS-Qi1 (Nikon Inc.) was used for digital imaging.

Results and Discussion

When the fluorescence images of a glass slide were checked at each step of bio-transducer preparation and immune reaction- raw slide and the APTMS-treated, glutaraldehyde-activated, coating antigen-immobilized and BSA-blocked, and nanoprobe-treated slides, the Qdot fluorescence was only found at the nanoprobe-treated bio-transducer (data not shown). This fact clearly indicated a false-positive interference signal might be negligible.

Effect of BSA treatment on interfering Qdot fluorescence

To the glutaraldehyde-activated glass slide of this study, the nanoprobe might bind non-specifically during the immune reaction due to its protein component having amino-functionality, resulting in a conspicuous false-positive signal. To minimize this kind of interference, BSA has been normally used to block the unoccupied surface of a bio-transducer (Jin et al., 2009; Wang et al., 2009). We compared the Qdot fluorescence of the blank slides and the bio-transducer without or with BSA blocking after the immune reaction with the nanoprobe solution. As shown in the panel A of Fig. 3, the blank slide without BSA treatment showed an intense Qdot

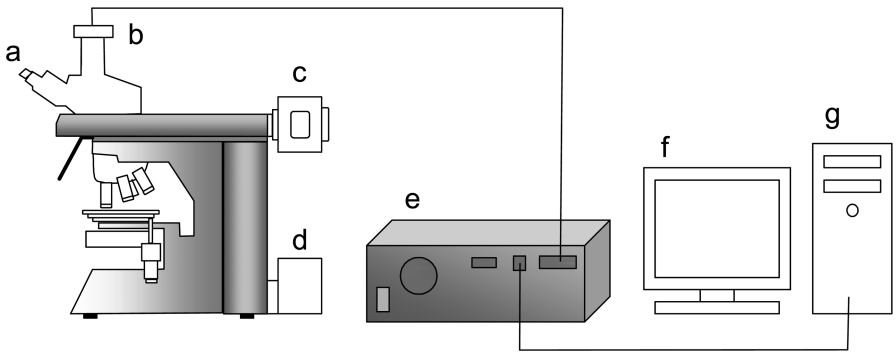

Fig. 2. Schematic representation of the microscopy system for fluorescence measurement. a, eye piece; b, camera head; c, mercury lamp house; d, halogen lamp house; e, camera control; f, monitor; g, PC.

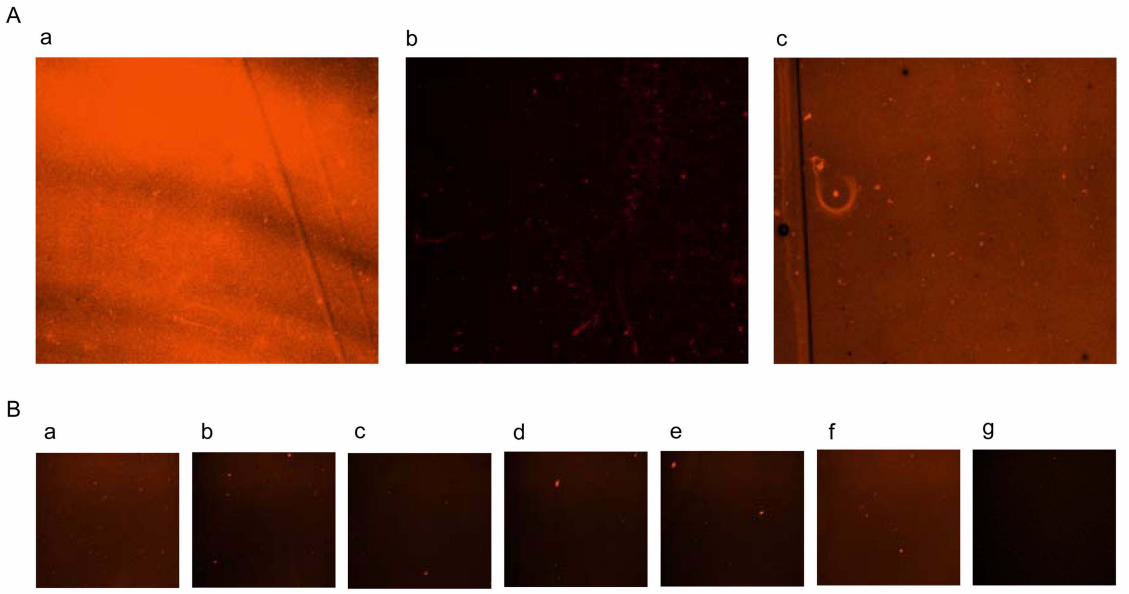

Fig. 3. BSA-blocking effect on the Qdot fluorescence after the immune reaction (A) and fluorescence images at varying CRP concentrations (B). In the panel A, the spotted solutions were the reaction buffer (a and b) and 0.1 mg/mL of the coating antigen (c), repectively. Whereas, the surface blocking was not done (a) or done with 1% BSA solution (b and c). In the panel B, the CRP concentrations in the pre-incubation mixture for IC immunosensing were 0.0005 (a), 0.01 (b), 0.05 (c), 1 (d) and 5 (e) ng/mL, respectively. The analyte concentration in the pre-incubation mixture for f (control) and g (blank) was zero. In all cases, the nanoprobe concentration in the pre-incubation mixture was 20 nM as Qdot basis.

fluorescence after the incubation with the nanoprobe solution. Whereas, the same slide treated with 1% BSA for 1 hr indicated no sign of fluorescence emission. This fact strongly indicated that non-specific binding of the nanoprobe over the slide surface was nearly excluded by this treatment (Jin et al., 2009; Wang et al., 2009). A prolonged BSA treatment over 1 hr was not necessary (data not shown). Therefore, we did the blocking of bio-transducer surface with 1% BSA for 1 hr for further experiments. At this condition, the bio-transducer immobilized with the coating antigen followed by BSA blocking showed a meaningful Qdot fluorescence after the immune reaction with the nanoprobe.

Concentration-dependent fluorescence images at varying CRP concentrations

The Qdot fluorescence of the bio-transducer after the IC immune reaction with 20 nM of the nanoprobe solution (Qdot basis) was determined at each CRP solution in the range of 0.0005~5 ng/mL. In the panel B of Fig. 3, the fluorescence images at some analyte concentrations (0.0005, 0.01, 0.05, 1 and 5 ng/mL) are depicted, together with those obtained with the blank and control slide. As expected, the Qdot fluorescence gradually decreased according to the increase in CRP concentration due to the IC binding between the immobilized and free CRP for the antibody in the nanoprobe (Adányi et al., 2007). In this case, the fluorescence images of the slide points having different concentrations of CRP were easily identifiable, which seemed to be used for a quantitative purpose.

Calibration curve

Based on the fluorescence images obtained, we tried to express empirically the concentration-dependent CRP

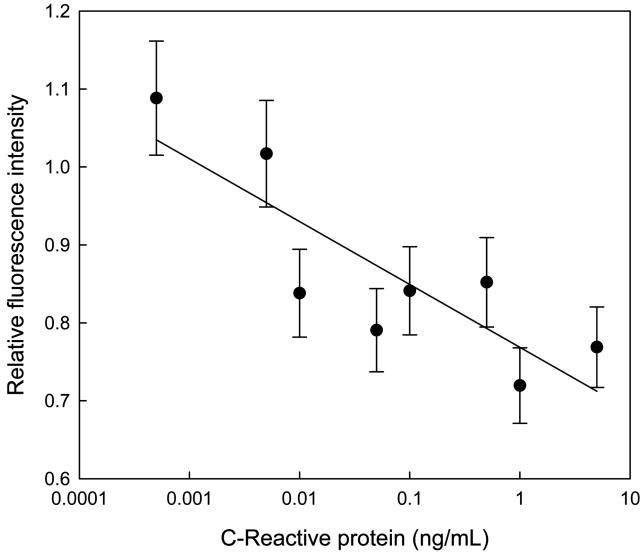

Fig. 4. Calibration curve for CRP in a semi-logarithmic scale.

responses as a linear relationship which routinely permits simple and efficient analyte quantification (Kuhlmeier et al., 2003). To do this, the intensities of fluorescence emission at different CRP concentrations were converted to the values of relative fluorescence intensity which is defined as the fluorescence intensity of a standard sample against that of the control which did not include CRP in the pre-incubation mixture. When we made a semi-logarithmic plot between CRP concentration and relative fluorescence intensity, we obtained the best fitting to a linear equation. For the linear response in Fig. 4, the regression equation of Y (relative fluorescence intensity) = – 0.0806 X ($\log_{10}$ CRP) + 0.7686 was obtained, together with the correlation coefficient (r) of 0.7981.

Taking into account the concentration-dependent relative fluorescence intensities, we presumed the limit of detection (LOD) of the present bio-transducer for CRP detection might be present in the concentration range of 0.1-1 ng/mL of CRP. It was presumed that a considerable serum dilution is required to cover the serum CRP level lower than 3 μg/mL for normal person and that higher than 3 μg/mL for high-risk person (Gabay & Kushner, 1999; Rifai & Ridker, 2001). This fact might have a positive effect with respect to the elimination of possible interference. The sensitivity of our method was as comparable as a sandwich-type chemiluminescent CRP ELISA that shows a LOD of 0.3 ng/mL (Yamada et al., 2006) and an immunometric assay on CRP which used fluorescent nanoparticles for signal transduction (Koskinen et al., 2004). On the other hand, the Qdot fluorescence-based immunosensor of this study held the sensitivity considerably higher than those of SPR and magnetic CRP immunosensors which have the LODs of 1 ng/m L-2 mg/mL (Hu et al., 2006; Meyer et al., 2006; Meyer et al., 2007).

Acknowledgement

This study was carried out as the part of the research project of Development of Food Nanotechnology, Korea Food Research Institute, Republic of Korea.

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