

Highly sensitive nano-porous lattice biosensor based on localized surface plasmon resonance and interference

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Abstract: We propose a design for a highly sensitive biosensor based on nanostructured anodized aluminum oxide (AAO) substrates. A gold-deposited AAO substrate exhibits both optical interference and localized surface plasmon resonance (LSPR). In our sensor, application of these disparate optical properties overcomes problems of limited sensitivity, selectivity, and dynamic range seen in similar biosensors. We fabricated uniform periodic nanopore lattice AAO templates by two-step anodizing and assessed their suitability for application in biosensors by characterizing the change in optical response on addition of biomolecules to the AAO template. To determine the suitability of such structures for biosensing applications, we immobilized a layer of C-reactive protein (CRP) antibody on a gold coating atop an AAO template. We then applied a CRP antigen (Ag) atop the immobilized antibody (Ab) layer. The shift in reflectance is interpreted as being caused by the change in refractive index with membrane thickness. Our results confirm that our proposed AAO-based biosensor is highly selective toward detection of CRP antigen, and can measure a change in CRP antigen concentration of 1 fg/ml. This method can provide a simple, fast, and sensitive analysis for protein detection in real-time.

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OCIS codes: (130.6010) Sensors; (240.6680) Surface plasmons.

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1. Introduction

Biotechnology is one of the fastest growing scientific fields because of the potential to improve the quality of human life. A primary goal of this field of research is to develop devices for real-time detection of small concentrations of biomolecules. Detection of antigen–antibody reactions in real-time is a particularly important goal in the biomedical field. Among the class of biosensor detection methods [1–3], optical biosensors offer high accuracy, sensitivity, and rapid response time. Such biosensors include optical fiber sensors, surface

plasmon resonance (SPR) biosensors [4], waveguide biosensors that measure changes in the refractive index, and interferometric biosensors [5–7].

In this study, we developed a biosensor with a gold-deposited nanoporous AAO substrate that allows us to use both optical interference and localized SPR (LSPR) to detect optical changes associated with detection of an antigen. LSPR is highly sensitive to changes in the refractive index of the sensing membrane, while a Fabry-Perot fringe pattern is generated when white light is directed perpendicularly to a nanoporous structure [8–10]. This property is useful in a biosensor intended to detect small molecules. The biosensor developed and reported herein has several advantages such as high sensitivity, not requiring chemical labels [11–14], small weight, and high portability. Moreover, the biosensor responds rapidly and continuously [15].

The proposed biosensor detects the human C-reactive protein (CRP), which is a blood serum marker for infections and inflammatory processes. Many reports have confirmed that the CPR level shows strong correlation with, inter alia, inflammatory bowel disease [16], colon cancer [17], and cardiovascular disease [18]. A medically useful measurement for CRP antigen (Ag) requires sensitivity of better than 10 fg/ml, which has not been achieved with prior measurement techniques [19, 20]. In addition, prior methods are difficult to apply in real-time, as secondary data analysis is required.

In this study, a biosensor using bioselective coatings on an anodic aluminum oxide (AAO) template is found to provide high selectivity, ~1 fg/ml sensitivity, and simple, real-time measurements of a wide variety of materials. Substrates with various pore depths are fabricated by a two-step anodizing method. After deposition of Au on the anodized surface, a CRP antibody (Ab) is immobilized on the surface of the substrate. In the presence of CRP antigen, the antigen–antibody reaction induces changes in the refractive index of a sensing membrane, which are measured as changes in the reflectivity of the sensing membrane.

2. Operating principles of the biosensor

To increase the sensitivity of the sensor system, we developed a sensor chip designed to detect CRP antigen using both interferometry and LSPR. Figure 1 shows a schematic diagram of the fabricated AAO chip and the optical antigen detection method. Au was deposited on a nanoporous structure to induce LSPR and to allow immobilization of a CRP antibody [14]. Reaction of the CRP antigen with the self-assembled monolayer (SAM) of the CRP antibody causes a change in the wavelength-dependent refractive index of the sensing membrane. The change in reflectance intensity and the wavelength shift are caused by interference. The target material is detected by analyzing the changes in the reflectance spectrum induced by changes in the refractive index and the effective optical thickness (L) of the membrane. Rossi [21] defines the effective optical thickness as the product of the membrane thickness and the refractive index, as given by Eq. (1).

$$m\lambda_1 = 2n_{\lambda_1} L \text{ and } (m+1)\lambda_2 = 2n_{\lambda_2} L \quad (1)$$

In Eq. (1), m is the order of a fringe that contains information on the optical path length difference and reflection-phase shift, and n_{λ} is the effective refractive index at wavelength λ [21, 22]. The symbols λ_1 and λ_2 denote the wavelengths of two neighboring fringes of the interference orders of m and $m+1$, respectively. The band gap of the AAO film is rather large; therefore, n_{λ} can be regarded as constant in the long-wavelength region. Thus, the relationship between fringe order m , λ_1 , λ_2 can be given by Eq. (2).

$$\lambda_1 / \lambda_2 = (m+1) / m \quad (2)$$

In order to enhance sensitivity of sensors, several researchers have mainly focused on nanoscale optical sensors such as nanoparticles and nanostructures. Thus, the proposed LSPR-based biosensor depends on the size and shape of the AAO nanostructure. The phenomenon

of LSPR can be understood using a model of the refractive-index response of the propagating surface plasmon on a metal surface [23].

$$\Delta\lambda_{\max} = m\Delta n[1 - e^{(-2d/l_d)}] \quad (3)$$

Here, $\Delta\lambda_{\max}$ is the wavelength shift, m is the refractive-index sensitivity, Δn is the change in refractive index induced by adsorption, d is the effective thickness of the adsorbate layer, and l_d is the characteristic electromagnetic-field decay length.

As shown in Eq. (1), the refractive index and effective optical thickness of the membrane change when a target molecule is bound through an antigen-antibody reaction on the nanoporous Au surface. In addition, according to Eq. (3), LSPR can detect a very small change in the interfacial refractive index of the surrounding media, which is greatly affected by the binding of biomolecules. Therefore, the proposed system causes a significant change in sensitivity, as observed by both optical interferometry and LSPR.

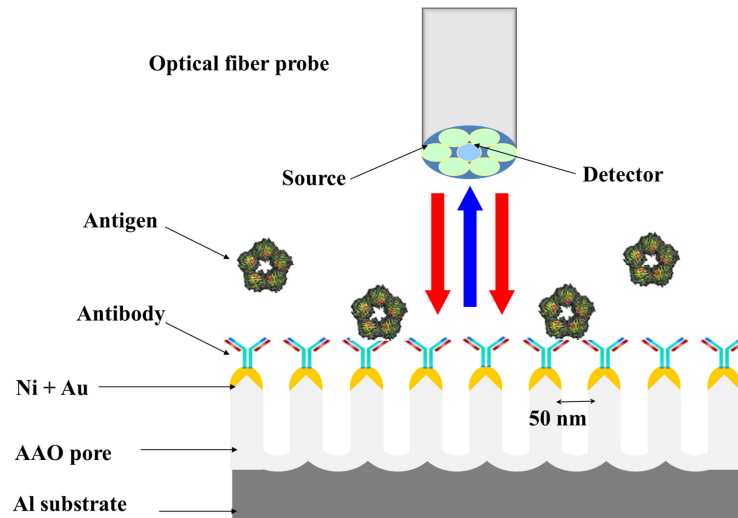


Fig. 1. Schematic diagram of fabricated AAO chip and antigen detection method.

3. Experiment and method

3.1 Fabrication of AAO chip

We used an anodizing method to grow the Al_2O_3 layer [24]. The principle underlying the growth AAO is that aluminum functions as the anode and the carbon electrode functions as the cathode, with the generation of a certain voltage in the Al_2O_3 layer as the aluminum ions combine with oxygen and hydrogen ions. Growth controlled by current is considered to have higher precision, but we controlled the growth of the nanostructure membrane by controlling voltage to simplify the manufacturing process. For improving the uniformity of the surface, we used a two-step anodizing method. The uniformity of two-step anodizing is thrice that of one-step anodizing [25, 26].

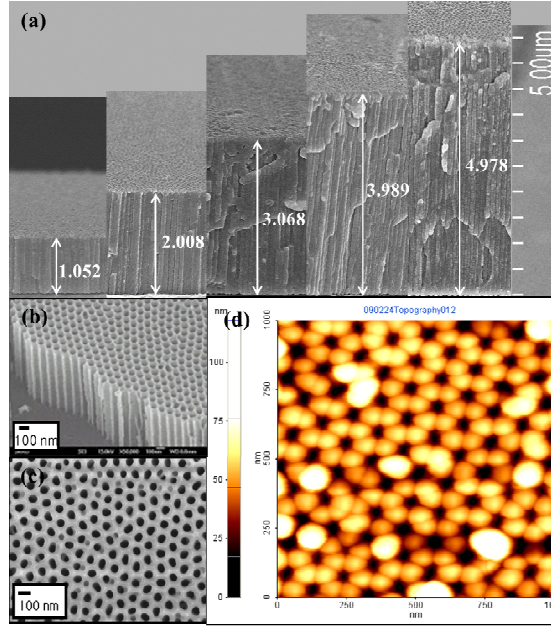


Fig. 2. SEM and AFM images of AAO chip: Cross-section SEM image of AAO chip in cross-sectional view (a), (b) and top view (c); AFM image of Au deposited AAO chip in top view (d).

The AAO chip was fabricated as follows: Aluminum foil (99.999% Al, Aldrich) was used as the substrate, and oxalic acid (0.3 M) was used as the electrolyte solution. The temperature was maintained at 9 °C. Initially, the aluminum foil was placed in a solution of ethanol (30%) and perchloric acid (70%) and subjected to a bias voltage of 20 V to remove impurities and the native oxide layer and to smoothen the surface of aluminum. Subsequently, the substrate was placed in an electrolyte solution with a bias voltage of 40 V for 30 min to grow an oxide layer. To remove the first anodized aluminum oxide layer, the aluminum foil was placed in a solution consisting of chromic acid (1.8 wt%) and phosphoric acid (6 wt%) for 90 min at 60 °C. After the removal of the first aluminum oxide layer, the foil was anodized again for various time periods to obtain the required pore depths. As shown in Fig. 2(b) and 2(c), pore diameter and pore depth were measured through scanning electron microscopy (SEM). Figure 2(d) shows the surface uniformity of the Au-deposited AAO chip observed through atomic force microscopy (AFM). Figure 2(a) indicates that five different depths of AAO (1–5 μm) were precisely fabricated through the anodizing method. As shown in Fig. 3, the depths depended on the various anodizing time periods (16, 33, 50, 67, and 83 min). The AFM images confirmed that the Au was uniformly deposited on the AAO surface. In addition, we obtained uniform pore diameters of 50 nm. On the basis of the above results, we analyzed the growth rate of AAO in terms of time and current (I) and determined a growth rate of 1 nm/s. We defined the growth rate as Eq. (4), where T is AAO film thickness.

$$T = I \times t \times 10^{-1} \mu m \quad (4)$$

As we expected, the sensor chip with 1 μm depth pores exhibited the best sensitivity because the changes in effective optical thickness became larger as the depth of the pores decreased. The fabrication of a sensor chip with pores less 1 μm depth compromised the surface uniformity.

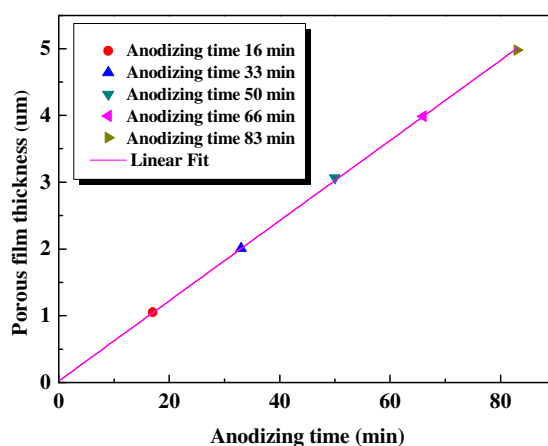


Fig. 3. Growth rate of AAO according to anodizing time.

3.2 Fabrication of sensing membrane

A 5 nm thick Ni layer and a 15 nm thick Au layer were deposited on the fabricated Al_2O_3 nanoporous structure through e-beam evaporation. The average deposition rate was $0.1 \text{ \AA/s}$ under vacuum of $4.0 \times 10^{-6} \text{ T}$. Here, the Ni layer is used as an adhesive interfacial layer to ensure uniform morphology of the Au layer in the sensing membrane [27]. In addition, this layer exhibits superior SPR as compared to the pure single metal layer such as the Au or Ag layer. Therefore, we incorporated a Ni interfacial layer to enhance the plasmon on the Au film on the Al_2O_3 template. In the deposited metal layer, the nanoporous Au layer induces LSPR and plays an important role in capturing and immobilizing the antibody. To form the SAM on the nanoporous Au layer, the Au-deposited AAO chip was immersed in a solution of 11-mercapto-1-undecanol (20 mM) in ethanol and incubated for 24 h [28, 29]. After the formation of the SAM, carboxylic groups were activated with N-hydroxysuccinimide (NHS) (50 mM) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (50 mM) for antibody immobilization through amino coupling. Finally, the CRP antibody (50 $\mu\text{g/ml}$) was immobilized through incubation at room temperature for 1 h. Unbound antibodies were removed by rinsing the surface with Tris-HCl buffered solution. After the surface of the sensing membrane was rinsed, the reflection spectrum of the immobilized antibody was measured using the detection system. Biomaterials were purchased from Sigma Aldrich. The commonly used bioreagent phosphate-buffered saline contains phosphoric acid, which can damage Al_2O_3 nanoporous membranes. Therefore, Tris-HCl buffered solution (pH 7.4) was used as the bioreagent in our experiments.

3.3 Experimental setup

As shown in Fig. 4, the developed biosensor system consisted of a white-light source, a reflectance optical probe, an antigen-antibody reaction chamber, a micropump, a spectrometer (Ocean Optics), and an AAO chip. CRP antibody had previously been immobilized on the AAO sensing membrane. Visible light was perpendicularly irradiated from an optical fiber bundle probe. The reflected light was detected using the same optical probe and measured as a reference spectrum by a spectrometer.

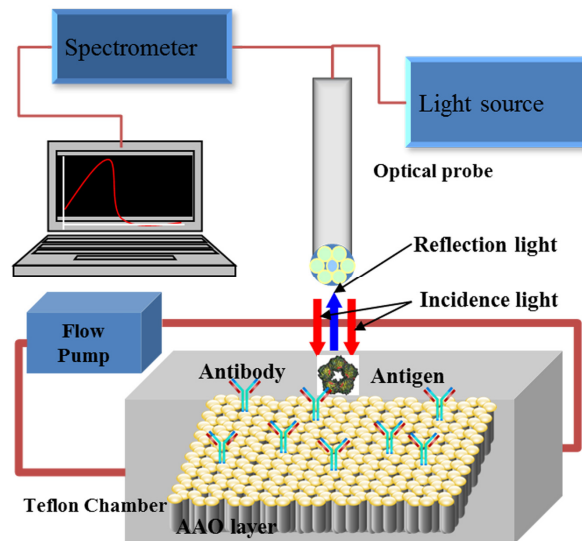


Fig. 4. Schematic diagram of real-time monitoring biosensor system.

4. Result

4.1 Optical characteristics of sensing membrane

Before applied to biosensor, the optical properties of nanoporous AAO substrate was tested with and without the Au layer. Prior to the main measurement, we confirmed that the spectrum shifted according to changes in the surrounding refractive index of the sensing membrane. We prepared a glycerin solution, which is commonly used as a refractive index standard for determining weight-percent variation. Four sets of aqueous glycerin solutions were prepared, with glycerin concentrations of 6, 9, 12, and 15%. An Abbe refractometer was used to measure the refractive index of each solution. In order of increasing concentration, the refractive indices were 1.3392, 1.3430, 1.3458, and 1.3491. Glycerin in various concentrations was then spin-coated onto the bare AAO chip and Au-deposited AAO chip, and then, its refractive index change was determined by measuring the shift in the reflection spectrum. In Fig. 5 (b), bare AAO chip showed a small wavelength shift by changes in the refractive index of surrounding media, whereas Au-deposited AAO chip wavelength shift was significant. Based on these results, bare AAO chip only showed wavelength shift by interferometric property while wavelength of Au-deposited AAO chip was shifted by both LSPR and interferometry. Therefore, it was confirmed that Au-deposited AAO chip has higher sensitivity than that of bare AAO chip. This confirms that our device can detect changes in the refractive index of the surrounding media that result from exposure to CRP antigen [30].

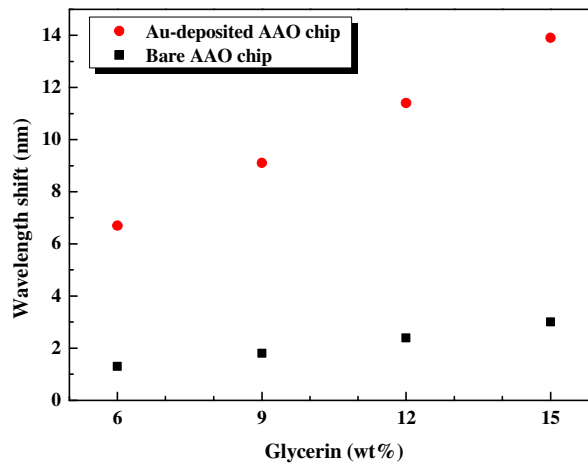


Fig. 5. The wavelength ($\lambda_{\max}$) shift of AAO chip with and without Au layer according to changes in refractive index of glycerin solutions.

4.2 Characteristics of LSPR-based biosensor

Deposition of the Au film on the surface of a high-uniformity AAO chip resulted in a Au structure similar to a nanoparticle array. The spectral wavelength of LSPR depends on the size and shape of the nanostructure. In other words, the resonance wavelength of an LSPR based biosensor with a nanoporous structure is defined by the neighboring pore distance. It is well known that plasmonic sensitivity is lower when the interspace between the neighboring nanoporous metals is less than 50 nm [31]. Therefore, we fixed the pore distance as 50 nm to improve the plasmonic sensitivity and subsequently performed the experiment. Finally, this chip was prepared for use through placement and immobilization of a monolayer of a CRP antibody on its surface. A quantitative CRP detection procedure was followed. After the immobilization of 300 $\mu\text{g/ml}$ CRP antibody on the Au-deposited nanoporous AAO chip, the membrane was rinsed with Tris-HCl buffered solution, and then, a reference spectrum was measured. In the ensuing steps, CRP was injected into a specially designed reaction chamber, where CRP reacted with the immobilized CRP antibody layer in an amount proportional to the concentration of CRP. The shift in the reflection spectrum of the membrane was then measured in real-time. The sensing membrane was rinsed with Tris-HCl buffered solution to remove nonreactive or saturated CRP antigen residue on the membrane after every antigen-antibody reaction was completed. No significant change in wavelength was observed after rinsing. The CRP antigen concentration was varied from 100 ag/ml to 100 $\mu\text{g/ml}$. As shown in Fig. 6(a), the reflected spectra from the membrane shifted in response to variations in CRP antigen concentration. The graph shows wavelengths from 600 nm to 780 nm. Increasing the CRP antigen concentration induced incremental increases in intensity, and the wavelength shifts in the reflectance spectra increased as the log of antigen concentration. No spectrum changes were observed following reaction with 100 ag/ml CRP solution, so the detection limit of the fabricated sensor chip was 1 fg/ml. Results can provide high sensitive detection for small biomolecules. Figure 6(b) shows the large dynamic range and high sensitivity of our chip with quantitative detection of CRP antigen. The $\lambda_{\max}$ of reflectance wavelength shifted dramatically with variations in CRP antigen concentration (from 1 fg/ml to 100 $\mu\text{g/ml}$) in CRP antigen-antibody reaction. As CRP antigen concentration increased over 100 $\mu\text{g/ml}$, no significant changes in the reflectance spectrum were measured. This indicated the saturation of the surface-immobilized antibody reacted to CRP antigen. We performed measurements

over ten times (indicated by error bars in Fig. 6(b)) to confirm the reliability of the results. This result shows the different error ranges in the case of repeated measurements, corresponding to different CRP concentrations. The increased concentration of CRP antigen is attributed to the increased probability of non-specific binding and physical absorption of CRP antigen on the sensing membrane. Even though the error term appears, the wavelength shifts are quite distinct from each other, owing to the variation in concentration.

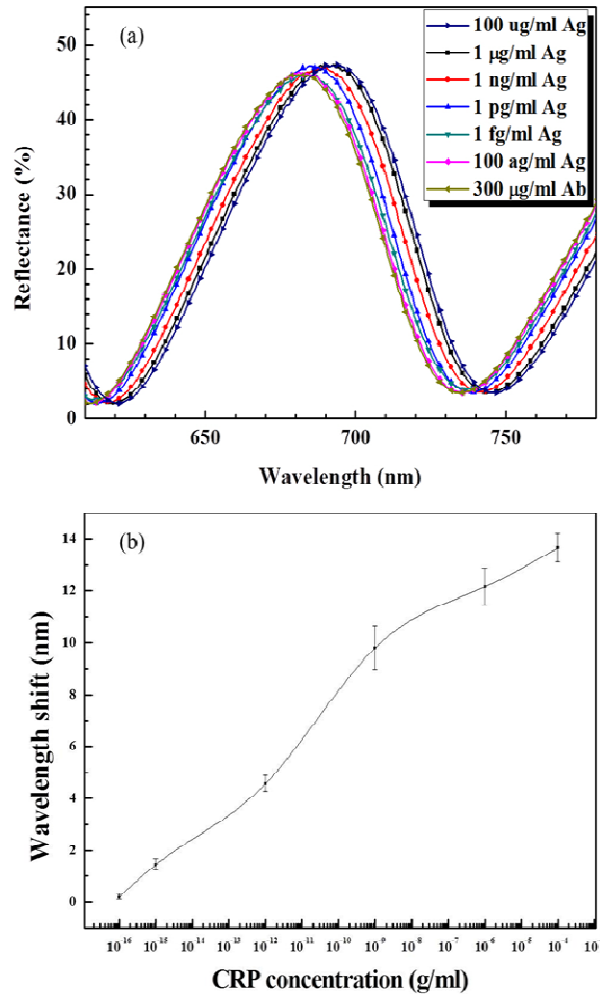


Fig. 6. Shift in the reflectance wavelength with variations in CRP antigen concentration (from 100 ag/ml to 100 $\mu\text{g/ml}$) in CRP antigen–antibody reaction: (a) fringe pattern of reflectance spectra; (b) Shift of λ_{max} in the reflectance wavelength.

Next, we evaluated selectivity of the sensor chip—specifically the ability to distinguish between CRP antigen and Troponin T antigen. Figure 7 shows the results of a set of selectivity measurements carried out with the sensor chip described earlier, at wavelengths ranging from 680 nm to 730 nm. Subsequently, we measured other kinds of antigens with the sensor chip in order to estimate its selectivity. After measuring the Tris-HCl buffered solution as a reference, we found that injecting 10 $\mu\text{g/ml}$ of Troponin T antigen did not induce a spectrum change. After rinsing the surface of the membrane with Tris-HCl buffered solution,

the 10 $\mu\text{g/ml}$ CRP antigen was introduced into the reaction chamber, after which the reflectance spectrum shifted. The fabricated sensor chip exhibited the predicted selectivity, reacting only with the design target materials.

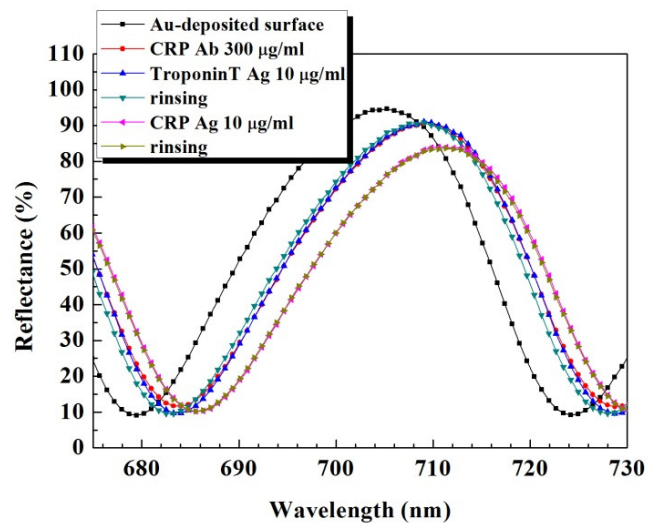


Fig. 7. Selectivity evaluation of the sensor chip: bare Au-deposited AAO (black line); 300 $\mu\text{g/ml}$ CRP antibody immobilized on Au-deposited AAO (red line); injected 10 $\mu\text{g/ml}$ Troponin T antigen (blue line); rinsed with Tris-HCl buffered solution (green line); injected 10 $\mu\text{g/ml}$ CRP antigen (pink line); rinsed with Tris-HCl buffered solution (yellow-green line).

5. Conclusion

We report on a biosensor chip using integrated interferometric and LSPR changes to monitoring antigen–antibody reactions in real-time. CRP was quantitatively detected with high sensitivity. The antigen–antibody reaction was detectable in real-time without the drying process, which is required by conventional methods. The sensor chip was able to detect only the desired biomarkers, as it showed no measurable change when exposed to antigens for which it was not designed. Our sensor chip demonstrated excellent monitoring sensitivity and selectivity. This sensor chip is simpler to apply and operate than the well-known SPR sensor system. Furthermore, the developed sensor system can also be used for detecting DNA hybridization and DNA–protein interactions. Our sensor chip represents a unique approach for the detection of biomarker that uses a very simple and portable biosensor system.

Acknowledgments

This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (No. 2011-0001081).