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Label-free CRP detection using optical biosensor with one-step immobilization of antibody on nitrocellulose membrane

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ABSTRACT

To assess the potential of using the MCLW sensor as a label-free optical biosensor system for clinical diagnosis, we evaluated the potential of detecting C-reactive protein (CRP) in a diluted real serum sample by using nitrocellulose as an adlayer. Nitrocellulose was coated on the MCLW sensor chip via spin coating and the CRP antibody was directly immobilized on the sensor surface by hydrophobic physical adsorption. Biosensing using the nitrocellulose surface is highly convenient and does not require any additional treatment for the immobilization of ligand. We optimized the condition for the deposition of nitrocellulose on the MCLW sensor chip with the goal of generating the largest signal difference of the sample against the control signal. In addition, label-free immunosensing of CRP was achieved using the nitrocellulose-coated MCLW sensor in real time. As a result, we were able to quantitatively detect CRP in a human serum within a concentration range of 0.1–10 μg/mL.

1. Introduction

Optical sensing technologies based on monitoring changes in the refractive index in the proximity of the sensor surface have been widely utilized as biosensing tools due to their detecting performance with reliability, high sensitivity, label-free and real-time monitoring capabilities. The biosensing with the metal clad leaky waveguide (MCLW) sensor is also a surface-sensitive measurement and this measurement is based on changes in the refractive index near the sensor surface. The fabrication of the MCLW sensor chip is relatively simple and the surface of the sensor is not vulnerable to environmental stress, such as oxidation because the outer sensing surface is a metal oxide film. However, the most unique feature of the MCLW sensor is the extensive penetration depth of the evanescent field arising from the sensor surface, which is significantly larger than other refractometry-based biosensors [1].

In the MCLW sensor, the incident light is resonantly reflected from the non-absorbing medium layer with a thin metal clad when the thickness and the refractive index of each layer are set to the appropriate values. This occurs when the incident angle is slightly larger than the critical angle for total internal reflection. When the incident light is internally reflected from the dielectric waveguide layer, an evanescent field is generated at the interface between the oxide waveguide layer and its outer medium (the analyzed layer), and the field intensity decays exponentially in the direction normal to the surface of the waveguide. Therefore, bio-substances adsorbed on the surface of the dielectric layer can be detected by observing the change in the refractive index near the surface exposed to the evanescent field, which is similar to SPR sensors. However, the penetration depth of the evanescent field from the surface is approximately 1.5 μm for the MCLW sensor [1], which is much larger than the 150–200 nm penetration depth for SPR [2,3]. Therefore, the MCLW sensor is adequate for sensing and monitoring the states of large particles, such as spores and cells. In previous studies, the MCLW sensor was utilized for sensing *Escherichia coli* spores [4,5].

However, the extensive penetration of the evanescent field in MCLW sensor decreases its sensitivity relative to SPR, especially for layers thinner than a hundred nanometers [6]. Therefore, the MCLW sensor has been considered to be inadequate to detect and analyze biochemical substances that are on the molecular scale. However, we recently showed that the current MCLW sensor could be utilized as a sensitive bio-analytical tool for clinical diagnosis, such as an

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immunosensor [7], where the performance of the MCLW sensor was estimated, based on the specific binding of human interleukin 5 (hIL5) to the sensor surface modified with anti-hIL5 in real time.

To increase the usability of the MCLW sensor as an immunosensing tool, here we utilized a membrane adlayer, which enabled a receptor molecule such as an antibody to be directly immobilized on the surface. In most biosensors, immobilization of a receptor that specifically recognizes a target molecule on the sensor surface requires additional steps, including the immobilization of linker molecules and activation of the functional groups as well as modification of the sensor surface. However, one can simplify the overall process for the immobilization of the receptor via coating an adlayer that can allow for direct immobilization. To achieve this goal, we utilized nitrocellulose (NC) as a receptor-immobilizing adlayer in this study. NC has been a preferred material to immobilize and capture biomolecules in a non-covalent but essentially irreversible manner and has been used in traditional blotting and binding applications for over 60 years. This macroporous film can adhere onto a glass slide directly via spin coating and protein and ligand molecules can become immobilized on the surface by hydrophobic physical adsorption and van der Waals forces [8]. However, one caveat of this approach is that the deposition of a dielectric adlayer with a uniform thickness on the sensor surface can cause a decrease in the sensitivity of the biosensor because the MCLW sensor utilizes the evanescent field near the sensor surface. However, since the MCLW sensor has a deep penetration depth of the evanescent field from the sensor surface, we hypothesize that using an NC adlayer with thickness of a few 10 nm will not lead to a significant deterioration in the sensitivity of the sensor, which was demonstrated based on the experimental data in this study. Finally, to demonstrate the usability of the NC-coated MCLW sensor, the C-reactive protein (CRP) was chosen as an analyte in the biosensing test using a real serum. CRP is an important biomarker for diagnosis of infection and inflammation as well as a potential risk predictor for cardiovascular disease. The concentration level of CRP in the blood is typically less than 3 μg/mL in a normal person and higher concentration of CRP has been used as an indicator of infection, inflammation and cardiovascular disease [9,10]. We quantitatively detected C-reactive protein (CRP) in human serum through one-step immobilization of the antibody on NC-coated MCLW sensor without the use of any labels.

2. Experimental

2.1. Materials

The nitrocellulose (NC) and amyl acetate were purchased from Ernest F. Fullam Inc. (USA). The CRP antibody (polyclonal, IgG) was purchased from abcam (UK) and human CRP antigen were obtained from the Oriental Yeast Co., Ltd., Japan. Human CRP free serum was purchased from Fitzgerald and skim milk was from BD. All other chemicals were purchased from standard chemical sources and were of analytical grade.

2.2. Instrumentation of a portable MCLW sensor for biosensing

The angle-interrogation method was employed in the present MCLW sensor system using a monochromatic light source. The incident angle of the maximum peak in the reflected angular spectrum was traced by monitoring the change in the pixel position of a 2-dimensional image sensor in real time. In this study, the optical system for biosensor was constructed as a portable system with a dimension of $31.5 \times 14 \times 9\,cm^3$ (Fig. S1 in the supplementary information). However, the optical components and detail configurations in the system were identical to the lab-top system reported in a previous study [7]. Briefly, the LED light ($\lambda_0 = 850$ nm), which passed through a linear polarizer, was focused on the surface of the MCLW sensor chip that was optically coupled to a hemi-cylindrical prism and the focusing angle was about 5°. In addition, the center angle (offset angle) of the measurement could be adjusted from 40° to 70° by adjusting the linear goniometer of the sensor system. The image internally reflected from the sensor surface was projected onto a CMOS detector and the bright line pattern corresponded to the resonance peak (Fig. S2 in the supplementary information). The projected image was acquired as the intensity value of each pixel and the resonance angle was defined as the spectral centroid of the peak in the intensity profile of the CMOS pixels [7]. The position of the centroid shifted and was used for detection of biomolecules that had adsorbed on the MCLW sensor surface.

2.3. Fabrication of the bare and nitrocellulose coated MCLW sensor chip

First, the glass substrate (BK7, 22 mm × 22 mm × 0.3 mm; Matsunami, Japan) was cleaned with sonication in absolute ethanol for 10 min and then washed with deionized (DI) water. Piranha solution (4:1 volume ratio of H_2SO_4 and H_2O_2) was used for cleaning the chip surface at 80 °C for 30 min, and the chip was the rinsed with DI water and dried under a nitrogen stream. The titanium and silicon dioxide films were deposited in sequence on the glass using an electron beam (E-beam) evaporator. The final thickness of titanium and SiO2 was 9 nm and 347 nm, respectively, which were optimized via a calculation based on a four-layer structure model (see Fig. S3 in the supplementary information; [7]).

Without any modification of sensor surface, 100 μl of 0.5–2% (v/v) NC in amyl acetate was deposited on the as-fabricated sensor chip by spin-coating (300 rpm for 5 s, followed by 4000 rpm for 35 s) to obtain a thin and uniform NC-coated sensor chip. Finally, the chip was dried on a hot plate at 200 μl for 1 h 30 min immediately after coating to remove excess solvents. The fluidic cell for injection of the sample solutions and washing the chip surface consisted of two channels with dimensions of 8.5 mm (l) × 1.1 mm (w) × 0.25 mm (d).

3. Results and discussion

3.1. Optimum condition of NC coating on the sensor surface

In this study, we used NC for easy immobilization of the antibody onto the MCLW chip surface. To determine the optimum condition for efficient immobilization of the antibody, we prepared MCLW sensor chips coated with different concentrations of NC (0.5, 0.75, 1 and 2%). A simple batch-type of sample handling system was used to immobilize the antibody onto the NC coated sensor surface and the CRP immunoassay was performed. Prior to the immobilization of the antibody, the NC coated sensor chip was rehydrated using phosphate buffered saline (PBS; pH 7.4) as the running buffer. The procedures used for the immobilization of the antibody and CRP immunosensing are illustrated in Fig. 1.

A 100 μg/mL of CRP polyclonal antibody in PBS (pH 7.4) was injected into a sample channel and reacted for 30 min on the stabilized sensor chip via rehydration. After incubation, the sensor surface was washed 4 times with 1× PBS to remove any unbound antibody and the unreacted NC surface was blocked with a 5% skim milk blocking solution for 30 min to reduce non-specific binding. At the same time, an identical blocking solution was injected into the control channel, followed by incubation for 30 min, which prevented the NC surface of the control channel from reacting with any biomolecules. After washing both channels, a 1 μg/mL of CRP in a CRP-free human serum that had been diluted by a factor of 10