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*Research Article*

# Detection of Biomarkers Using LSPR Substrate with Gold Nanoparticle Array

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In the biosensing platform, label-free detection technique provides advantages such as the short analysis time and the cost-effectiveness. In this study, we showed the feasibility of the LSPR substrate with gold nanoparticle array for detecting low density lipoprotein (LDL) and high density lipoprotein (HDL) without labeling. The LSPR substrate was fabricated through the lift-off process with the anodized alumina mask, and its LSPR phenomenon was observed by measuring the optical transmission of substrate. The antibodies were immobilized on the gold nanoparticle array via the chemical binding, in which the 11-MUA was used as the linker to bind the antibodies. The binding of antibodies was confirmed by observing the shift of LSPR peak of the substrate. Finally, with the LSPR substrates with the antibodies immobilized, the detection of LDL and HDL was investigated. As a result, LDL and HDL could be detected in the clinically available concentration range, respectively.

## 1. Introduction

Immunoassay based on antigen-antibody binding is prevalently used for detecting several kinds of analytes such as protein, pathogens, or small molecules. As the method can be quite rapid, it has been applied to develop a point-of-care diagnostic device of which the operation should be simply implemented [1].

The labeling-based detection techniques have been widely employed for detecting the antibody-antigen binding in the point-of-care diagnostic device [1, 2]. For example, a fluorescence detection technique has been used to detect antibody-antigen binding on a solid surface. In the technique, the labeling step, in which the complex of antibody-antigen binding is labeled with the probe antibody, conjugated with fluorescence molecule. Basically, the labeling techniques involving the use of fluorescence, ELISA, and isotropic labeling require one or two intermediate steps for labeling. So, the simpler detection technique should be developed for implementing the point-of-care diagnostic device.

Label-free detection technique would provide a direct approach to the detection of protein-protein binding. Electrochemical impedance technique, surface plasmon resonance (SPR), and mass-sensitive cantilever detection have all been applied to label-free detection methods [3–5]. In particular, the SPR technique has been used as a versatile detection tool for the study of the kinetics of receptor-ligand interaction [6]. In the conventional SPR technique, the plasmon resonance is excited by the light beam incident onto the interface between a metal and a dielectric medium, and the plasmon resonance condition such as the incident angle is attributed to the refractive index in the dielectric medium. So, the complicated optical system or mechanical structure is required to implement the SPR device [7]. On the other hands, the localized surface plasmon resonance (LSPR) is observed in the metal nanoparticle, not presented in the bulk metal [8]. When light is incident on a surface with metal nanoparticles smaller than the wavelength of light, the collective oscillation of conduction electron in them with a resonant frequency is induced. The LSPR is

dependent on the dielectric properties of the local environment surrounding the nanoparticles [9]. This property allows the LSPR to provide the label-free detection method for an antigen-antibody reaction [10]. Also, as the LSPR of metal nanoparticle is easily observed by measuring the adsorption spectrum, the biosensor platforms based on the LSPR can be simply implemented.

The substrate, onto which the nanosized metal structures exhibiting the LSPR phenomenon are deposited, should be developed for the LSPR-based biosensor. There have been several methods reported to fabricate the LSPR substrate, such as the direct deposition of nanoparticles or nanorods in solution onto a solid surface, the growth of nanoparticles on the surface, nanosphere lithography, and nanoimprint lithography [9–14]. Recently, we reported the fabrication of gold nanoparticle array on solid surface [15]. The gold nanoparticle arrays were simply fabricated via the lift-off process, and the absorption peak showing the maximum absorbance in the absorption spectrum (defined as the LSPR peak) could be regulated in the wavelength range of 500~700 nm with the control of thickness.

In this study, we demonstrate the application of the gold nanoparticle array fabricated by the lift-off process to detection of two biomarkers, low density lipoprotein (LDL) and high density lipoprotein (HDL). They are the important biomarkers for diagnosing the cardiovascular diseases [16]. The immobilization of antibody onto the gold nanoparticle array was confirmed by observing the change of the LSPR peak. In addition, we showed the applicability of the gold nanoparticle array to the detection of biomarkers by measuring the change of the LSPR peak with the increasing concentration of the biomarkers.

## 2. Materials and Methods

*2.1. Materials.* LDL and antibody against LDL and HDL and antibody against HDL were purchased from Abcam plc company. 11-mercaptoundecanoic acid (11-MUA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC), and human serum were purchased from Sigma-Aldrich company. The other chemicals used in this study were reagent grade and were obtained commercially.

*2.2. Fabrication of Gold Nanoparticle Array.* The gold nanoparticle array was fabricated by the method reported previously [15]. After a glass substrate (size: 24 × 24 mm) was cleaned with the piranha solution (1 : 4 of hydrogen peroxide and sulfuric acid), 1 $\mu$m thick aluminum film was deposited by electron beam evaporation (EI-5, ULVAC Co., USA). A two-step anodization process was executed to form the alumina mask on the substrate as described elsewhere [17, 18]. In the first anodization process, after the substrate and the carbon electrode were placed in a beaker of 0.3 M oxalic acid, 40 V was applied between the substrate and the carbon electrode to anodize the aluminum film for 13 minutes at 5°C. The alumina film formed on the substrate was removed by immersing it in a solution of 6 wt% $H_3PO_4$ and 1.5 wt% $CrO_3$ at 60°C. After the substrate was rinsed and dried, the second anodization process was executed with the aluminum film remaining on the substrate under the same conditions as the first anodization. Finally, the substrate was immersed in a beaker of 5 wt% $H_3PO_4$ to remove the barrier layer and widen the diameter of the pores formed in the alumina film [19].

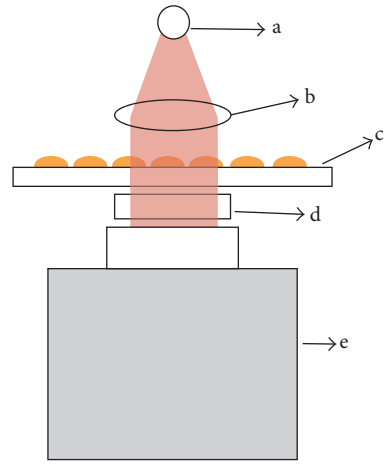
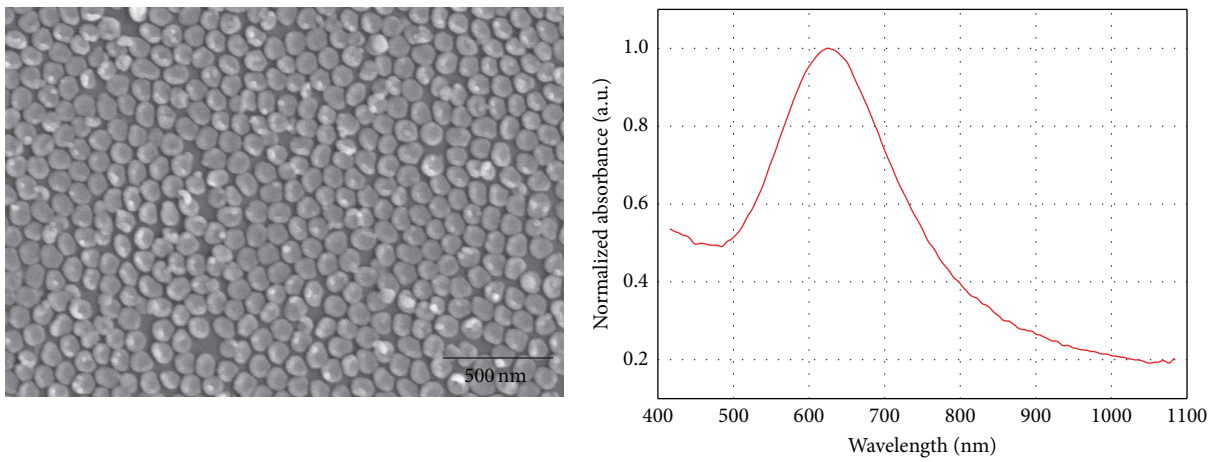



Figure 1: Hand-made equipment for measuring the optical transmission of substrate (a: halogen lamp, b: collimator, c: substrate with gold nanodot array, d: optical attenuator, and e: CCD-type spectrophotometer).

The gold nanoparticle array was fabricated using the alumina mask lift-off process. To fabricate the gold nanoparticle array, a 2 nm thick layer of chromium and a gold layer were sequentially deposited via electron beam evaporation onto the substrate. After depositing the gold layer, the gold nanoparticle array on the substrate was completed by removing the alumina mask by immersing the substrate in 5 wt% $H_3PO_4$ at 30°C for 60 minutes.

*2.3. Immobilization of Antibody.* The antibodies against biomarkers were covalently immobilized on the surface of each gold nanoparticle by the method reported elsewhere [20]. The glass substrate with the gold nanoparticle array was cleaned with ethanol, followed by the oxygen plasma treatment. The monolayer of 11-mercaptoundecanoic acid (11-MUA) was deposited on the gold nanoparticle array by submersing the substrate in the ethanol solution containing 150 mM 11-MUA for at least 12 hours. To bind the antibody covalently, the carboxylic group of the monolayer was activated by submerging the substrate modified with 11-MUA into a solution of 10% EDAC in water/ethanol (10/1 v/v) for 2 hours at room temperature. The immobilization process of antibody was completed by applying the activated surface to 20 $\mu$g/mL antibody solution in the phosphate buffer saline (PBS) buffer for at least 2 hours, followed by cleaning the surface.

*2.4. Experimental Setup.* Absorption spectrums of LSPR substrate were acquired with the hand-made device equipped with the commercial components as shown in Figure 1. The device is composed of the CCD-array type spectrophotometer (BRC115A, B&W Tek Inc.) and the 10 W tungsten lamp (Osram Co.) connected with the collimator and configured to




(a) (b)

Figure 2: (a) Scanning electron micrograph of 10 nm thick gold nanodot array formed on glass substrate. (b) Typical absorption spectrum of the substrate with gold nanodot array.

measure the transmission of the substrate. The transmission value measured was converted into the absorbance value by Beer's law.

## 3. Results and Discussion

*3.1. Gold Nanoparticle Array.* As mentioned above, the gold nanoparticle array on the glass substrate was fabricated with the lift-off process of the alumina mask [15]. Previously, we reported that the sensitivity of LSPR substrate defined as the ratio of the shift of LSPR peak to the refractive index unit (RIU) of the medium surrounding the gold nanoparticle array was changed with the thickness of gold layer deposited, and the maximum sensitivity was at the deposition thickness of 10 nm. Here, we used the gold nanoparticle array with the deposition thickness of 10 nm for the LSPR biosensing as shown in Figure 2(a). The diameter of each gold nanoparticle was 94.9 ± 5.9 nm, and the typical absorption spectrum of the substrate was shown in Figure 2(b), in which the position of LSPR peak of the gold nanoparticle array was 626.7 ± 5.9 nm, which is calculated with the centroid algorithm [21].

*3.2. Immobilization of Antibody.* Several techniques for binding antibody to the substrate have been reported, and are divided into two categories: the physical binding and the chemical binding. The chemical binding technique, in which antibody is covalently immobilized onto the substrate, has been found to show good reproducibility and coverage [22]. In this study, the chemical binding in which the monolayer of 11-MUA was used as the linker to bind antibody was used in order to immobilize the antibody on the gold nanoparticle array. Figure 3 shows the scheme of the immobilization of antibody used in this study. The monolayer of 11-MUA with the carboxylic acid as the terminal group was self-assembled on the surface of each gold nanoparticle. The substrate modified with the 11-MUA monolayer was treated with EDAC to allow the chemical binding between the lysine residues of antibody and the carboxylic group via the amide

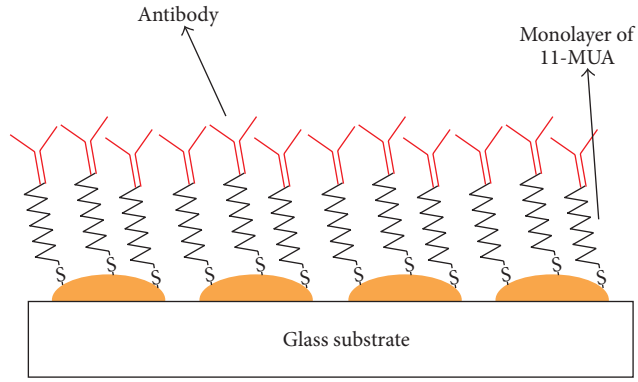



Figure 3: Scheme for immobilizing the antibody on the substrate.

bonding. Figures 4(a) and 4(b) show the change of absorption spectrums according to sequentially depositing the 11-MUA and antibody onto the substrate. Following the deposition of the monolayer of 11-MUA, the position of LSPR peak was shifted from 626.7 ± 5.9 nm (the LSPR peak position of substrate without the monolayer) to 641.6 ± 4.3 nm. It is known that the closely packed 2-dimensional molecular layer of 11-MUA is formed on the substrate by the van der Waals attractive force among the long alkyl chains [23]. Therefore, the shift of peak position is attributed to the change of RIU near the gold nanoparticle array. After immobilizing the antibody against LDL onto the monolayer of 11-MUA, the LSPR peak was shifted to 676.8 ± 4.2 nm. Also, in case of immobilization of antibody against HDL, the LSPR peak of substrate was shifted to 671.1 ± 5.6 nm.

*3.3. Detection of Biomarkers.* LDL is the major carrier of cholesterol in the blood, and the accumulation of it on the vessel wall constitutes one of the initial steps in arteriosclerosis [24]. So, the increased level of LDL in blood is associated with atherosclerosis. On the other hand, HDL is

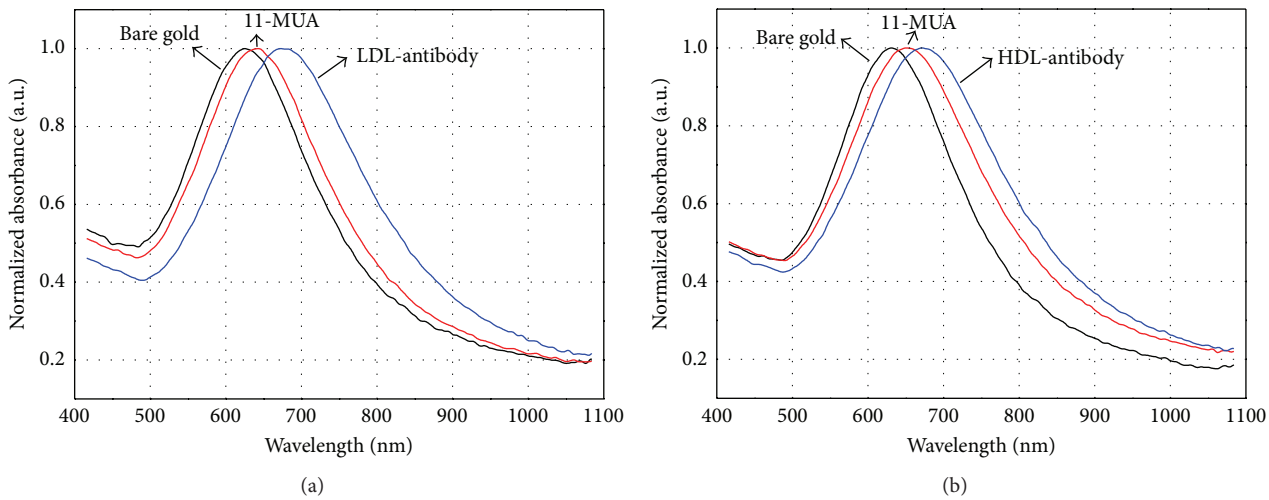



Figure 4: Absorption spectrums of substrate with depositing the 11-MUA monolayer and immobilizing the antibodies on the monolayer. (a) Immobilization of the antibody against LDL. (b) Immobilization of the antibody against HDL.

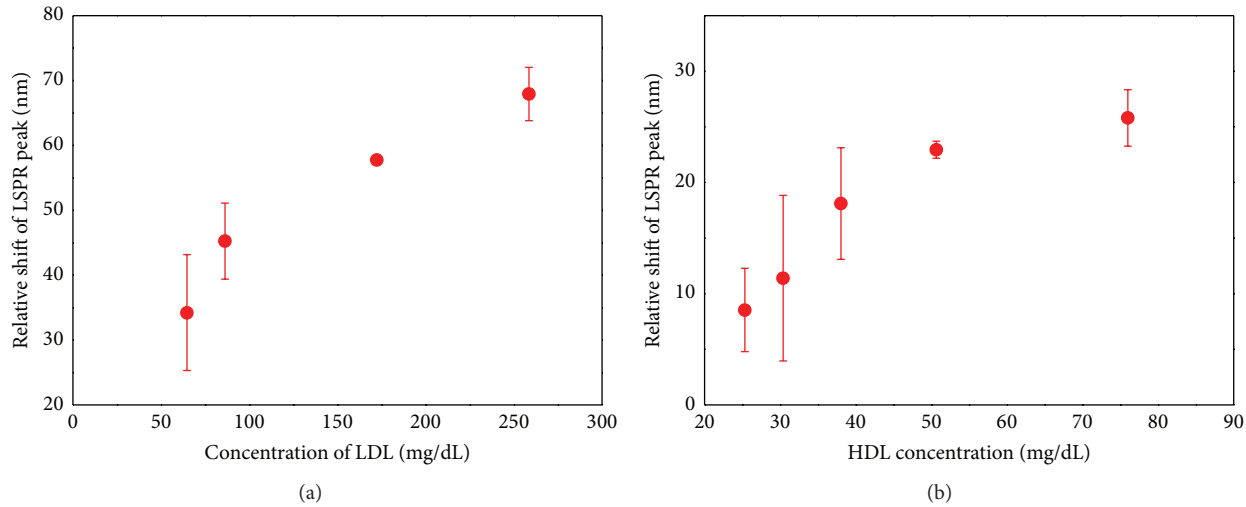



Figure 5: Relative shifts of LSPR peak corresponding with reacting with the varying concentration of biomarkers: LDL (a) and HDL (b).

the lipoprotein which has been shown to be able to prevent and reverse arteriosclerosis [16]. So, the level of HDL in blood should be higher than the normal concentration level. Therefore, as LDL and HDL are the import biomarkers to diagnose or monitor the cardiovascular diseases, the detections of them are routinely executed in the laboratory medicine. Here, the detection of LDL and HDL with the LSPR of the gold nanoparticle array substrate was demonstrated.

In the experiment, the LDL and HDL were diluted into the varying concentrations in the human serum purchased from Sigma-Aldrich Co. Then, the substrate with the antibody immobilized was incubated in the biomarker solutions with the varying concentration for 1 hour with gently stirring. After being incubated, the substrate was cleaned with the water, followed by drying under the nitrogen gas. The absorption spectrum of substrate was acquired with the equipment described above. Figure 5(a) shows the relative shift of LSPR peak as a function of the concentration of LDL. The relative shift of LSPR peak in the vertical axis of the graphs meant the amount of the shift of LSPR peak from the LSPR peak of substrate with the antibody immobilized. The concentrations of LDL prepared in the experiment were 64.6, 86.2, 172.3, and 258.5 mg/dL, respectively. In the range, the relative shift of LSPR peak increased with the increasing concentration of LDL. According to the American Heart Association, the optimal level of LDL in blood is lower than 100 mg/dL, and the value higher than 200 mg/dL is very high LDL level, corresponding to highest increased rates of cardiovascular disease event [25]. The experimental result shows that our LSPR substrate can be used to detect the LDL in the clinical available range of concentration. Figure 5(b) shows the relative shift of LSPR peak as a function of the concentration of HDL. Similar to the experiment of LDL detection, the LSPR peak was positively shifted with the

increasing concentration of HDL. In particular, the relative shift of LSPR is linearly proportional to the concentration in the low concentration range. Contrary to the LDL level, it is known that the optimal level of HDL in blood is higher than 60 mg/dL, and the values under 40 mg/dL for men and under 50 mg/dL for women are risky for heart diseases [25]. The experimental result shows the LSPR substrate with the gold nanoparticle array can be applied to detect the HDL.

In this study, we showed the applicability of the substrate with the gold nanoparticle array to detect the LDL and HDL. The LSPR substrate for the development of biosensor provides two advantages over the conventional analysis [26]. At first, the detection time is short because the step for labeling fluorophores or ELISA is omitted. Moreover, as the probe antibody used for labeling is not needed, it is cost-effective. Second, the equipment for readout is simple. As described above, the antigen-antibody binding can be simply detected by measuring the optical transmission of substrate. In reality, we constructed the equipment for measuring the transmission with the engineering technique not complicated. Our gold nanoparticle array and its fabrication method provide the simple and powerful solution to develop the LSPR-based biosensor. However, there are several hurdles to overcome to apply the LSPR substrate in the clinical area. The sample for analyzing in the clinical diagnostics is the blood, which is the complex of white and red blood cells, and the serum with several proteins. In particular, as the high concentration of red blood cells in blood affects the measurement of transmission, only the plasma should be extracted from blood before analyzing the biomarker. Recently, the microfluidics technology for extracting the plasma from the blood has been reported [27, 28]. In our opinion, the point-of-care device for biosensing LDL and HDL can be developed with the strategy of allocating the LSPR substrate in the downstream of plasma in the microfluidic device.

## 4. Conclusions

In this study, we showed the feasibility of the LSPR substrate with gold nanoparticle array to detect LDL and HDL, which are the biomarkers for diagnosing and monitoring the cardiovascular diseases. The LSPR substrate was fabricated through the lift-off process with the anodized alumina mask, and its LSPR phenomenon was observed by measuring the optical transmission of substrate. The antibodies were immobilized on the gold nanoparticle array via the chemical binding, in which the 11-MUA was used as the linker to bind the antibodies. The binding of antibodies was confirmed by observing the shift of LSPR peak of the substrate. Finally, with the LSPR substrates with the antibodies immobilized, the detection of LDL and HDL was investigated. As a result, LDL and HDL could be detected in the clinically available concentration range, respectively.

When the LSPR substrate is used for the development of biosensor, it provides the advantages such as the fast detection protocol and the cost-effectiveness. In particular, by fusing the LSPR substrate and the microfluidic device, the development of point-of-care diagnostic device based on the immunoassay can be implemented in the view of practice.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Acknowledgment

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