Journal of Bioscience and Bioengineering
VOL. 119 No. 2, 195–199, 2015

www.elsevier.com/locate/jbiosc

Reflectometric interference spectroscopy-based immunosensing using immobilized antibody via His-tagged recombinant protein A

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Received 23 January 2014; accepted 25 June 2014
Available online 22 July 2014

The proposed approach demonstrated in this study provides an immunosensing system based on reflectometric interference spectroscopy (RIfS) in combination with an antibody immobilization method using histidine-tagged recombinant protein A. Carboxymethyldextran (CMD) was immobilized on a 3-aminopropyltriethoxysilane-treated a silicon nitride-coated silicon wafer, followed by chelating histidine-tagged recombinant protein A with copper (II) ions. The CMD-layer was found to be advantageous in terms of not only immobilization of histidine-tagged recombinant protein A-mediated an antibody against myoglobin (anti-Myo) but also prevention of non-specific binding of myoglobin. Myoglobin was repeatedly detected, and the apparent detection limit was 0.1 μg mL^{-1}. The proposed RIfS-based protein sensing system, in conjunction with the easy preparation of silicon-based inexpensive immunosensing chips, is expected to be applicable for label-free optical detection for other proteins in various fields.

[**Key words:** Immunosensor; Immunosensing; Reflectometric interference spectroscopy; Myoglobin; Histidine-tagged recombinant protein A; Antibody]

Diverse biosensors based on electrochemical, optical and micromechanical detection principles, have been developed as simple and convenient tools for detection of target molecules in complicated matrices (1–3). Among them, sensing systems for antigen–antibody interaction are of growing interest in bioanalytical fields such as clinical examination, biochemical analysis and proteomics (4–6). Although labeled antibodies and antigens have been used as immunosensing probes (5), the complicated and time consuming labeling process often leads to physiologically irrelevant binding information and the denaturation of the labeled antibodies and antigens (6).

Label-free optical biosensing techniques have been increasingly attractive for antigen–antibody interaction analyses, which allow label-free and time resolved detection (7,8). Recently, reflectometric interference spectroscopy (RIfS)-based sensing system has been used for label-free detection of biomolecules (9–12). RIfS is based on interference of reflection wave by a multiple thin layer on silicon-based substrates bearing a different refractive index each other. When a molecular recognition element is immobilized onto the sensor chip and an analyte is adsorbed, increase in physical thickness and change in reflective index can occur in the surface layer, resulting in the wavelength shift of the minimum value of reflectance spectra in RIfS system (defined as Δλ) (13). As a result, the quantification of analytes can be inferred from changes in reflectometric interference spectrum. In our previous studies, a RIfS microfluidic flow system was applied for detection of antigen–antibody interaction, equipped with a silicon nitride (SiN)-coated silicon wafer on which anti-alfa-fetoprotein monoclonal antibody was immobilized by a conventional coupling method using triethoxysilylpropylmaleamic acid-treated SiN sensor chip (14). Since the number of hydroxyl groups located on the SiN layer are relatively small for immobilization of silane coupling agent, we developed the immobilization method by a pre-treatment with trimethylsilyl chloride (TMS), followed by UV-light irradiation to *in situ* generation of homogeneous silanols on the surface: An anti-C-reactive protein antibody (anti-CRP) was effectively immobilized by protein A-mediated binding of anti-CRP (15).

In order to construct label-free immunosensing system, various antibody immobilization have been carried out on the substrate (16,17). Commonly used immobilizing methods have involved amine-coupling reaction by using coupling agents such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) with a combined use of *N*-hydroxysuccinimide (NHS) that facilitate the formation of covalent bonds between amino groups on antibodies and carboxy groups on sensor chip surface (18,19). For examples, Pfeifer et al. reported that myoglobin was detected by a surface plasmon resonance (SPR) sensor with anti-myoglobin (anti-Myo) immobilized sensor chip (20). Masson et al. (21) immobilized anti-Myo via coupling with carboxymethyldextran (CMD) layer by using EDC–NHS coupling reagent, which allows 2.9 ng mL^{-1} of the detection limit for myoglobin by using a fiber-optic-based SPR system. In this case, CMD layer provides immobilization sites with antibody, so that the number of the immobilized antibody increases with increasing molecular weight. However, when molecular weight is over 5 MDa, amount of useful antigen binding sites decreases due to beyond the evanescent field (21). On the contrary, if CMD layer is immobilized on silicon nitride (SiN)-coated silicon wafer for RIfS, influence of the evanescent field is negligible

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2014.06.017

because the optical thickness change is detectable over effective distance of the evanescent field on gold surface. This is a merit of RIfS for immunosensing as compared with SPR-based immunosensing (13). However, as far as we know, there are no reports of RIfS-based immunosensing for myoglobin detection.

Unless using the chemical reagents, antibodies may not be oriented toward reaction medium side, which often brings some problems such as low sensitivity and specificity. One of the challenges to enhance the activity is that direction of immobilized antibody is controlled by using a binding protein for the Fc region of antibody such as protein A (22–24). Currently recombinant protein A is available, which possesses six-consecutive histidines, called His-tag, for the purification by chelation with nickel (II), copper (II) and other transition metals in the presence of ligands such as nitrilotriacetic acid (NTA) and ethylene diamine triacetate (EDTA) (25,26). When the recombinant protein A was immobilized on sensor chips via the chelation, the direction of immobilized protein A could be oriented due to the fixed position of His-tag on protein A. This should also result in orientedly immobilized antibodies on the surface of sensor chips when the immobilized protein A captured antibodies, making the immobilized antibody's activity enhanced (27,28).

Here, we developed the RIfS-based immunosensing system, where a His-tagged recombinant protein A was immobilized on a SiN-coated silicon-based sensing chip having copper(II)-chelated EDTA functionalized surface, followed by immobilizing monoclonal antibody via protein A-Fc region interaction (Fig. 1). To the best of our knowledge, this is the first report of the RIfS-based immunosensing using SiN-coated silicon wafer on which an antibody is immobilized by using the His-tagged recombinant protein A. The most important point in this system is to immobilize *N*-((3-trimethoxysilyl)propyl)ethylene diamine triacetic acid trisodium salt (EDTAS) on CMD-modified SiN-coated chip. It is expected that myoglobin is detected by optical thickness change when bulky CMD- layer is covered on the SiN-coated silicon wafer. Myoglobin was used as a model target. Myoglobin, which is the iron- and oxygen-binding protein, exists in muscle tissue of vertebrates in mammals, and is applicable for diagnosis related to muscle diseases and a potential marker for heart attack (29–31). When muscle tissues are damaged, myoglobin will be released and filtered by kidneys. Myoglobin in serum of healthy people extends in the range of 0.008–0.1 μg mL^{-1}. However, when muscle tissues are damaged, the value of myoglobin is increased to 80 μg mL^{-1} (30).

MATERIALS AND METHODS

Reagents and chemicals Trimethylsilyl chloride (TMS), 2-(4-2-(hydroxyethyl))-1-piperazinyl ethanesulfonic acid (HEPES), and sodium chloride (NaCl) were purchased from Nacalai Tesque Co. Ltd. (Kyoto, Japan). 3-Aminopropyltriethoxysilane (APTES) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were obtained from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). *N*-((3-Trimethoxysilyl)propyl)ethylene diamine triacetic acid trisodium salt (EDTAS, 35% water solution) was purchased from Gelest, Inc. (Morrisville, PA, USA). Carbomethyldextran (CMD, Mw 40,000) was kindly provided by Meito Sangyo Co. Ltd. (Nagoya, Japan). Recombinant protein A was purchased from BioVision Inc. (Mountain View, CA, USA). Anti-myoglobin (monoclonal, mouse) (anti-Myo) was purchased from Mikurilab Co. Ltd. (Osaka, Japan). Myoglobin and *N*-hydroxysuccinimide (NHS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Copper (II) chloride dehydrate ($CuCl_2$) and bovine serum albumin (BSA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Other reagents and solvents were used without further purification.

Apparatus A RIfS-based molecular interaction analyzer, MI-Affinity LCR-01 was purchased from Konica Minolta Opto, Inc. (Tokyo, Japan). A pump (PU-980, Jasco, Tokyo, Japan), a degasser (DG-980-50, Jasco) and an auto-sampler (AS-950-10, Jasco) were used to construct an automated flow system for the RIfS device.

Silicon nitride (SiN) chips (L 9 × W 9 × H 0.725 mm) and PDMS-based microfluidic cells (cell size: L 5 × W 1 × H 0.02 mm, cell volume: 1 μL) were purchased from Konica Minolta Opto, Inc.

TMS-coated silicon nitride chip Trimethylsilyl chloride (TMS, 1% (v/v)) was dissolved in toluene (5 mL) under nitrogen atmosphere and mixing for 15 min. A SiN chip was cleaned by using a UV–O_3 cleaner (Bioforce nano science) for 15 min. The SiN chip was soaked in the TMS solution and incubated for 10 min at room temperature (15). The SiN chip was washed with toluene and distilled water, then dried by N_2 blowing.

Amination on the silicon nitride chip Amination of the SiN surface was performed by APTES. APTES (0.1 mL) was added to 10 mL 95% ethanol, and the solution was stirred for 30 min at room temperature. The TMS coated SiN chip was treated by using the UV–O_3 cleaner for 30 min. The TMS-treated SiN chip was immersed in 1% APTES solution for 60 min. Subsequently, the sensor chip was placed in the oven (As One, EO-600B) for 60 min at 80°C to obtain an aminated SiN chip.

Immobilization of CMD on the silicon nitride chip For immobilization of CMD via covalent bonding on the aminated SiN surface, CMD (0.1 mg mL^{-1}) was dissolved in distilled water. Carboxyl groups in CMD were converted to the activated esters by EDC and NHS (0.2 M and 0.05 M, respectively) for 15 min, and then the aminated SiN chip was immersed in CMD solution for 15 min. The CMD-immobilized SiN chip was washed with distilled water to remove any excess reagents and dried by N_2 blowing.

Surface modification of EDTAS on the CMD-immobilized silicon nitride chip The ethylenediamine triacetic acid groups-functionalized SiN sensor chip was performed by EDTAS coupling agent. EDTAS (0.142 μL) was dissolved in water (5 mL) including 2% acetic acid for hydrolysis, and the solution was stirred for 30 min. The CMD-modified SiN chip was immersed in 1% (v/v) EDTAS solution for 60 min at room temperature. Subsequently, the sensor chip was washed with distilled water, and dried by N_2 blowing. Finally, the SiN chip was placed in the oven (As One, EO-600B) for 60 min at 80°C.

EDTA groups functionalized on TMS-treated SiN surface also were performed in the same manner. Before modifying surface-functional groups to ethylene diamine triacetate moiety, TMS-treated SiN chip was converted into silanol groups by UV irradiation for 30 min and soaked into the 1% EDTAS solution (29).

Immobilization of anti-Myo on the EDTAS-treated sensing surface via His-tagged recombinant protein A Immobilization of anti-Myo was performed by the RIfS system, where the PDMS-based micro-flow-cell (cell volume 1 μL) was equipped to the RIfS apparatus, and 10 mM HEPES (pH 7.4) containing 140 mM NaCl, was used as a running buffer. Copper (II) chloride ($CuCl_2$) dissolved in water (10 mM, 100 μL) was injected for chelation on the surface treated by EDTAS. Then, His-tagged recombinant protein A (100 μg mL^{-1}) and anti-Myo dissolved in the running buffer, were consecutively injected on the sensor chip at a flow rate of 20 μL min^{-1}. Finally, 100 μL of BSA (0.1 mg mL^{-1}) was injected for blocking.

Detection of myoglobin by the RIfS-based label-free sensing system In order to detect the myoglobin on the anti-Myo-immobilized sensor chip via His-tagged recombinant protein A, a variety of myoglobin solutions (0.001, 0.005, 0.01,

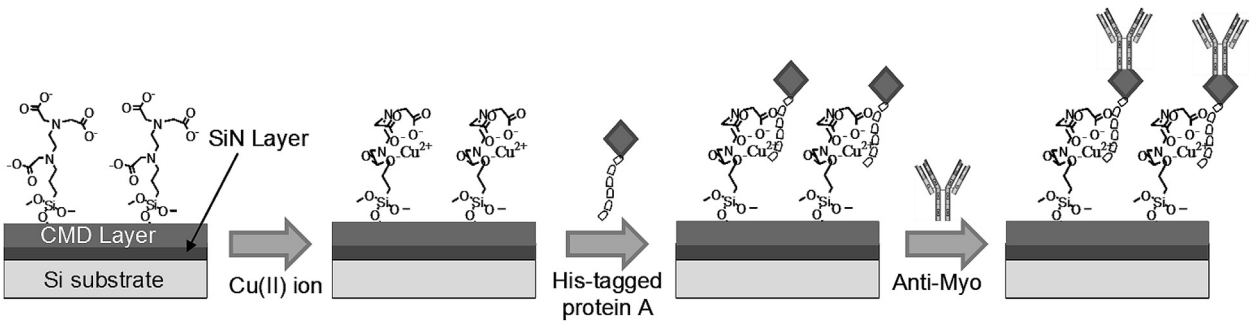

FIG. 1. Illustration of the immobilization of anti-Myo on the EDTAS-treated sensing surface via His-tagged recombinant protein A.