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Biotech Method

Label-free optical detection of C-reactive protein by nanoimprint lithography-based 2D-photonic crystal film

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The development of high-sensitive, and cost-effective novel biosensors has been strongly desired for future medical diagnostics. To develop novel biosensors, the authors focused on the specific optical characteristics of photonic crystals. In this study, a label-free optical biosensor, polymer-based two-dimensional photonic crystal (2D-PhC) film fabricated using nanoimprint lithography (NIL), was developed for detection of C-reactive protein (CRP) in human serum. The nano-hole array constructed with NIL-based 2D-PhC (hole diameter: 230 nm, distance: 230, depth: 200 nm) was fabricated on a cyclo-olefin polymer film (100 μm) using thermal NIL and required surface modifications to reduce nonspecific adsorption of target proteins. Antigen-antibody reactions on the NIL-based 2D-PhC caused changes to the surrounding refractive index, which was monitored as reflection spectrum changes in the visible region. By using surface modified 2D-PhC, the calculated detection limit for CRP was 12.24 pg/mL at an extremely short reaction time (5 min) without the need for additional labeling procedures and secondary antibody. Furthermore, using the dual-functional random copolymer, CRP could be detected in a pooled blood serum diluted 100× with dramatic reduction of nonspecific adsorption. From these results, the NIL-based 2D-PhC film has great potential for development of an on-site, high-sensitivity, cost-effective, label-free biosensor for medical diagnostics applications.

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1 Introduction

Novel biosensors for detection of target molecules with high sensitively need to be developed rapidly and cost-effectively for medical applications [1, 2]. Diagnosis of many diseases such as cancers [3], infective diseases [4], and neurodegenerative disorders [5], require detection of low concentrations of target molecules in body fluids. High-sensitivity detection of target molecules is currently performed using several kinds of biosensors and conventional bioanalytical methods, such as an enzyme-linked immunosorbent assay (ELISA), using different detection principles such as electrochemical [6] and optical [7] systems, which have been widely used. These biosensors enable rapid and cost-effective detection of target molecules with high sensitivity. However, for detection of target molecules in body fluids with high sensitivity, previously reported biosensors and conventional bioanalytical methods have several disadvantages such as time-to-result, sophisticated step-by-step liquid handling, and labeling procedures. To overcome these disadvantages, we have been developing a novel optical biosensor based on specific optical characteristics of nanostructures [8–10]. Nanostructures, such as nanoparticles, possess observable optical characteristics such as light absorption [11], and diffraction [12], which depend on the size and physical properties of base materials. Furthermore, surrounding refractive index changes due to the biomo-

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Abbreviations: CRP, C-reactive protein; **NIL**, nanoimprint lithography; **PhC**, photonic crystal; **SEM**, scanning electron microscope

lecular interactions such as antigen-antibody reaction and DNA hybridization may change these specific optical characteristics of nanostructures.

Based on these specific optical characteristics of nanostructures, we have developed a novel optical chemical/biosensor using nanoimprint lithography (NIL)-based two-dimensional photonic crystals (2D-PhC) [13]. A NIL-based 2D-PhC is a nanostructure-based optical device constructed of a periodic nano-array of dielectric materials such as polymers and metal oxides. In addition, NIL-based 2D-PhC can diffract and reflect at specific wavelengths of light. The reflection peak wavelength and intensity will be changed by the surrounding refractive index change due to the antigen-antibody reaction or DNA hybridization. By monitoring the reflection peak wavelength and intensity change due to the biomolecular interactions, we can determine target molecule concentrations with high sensitivity without time-consuming labeling procedures using fluorescent dyes and enzymes.

Furthermore, using NIL, a 2D-PhC-based optical biosensor can be fabricated cost-effectively using a polymer film as a base material. NIL is a high reproducible fabrication technique for producing nanostructures on polymer films using a mold [14]. Generally, for optical telecommunication application, 2D-PhC have been fabricated on silicon or glass substrates using high-cost fabrication instruments such as electron beam lithography (EBL), focused ion beam (FIB), and reactive ion etching (RIE) in a clean room [15]. In contrast, using NIL, 2D-PhC can be fabricated without high-cost fabrication instruments and clean rooms. Based on the advantages of NIL, this fabrication technique to produce nanostructure-based optical devices using NIL is referred to as "printable photonics" [16].

In this study, determination of C-reactive protein (CRP) in human serum using 2D-PhC-based optical biosensor was performed. In addition, for high sensitive detection of CRP in human serum, the dual-functional random copolymer [17] was modified onto a 2D-PhC surface to reduce nonspecific adsorption of foreign substances. By using dual-functional random copolymers, the efficiency of CRP detection in pooled blood serum was evaluated.

2 Materials and methods

2.1 Materials

For determination of CRP using 2D-PhC, anti-CRP antibody (ab32412) and human CRP full-length protein (ab111647) were purchased from Abcam (Cambridge, MA, USA). To immobilize the anti-CRP antibody onto the 2D-PhC surface, dual-functional random copolymer was synthesized using Poly(ethylene glycol) methyl ether methacrylate (PEGMA) (average M_n = ~475), 3-(trimethoxysilyl)-propyl-methacrylate (TMSMA), *N*-acryloxysuccinimide (NAS), and 2,2'-azobisisobutyronitrile (AIBN), which were purchased from Aldrich Chemical Co. (Milwaukee, WI). For modification of dual-functional random copolymer, dimethyl sulfoxide (DMSO) was purchased from WAKO pure chemicals (Osaka, Japan). For evaluation of nonspecific adsorption efficiency, anti-rabbit IgG (whole molecule)-tetramethylrhodamine (TRITC) antibody (T6778-1ML) and IgG-fluoresceinisothiocyanate (FITC) from human serum (F9636) were purchased from Sigma-Aldrich Japan K. K. (Tokyo, Japan). In addition, ultra-pure water (18.2 MΩ cm) from Sartorius Stedim Biotech (Aubagne, Cedex, France) was used in all sample preparations.

2.2 Apparatus

For the fabrication of 2D-PhC, NIL was performed using a nanoimprint apparatus (X-300, SCIVAX Corp., Kanagawa, Japan). Evaluation of optical characteristics was done using the handy type spectrophotometer (USB-4000-UV-VIS, wavelength range: 200–1100 nm) with a tungsten halogen light source (LS-1, wavelength range: 360–2000 nm), which were purchased from Ocean Optics (Dunedin, USA). The bifurcated fiber bundle (BFY600HS02, fiber core diameter: 600 μm, wavelength range: 300–1200 nm) was purchased from Thorlabs (Tokyo, Japan).

For observation of the surface construction of 2D-PhC, scanning electron microscopy (SEM) was used from Keyence (VE-9800, Osaka, Japan). In addition, for evaluation of the nonspecific adsorption, Fluorescent microscopy was used from Keyence (VB-G25, Osaka, Japan).

2.3 Detection principle

Schematic illustration of experimental procedure with photograph of 2D-PhC and chemical structural formula of dual-functional random copolymer are shown in Fig. 1. Based on our previous reports using 2D-PhC-based optical biosensors [18, 19], the reflection peak, which depends on the periodicity and size, can be observed based on Bragg's law. Furthermore, to perform the label-free detection of antigen-antibody reaction on the 2D-PhC surfaces, surrounding refractive index (RI) change on the 2D-PhC surface by an antibody-antigen reaction can be monitored as a reflection peak intensity change based on the Fresnel reflection.

When the modification of random copolymer (Fig. 1b), antibody immobilization (Fig. 1c), and antigen-antibody reactions (Fig. 1d) occurred on the 2D-PhC surface, the average RI on the 2D-PhC changed. As a result, the reflection intensity-based on Fresnel reflection also changed. Hence, a fractional surrounding RI change was determined as a reflection peak intensity change. In this study, for optical biosensor fabrication, NIL-based 2D-PhC with a triangular configured nano-hole array 2D-PhC (hole diameter and distance: 230 nm, hole depth: 200 nm) was

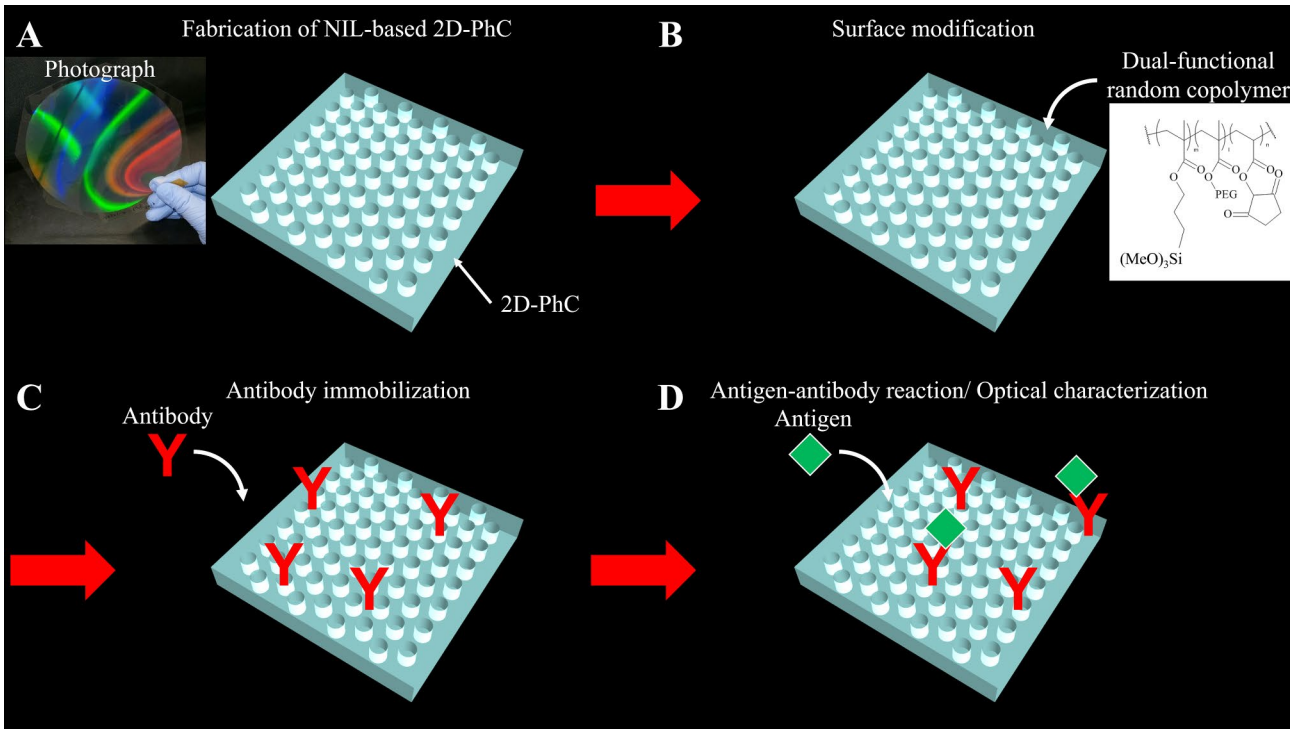

Figure 1. Schematic illustration of experimental procedure, photograph of NIL-based 2D-PhC (4-inch), and chemical structural formula of dual-functional random copolymer. After the fabrication of 2D-PhC using NIL (**A**), surface modification (**B**) and antibody immobilization were carried out (**C**). Different concentrations of antigen solutions were introduced onto antibody immobilized on 2D-PhC surfaces (**D**). The surrounding refractive index was changed by the antigen-antibody reaction, which depends on the antigen concentration. As a result, reflection peak intensity changes dependent on antigen concentrations can be observed.

fabricated onto cyclo-olefin polymer (COP) film (4 inches, 100 µm film thickness) using thermal NIL. Based on Bragg's law, by using this configuration of 2D-PhC, the structural color (blue to green) can be observed in naked eye (Fig. 1).

Based on this detection principle, for evaluation of optical characteristics of NIL-based 2D-PhC, the white light from the bifurcated fiber bundle was irradiated from the perpendicular direction. Reflection spectra were monitored from 400 to 800 nm on the handy type spectrophotometer at room temperature (RT).

2.4 Synthesis of dual-functional random polymer for reduction of nonspecific adsorption

For the development of future medical diagnostic devices using NIL-based 2D-PhC, target molecules need to be detected in body fluids such as serum, plasma, and saliva. These body fluids contain a high concentration of foreign substances such as immunoglobulin and albumin, which can increase background interference through nonspecific adsorption, impeding the highly sensitive detection of target molecules in body fluid.

For specific detection of the target molecules in body fluid with high sensitivity, dual-functional random copolymer was synthesized with the aim of reducing nonspecific adsorption of foreign substances through modification of the NIL-based 2D-PhC surface [20]. In a previous report, using this dual-functional random copolymer, high sensitive detection of influenza virus in human saliva could be achieved without nonspecific adsorption onto the NIL-based 2D-PhC [17].

The dual-functional random copolymer has several different functional groups (trimethoxy-silane, polyethylene glycol [PEG], and NAS), which have different functions. For the preparation of a dual-functional random copolymer, a previously reported copolymer was synthesized. In this study, the synthesis was performed as follows. PEGMA (1.425 g, 3 mmol), TMSMA (0.744 g, 3 mmol), NAS (0.507 g, 3 mmol) and AIBN (16.5 mg, 0.1 mmol) were placed in a glass vial and dissolved in tetrahydrofuran (THF, WAKO pure chemicals, Osaka, Japan) (10 mL). After preparation of the mixture, polymerization was performed at 70°C for 24 h under a nitrogen atmosphere. After the evaporation of the solvent, the polymer was obtained as a viscous liquid (Mw = 20 200). The polymer was dissolved in DMSO (100 mg/mL). Subsequently, the polymer solution was recrystallized with hexane to remove the unreacted reagents such as an AIBN. After the synthesis and purification of the dual-

functional random copolymer, surface modification and antibody immobilization was performed.

2.5 Detection of CRP using antibody immobilized on NIL-based 2D-PhC

For label-free detection of CRP using NIL-based 2D-PhC based on antigen-antibody reactions, the antibody was immobilized onto the NIL-based 2D-PhC surfaces via a modification of the dual-functional random copolymer.

For modification of the dual-functional random copolymer on the NIL-based 2D-PhC surface, O_2 plasma treatment (gas flow rate: 1526 sccm, power: 80 W) using an O_2 plasma cleaner (CUTE-1MP/R, Femto Science, Inc., Gyeonggi-Do, Korea) was performed on the NIL-based 2D-PhC surface for 25 min at RT to obtain the hydrophilized surface. After the O_2 plasma treatment, the NIL-based 2D-PhC was immersed into a dual-functional random copolymer solution diluted with ultra-pure water (1 ng/mL) for 30 min at RT, followed by washing with ultra-pure water. The dual-functional random copolymer-modified NIL-based 2D-PC film was then cured at 100°C for 1 min to ensure covalent bond formation via dehydration between the silane groups in the copolymer and the hydroxyl groups of the hydrophilized NIL-based 2D-PhC surface. By using this dual-functional random copolymer, troublesome liquid handling and operation steps such as blocking procedures and washing steps for biosensor fabrication were reduced.

For immobilization of anti-CRP antibody onto the dual-functional random copolymer modified NIL-based 2D-PhC surface, an anti-CRP antibody solution (1 μg/mL in 20 mM phosphate buffer, pH 7.4) was prepared. Subsequently, the antibody solution was introduced onto the NIL-based 2D-PhC surface for 2 h at RT. After the immobilization of antibody, antibody-immobilized on NIL-based 2D-PhC was immersed in a borate buffer (100 mM, pH 9.5) for 1 h to hydrolyze the unreacted NAS on the surface. From these antibody immobilization procedures, the antibody-immobilized on NIL-based 2D-PhC was used for label-free detection of CRP.

For detection of CRP using antigen-antibody reaction, different concentrations (100 fg/mL–100 ng/mL) of CRP solutions were introduced onto the anti-CRP antibody immobilized on NIL-based 2D-PhC for 5 min at RT for antigen-antibody reaction. After the antigen-antibody reaction, NIL-based 2D-PhC was washed with ultra-pure water to remove unreacted CRP. After the washing procedure, 2D-PhC was dried up using an air blower. Subsequently, the reflection intensity changes with the CRP concentrations were monitored using spectrophotometer.

On the other hand, in this study, to confirm the usability of dual-functional random copolymers, reduction efficiency of nonspecific adsorption was evaluated. For evaluation of reduction efficiency of nonspecific adsorption, a 100-fold diluted pooled blood serum (12181201, Kohjin Bio Co. Ltd., Saitama, Japan) was introduced onto the anti-CRP antibody immobilized on NIL-based 2D-PhC for 5 min at RT. After the antigen-antibody reaction, the reflection peak intensity changes were monitored.

2.6 Evaluation of reduction efficiency of nonspecific adsorption using dual-functional random copolymer modified NIL-based 2D-PhC

To confirm the nonspecific adsorption on the NIL-based 2D-PhC, fluorescence imaging by adsorption of fluorescent-dye conjugated protein was observed concurrently. For observation of fluorescence images, anti-rabbit IgG-TRITC antibody was immobilized onto the dual-functional random copolymer modified NIL-based 2D-PhC surface following a previously described immobilization procedure. On the other hand, to compare the nonspecific adsorption efficiency, anti-rabbit IgG-TRITC antibody was immobilized onto the NIL-based 2D-PhC directly via physical adsorption for 2 h at RT. After the immobilization, bovine serum albumin (BSA) solution was introduced as a blocking agent for 1 h at RT. Then, IgG-FITC from a human serum solution (2 μg/mL) was introduced onto the anti-rabbit IgG antibody immobilized on 2D-PhC surfaces for 5 min at RT. After the introduction and incubation of IgG-FITC from human serum solution, the 2D-PhC was dried after the washing procedure. Fluorescence images were obtained using fluorescent microscopy. The fluorescence images analysis was carried out using image analysis software (Image J).

3 Results and discussion

3.1 Detection of CRP using antibody immobilized on NIL-based 2D-PhC

The reflection spectrum of NIL-based 2D-PhC is shown in Fig. 2a. Using the NIL-based 2D-PhC, two reflection peaks could be observed at 433.7 and 487.4 nm. As shown in Fig. 2a, obtained reflection peak wavelengths were different from calculated reflection peak wavelengths based on Bragg's law. Based on Bragg's law, the expected reflection peak wavelength is 451.5 nm. These differences may be a result of the optical setup such as numerical aperture of the optical fiber bundle (NA = 0.39), wavelength dependency of light intensity, and the shape of the NIL-based 2D-PhC.

In addition, reflection spectra with different CRP concentrations were shown in Fig. 2b. Reflection peak intensity changes were observed with CRP concentrations. The different concentrations of CRP increased the surrounding refractive index and surface roughness, which is dependent upon the CRP antigen concentrations. As a result, reflection peak intensity ratios also decreased (Fig. 2b). In this study, antigen-antibody reaction time

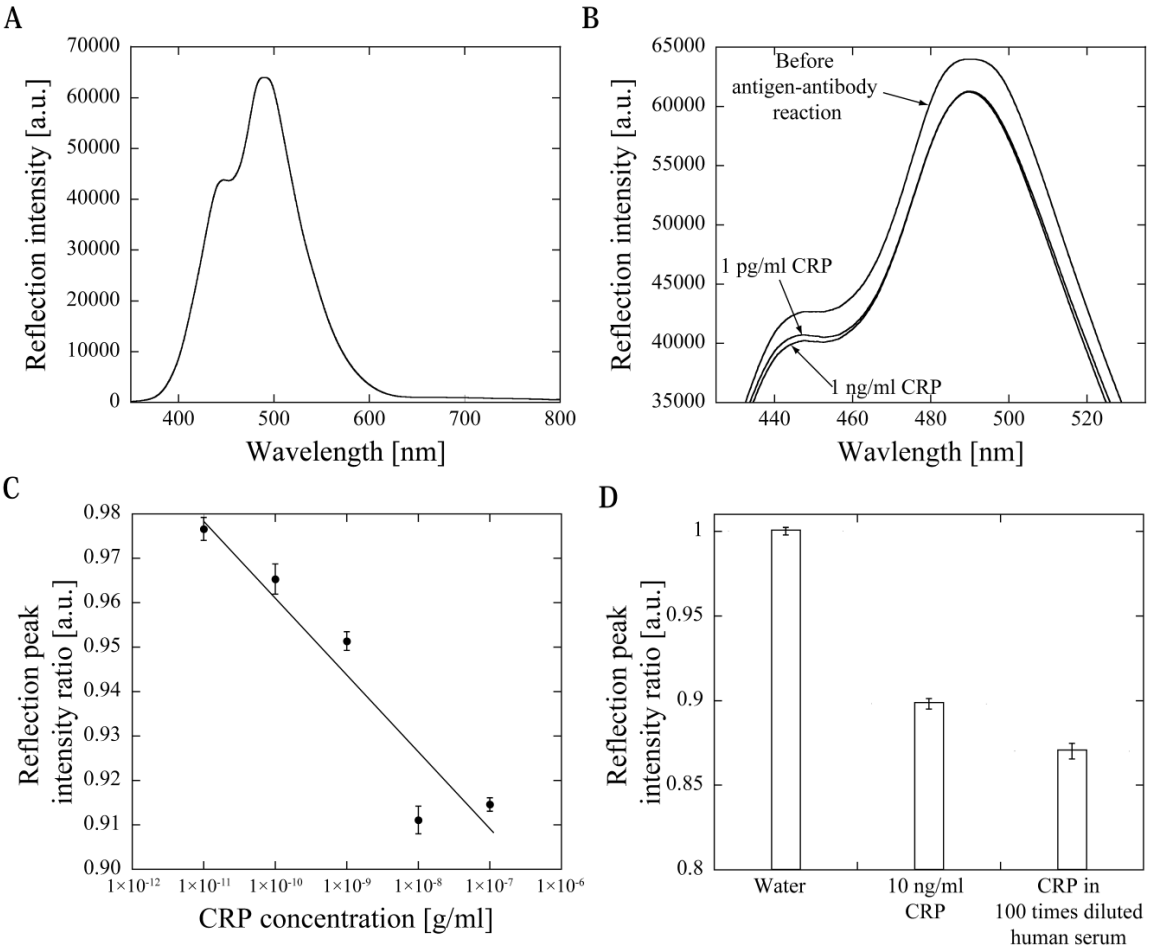

Figure 2. NIL-based 2D-PhC biosensor for detection of CRP using an antigen-antibody reaction. By using NIL-based 2D-PhC, the reflection peak could be observed at 433.7 and 487.4 nm. After the antibody immobilization, when the antigen solutions were introduced, reflection peak intensity changes dependent on the antigen concentrations were observed. (**A**) Reflection spectrum of NIL-based 2D-PhC. (**B**) Reflection spectrum change by antigen-antibody reaction. (**C**) Calibration curve for CRP using antibody immobilized on NIL-based 2D-PhC ($N = 5$). Error bars indicate standard deviation of five samples. (**D**) Comparison of reflection peak intensity ratios by introduction of CRP solution or CRP contained in 100-fold diluted human serum.

was set at 5 min. For optimization of reaction time, different reaction times were applied for similar experimental conditions. We could observe the reflection intensity change at 10 pg/mL at 5 min (data not shown). Hence, in this experiment, antigen-antibody reaction was carried out for 5 min. The reflection intensity change ratio was calculated from the reflection spectrum obtained from different concentrations of CRP at 433.7 nm. As shown in Fig. 2c, using antibody immobilized on NIL-based 2D-PhC; the calculated detection limit (3σ) for CRP was 12.24 pg/mL. Moreover, from these experimental results, change in construction of the hole and 2D-PhC surface also affected the reflection peak intensity change. The flatness of NIL-based 2D-PhC was decreased by surface modification of dual-functional random copolymer, antibody immobilization, and antigen-antibody reaction with different concentrations of antigens. The loss of flatness of NIL-based 2D-PhC resulted in a decrease in the reflection efficiency based on Bragg's law. Furthermore, when 100-fold diluted pooled blood serum was introduced onto the antibody immobilized on NIL-based 2D-PhC surface, reflection peak intensity changes could be observed. In human serum, high concentrations of CRP are present (average level of CRP concentration in human serum: 1–3 mg/L) [21]. From the reflection peak intensity change ratio and calibration characteristics, similar reflection change ratios were indicated. These results suggest that the antibody immobilized on NIL-based 2D-PhC enabled determination of CRP in bodily fluids (Fig. 2d).

In addition, the SEM observation and confirmation of decrement of flatness of NIL-based 2D-PhC by surface modification of dual-functional random copolymer was carried out. However, by the SEM observation, we could not observe the drastic surface morphology change (data not shown). On the other hand, contact angle was measured to confirm the surface modification of dual-function-

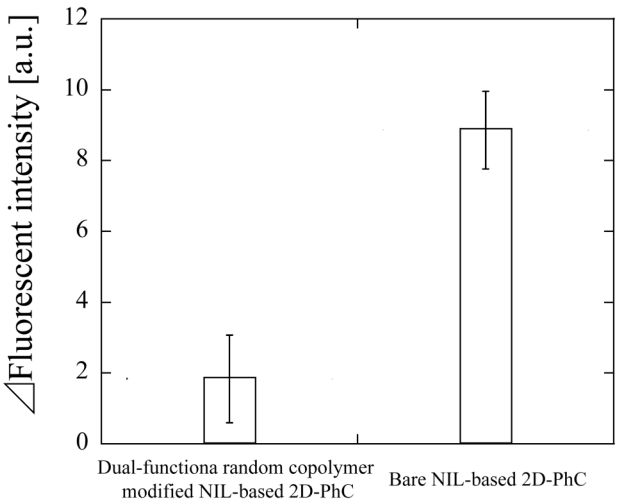

Figure 3. Fluorescence intensity change by nonspecific adsorption ($N = 5$). Error bars indicate standard deviation of five samples. By introduction of anti-rabbit IgG-TRITC antibody, there are no differences between surface modified NIL-based 2D-PhC and bare NIL-based 2D-PhC. However, by introduction of IgG-FITC, fluorescent intensity change by nonspecific adsorption could be observed using bare NIL-based 2D-PhC. It was concluded that dual-functional random copolymer enabled the reduction of nonspecific adsorption.

al random copolymer. As a result, contact angle change (20° to 64°) could be observed. Based on the contact angle measurement, it appeared that a thin polymer layer was formed on the NIL-based 2D-PhC.

3.2 Evaluation of reduction efficiency of nonspecific adsorption

For evaluation of reduction efficiency of nonspecific adsorption, fluorescence image analyses by immobilization of anti-rabbit IgG-TRITC antibody and after introduction of IgG-FITC from human serum were carried out. In the case of immobilization of anti-rabbit IgG-TRITC antibody using dual-functional random copolymer modified NIL-based 2D-PhC or bare NIL-based 2D-PhC, a similar fluorescence intensity by immobilization of anti-rabbit IgG-TRITC antibody could be measured. Conversely, by introduction of IgG-FITC from human serum, fluorescent intensity change using dual-functional random copolymer modified NIL-based 2D-PhC was lower than bare NIL-based 2D-PhC (Fig. 3). From the results of fluorescence intensity change, nonspecific adsorption could be reduced (reduction efficiency: 79.27%) by modification of the dual-functional random copolymer. PEG acted as a blocking reagent in the modified dual-functional random copolymer. However, introduction of BSA as a blocking reagent decreased adsorption homogeneity on the NIL-based 2D-PhC surface. Hence, nonspecific adsorption occurred by using bare NIL-based 2D-PhC surface. From these experimental results, using dual-functional random copolymer enabled a drastic reduction in nonspecific adsorption.

4 Summary and concluding remarks

In this study, by using NIL-based 2D-PhC, highly sensitive, label-free detection of CRP with low nonspecific adsorption using an antigen-antibody reaction was achieved in an extremely short reaction time. In addition, CRP could be detected in pooled blood serum specifically and rapidly. The 2D-PhC was fabricated easily and cost effectively based on printable photonics technology. NIL-based 2D-PhC and dual functional random copolymers have possibilities and advantages for various biosensing applications in real samples with highly concentrated foreign substances. In addition, more simple, rapid, and high-sensitivity biosensing systems may be developed for medical diagnostics applications using a simple and cost effective optical setup, such as a smartphone.

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The authors declare no financial or commercial conflict of interest.

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Cover illustration

This special issue, in collaboration with the Asian Federation of Biotechnology and edited by Professors Eiichi Tamiya and Chunhai Fan, covers advances in biosensors. This issue includes articles on optimization of biosensors for better sensitivity, exploration of the graphene interface for DNA analysis, aptamers for developing biosensors, etc. Image: © kentoh – Fotolia.com

Biotechnology Journal – list of articles published in the June 2016 issue.

Research Article

Quantification of total phosphorothioate in bacterial DNA by a bromoimane-based fluorescence method

Lu Xiao and Yu Xiang

http://dx.doi.org/10.1002/biot.201500432

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Label-free optical detection of C-reactive protein by nanoimprint lithography-based 2D-photonic crystal film

Tatsuro Endo, Hiroshi Kajita, Yukio Kawaguchi, Terumasa Kosaka and Toshiyuki Himi

http://dx.doi.org/10.1002/biot.201500440

Biotech Method

Imaging of enzyme activity using bio-LSI system enables simultaneous immunosensing of different analytes in multiple specimens

Toshiki Hokuto, Tomoyuki Yasukawa, Ryota Kunikata, Atsushi Suda, Kumi Y. Inoue, Kosuke Ino, Tomokazu Matsue and Fumio Mizutani

http://dx.doi.org/10.1002/biot.201500559

Rapid Communication

Aptamer-aptamer linkage based aptasensor for highly enhanced detection of small molecules

Van-Thuan Nguyen, Bang Hyun Lee, Sang Hoon Kim and Man Bock Gu

http://dx.doi.org/10.1002/biot.201500433