

Detection of tumor markers based on extinction spectra of visible light passing through gold nanoholes

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The authors demonstrate that nanometric holes in optically thin gold films on glass slides can be used as biochemical sensors based on extinction spectra of visible light passing through these holes. Binding and adsorption of biomolecules to the surfaces of gold and glass result in a redshift of the localized surface plasmon resonance peak in the extinction spectrum of the gold holes. Selective sensing of antigens such as cancer antigen 19-9 (of less than 1 pg on an ~ 0.1 mm² probing area) can be realized using this type of devices after functionalizing the gold and glass surfaces with bioreceptors. © 2007 American Institute of Physics. [DOI: 10.1063/1.2535919]

The development of optical biosensors has drawn extensive attention for applications in the areas of medical and environmental diagnostics.¹⁻³ Biosensors based on the surface plasmon resonance (SPR) phenomenon have been widely applied to monitor and characterize protein-binding events near noble metal surfaces. SPR sensing devices can be mainly categorized into two types: (i) devices based on the measurement of changes in the resonant angle induced by the SPR (angular SPR)⁴ and (ii) devices based on measurements of changes in resonant wavelength induced by the SPR (spectral SPR).⁵ Commercial SPR systems are typically operated in a total internal reflection mode using the Kretschmann configuration.⁴ Such systems have been used for highly sensitive, real-time, and label-free detection of a variety of biomolecules by functionalizing the metal surface with bioreceptors. However, one drawback of the Kretschmann configuration is the difficulty to performing high-throughput analysis. Recently, there have been reports describing SPR systems operated in a transmission configuration, which have a simpler collinear optical arrangement and smaller probing area when compared with the Kretschmann configuration. These methods are promising for array-based high-throughput sensing.^{6,7} Progress has been made in fabricating SPR sensors in a transmission configuration from nanoparticles and islands of noble metals on glass.^{6,7} Nevertheless, significant improvement and additional study are required to achieve reliable sensing devices with these techniques.

Recently, it has been shown that nanometric holes in optically thin Au films exhibit a localized surface plasmon resonance (LSPR) phenomenon.⁸ When the holes are in a random pattern (thus avoiding the long-range high-order diffractive coupling effects), the LSPR of such holes results in a resonance peak in the extinction spectrum, and the position of the resonance peak has been found to be a function of the refractive index of the adjacent medium.⁸ This suggests that a device made of nanometric Au holes provides the opportunity

for designing SPR-based sensors to monitor analytes positioned around the Au holes. Most recently, this device has been employed to monitor lipid-membrane-mediated biorecognition events.⁹

In this letter, we report the application of this type of device for the specific detection of proteins, such as tumor markers, from the changes in extinction spectra of visible light passing through the holes. We expect that this technique could potentially be developed into a simple and inexpensive platform for fabrication of label-free and array-based biosensors.

The Au nanoholes were fabricated using the colloidal lithography technique.⁸ Briefly, the glass slides (75 × 25 mm², Fisher Scientific) were first cleaned in a mixture of NH₃·H₂O and H₂O₂ (at a volume ratio of 2:1). After thoroughly rinsed with de-ionized (DI) water, the slides were functionalized with amine groups by sonication in 5% (weight) 3-aminopropyl triethoxysilane (C₉H₂₃NO₃Si, Sigma Aldrich) aqueous solution for 20 min. The slides were baked at 80 °C for 1 h afterwards. The positively charged glass slides were coated with negatively charged polystyrene nanoparticles (60 nm in diameter, Interfacial Dynamics Corp., Tualatin, OR) by dipping the slides in a mixture of nanoparticles and water, which was made by mixing 0.2 ml concentrated stock sample with 40 ml DI water. The coated slides were rinsed with DI water and placed in boiling water for 30 s before they were dried in air. Then, an ~ 20 nm thick Au film was thermally evaporated onto the glass slides. The particles were then stripped from the slides using a tape, which left nanometric holes in the Au films.

Figure 1(a) shows a scanning electron microscopy (SEM) image of the holes. These holes are about 60 nm in diameter and randomly placed on the glass slide. The slide was cleaned in a mixture of aqueous ammonia and hydrogen peroxide, and dried in air afterwards. An extinction spectrum was taken in air using a fiber spectrophotometer (Ocean Optics, HR 4000) with a wavelength resolution of 0.7 nm and, as shown in Fig. 1(b), the spectrum exhibits a resonance peak around a wavelength of 600 nm, which is consistent with previous observations.⁸ When the slide was placed in solvents of increasing refractive index the resonance peak

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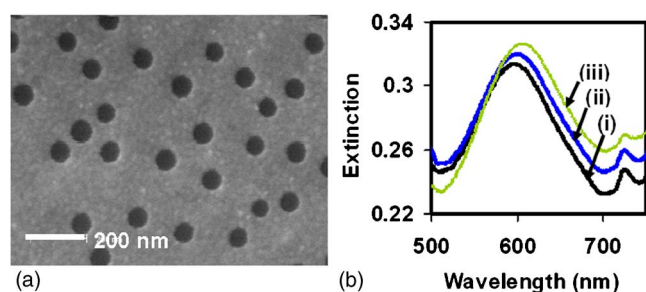


FIG. 1. (a) (Color online) SEM image of nanoholes in an optically thin Au film. The holes are ~ 60 nm in diameter, and the Au film is ~ 20 nm thick. (b) Extinction spectra of Au nanoholes on a glass slide. The spectra were taken after the surfaces of Au and glass were (i) cleaned in a mixture of $\text{NH}_3 \cdot \text{H}_2\text{O}$ and H_2O_2 , (ii) modified with SAM coatings of DT and OTS, and (iii) further modified by BSA adsorption. The resonance peak shifted about 1.5 and 7 nm to the right after steps (ii) and (iii), respectively.

shifts to longer wavelengths with almost a linear correlation at a rate of approximately 110 nm per refractive index unit.

A shift in the resonance peak position can also be induced by surface binding of material to the Au surface and/or the glass surface at the bottom of the Au holes. This was seen from the extinction spectrum after coating the Au surface and the inside glass surface with self-assembled monolayers (SAMs) formed from precursors 1-dodecanethiol¹⁰ [DT, $\text{CH}_3(\text{CH}_2)_{10}\text{CH}_2\text{SH}$] and octadecyltrichlorosilane [OTS, $\text{CH}_3(\text{CH}_2)_{17}\text{SiCl}_3$],¹¹ respectively. As a result of the SAM coatings, the resonance peak in the extinction spectrum of the Au holes shifts about 1.5 nm [Fig. 1(b)]. The surfaces of Au and glass were further modified by adsorbing bovine serum albumin (BSA). The slide was immersed in 10 mg/ml BSA in phosphate buffered saline (PBS, pH 7.4) solution for 20 min, rinsed with PBS, and dried in air. The adsorption of PBS to the Au and glass surfaces caused the resonance peak to shift about another 7 nm [Fig. 1(b)]. These results suggest that both covalent chemical modification and physical adsorption on the glass and Au surfaces may be detected by changes in the extinction spectrum of this device, which is the basis for the construction of a biosensor.

To demonstrate the use of this device as a label-free biosensor, an antibody to cancer antigen 19-9 (CA 19-9; CA 19-9 and its antibody were purchased from Fitzgerald Industries International, Inc., Concord, MA), a tumor marker involved in the diagnosis of liver cancer,¹² was immobilized to the Au and glass surfaces. The immobilization was accomplished through an elastin fusion protein consisting of an elastin domain and an antibody-binding domain, protein L.¹³ Figure 2 schematically shows the procedure for the immobilization of the antibody and the detection of the tumor marker using this device. Because it has been reported that binding or adsorption of molecules to the surfaces both inside and outside the holes may result in peak position change in the extinction spectrum,^{8,9} in our experiment the surfaces both inside and outside the holes are functionalized with antibodies to reduce the effect of nonspecific adsorption on the sensor performance. The antibody was first conjugated to the protein L domain of the fusion protein to form an elastin-protein L-antibody complex. After purification using a temperature cycle,¹³ the complex was immobilized onto the hydrophobic surfaces of SAM-coated Au and glass through hydrophobic interactions.¹⁴

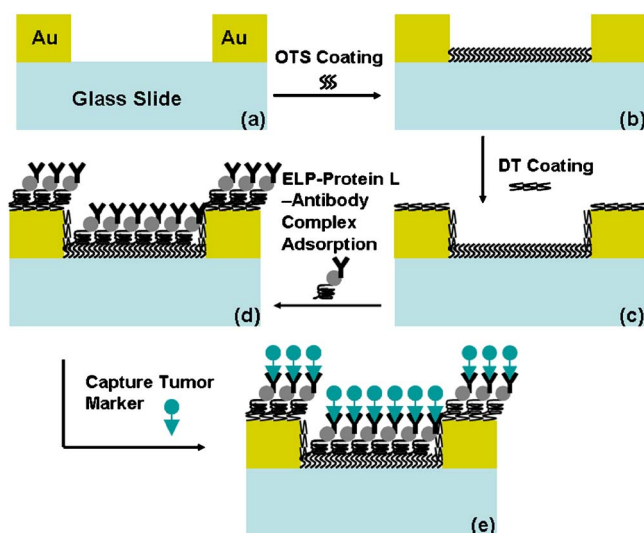


FIG. 2. (Color online) Schematic procedure to immobilize the antibody and detect the tumor marker CA 19-9. (a) Au nanoholes on a glass slide with clean Au and glass surfaces. (b) The glass surface inside the Au holes was modified with OTS SAM. (c) The Au surface was modified with DT SAM. (d) The antibody was conjugated with the ELP-protein L fusion to form a complex, which was immobilized onto hydrophobic surfaces of glass and Au through hydrophobic interactions. (e) Tumor marker CA 19-9 was captured by the antibody immobilized on the Au and glass surfaces.

As expected, the immobilization of the complex to the Au and glass surfaces resulted in a redshift of the resonance peak in the extinction spectrum. The peak shifted to the right gradually as the surface coverage of the complex increased. The peak position stabilized after about 0.6 ng/mm^2 complex was loaded to the slides and about 10 nm redshift was observed (Fig. 3). Then, BSA (10 mg/ml) was applied to the slide surface, which did not cause an obvious shift of the resonance peak (Fig. 3). This was likely because the hydrophobic surfaces of Au and glass were almost completely covered by the elastin-protein L-antibody complex.

Before exposing the device to the CA 19-9 antigen which was the targeted analytes in this experiment, the sens-

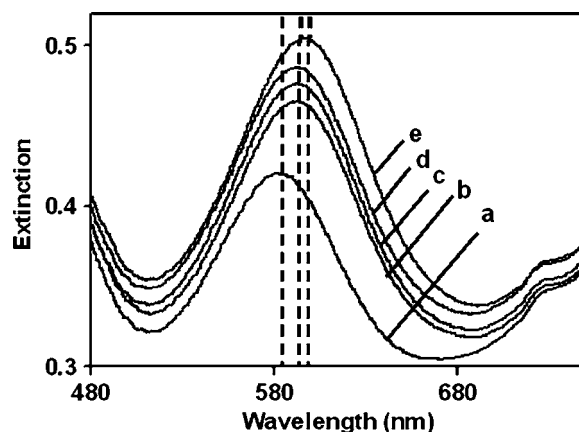


FIG. 3. Extinction spectra of Au nanoholes on a glass slide after the following steps in sequence. (a) The Au and glass surfaces were modified with DT and OTS SAM, respectively; (b) The ELP-protein L-antibody (to CA 19-9) complex was immobilized onto the Au and glass surfaces; (c) BSA was loaded to the device; (d) Tumor marker CEA was loaded to the device; (e) Tumor marker CA 19-9 was loaded to the device. The resonance peak of the spectrum shifted to the right about 10 nm after step (b) and 3 nm after step (e), respectively. No significant shift was observed after steps (c) and (d). Spectra (b), (c), (d), and (e) have been shifted along the extinction axis by 0.01, 0.02, 0.03, and 0.04 units, respectively.

ing selectivity of the device was tested by loading another antigen, tumor marker carcinoma embryonic antigen (CEA, purchased from Fitzgerald Industries International, Inc.) which had also been used in the diagnosis of liver, ovarian, and choriocarcinoma cancers, at a concentration of 1 mg/ml. As seen from Fig. 3, no obvious peak position shift was observed after exposure to CEA (the total loading on the slide is about 15 ng/mm²). We then introduced antigen CA 19-9 (at a concentration of 2 μg/ml) to the device (the total loading on the slide is about 6.4 × 10⁻³ ng/mm²), which caused the resonance peak to shift about 3 nm to the right. This experiment shows the detection specificity of the antibody-antigen binding event. Because the area illuminated by the fiber optic spectrophotometer was about 0.1 mm², we estimate that less than 1 pg (10⁻¹² g) of antigen was detected on the probing area. These results clearly demonstrate that this SPR device based on Au nano-holes can serve as a platform for biosensor development.

It should be noted that arrays of *ordered* nanometric holes in *optically thick* Au films have also been used for biochemical sensing purposes, where the LSPR peak position in transmission spectra is observed to shift when SAM coatings or BSA are covalently linked to or adsorbed onto the Au surface.¹⁴ In this approach, the peak whose position is used for sensing is caused by the *enhanced* light transmission. In contrast, the method reported here used the peak caused by *diminished* light transmission (or enhanced light extinction). Another significant difference between the technique using ordered arrays of Au holes and the current one is that, from the practical point of view, the fabrication of *ordered* arrays requires utilization of focused ion beam equipment, which is much more expensive and time consuming when compared with the colloidal lithography technique used here to fabricate the *random* arrays of holes. The sensitivity of SPR devices have been compared using $d\lambda/dc$ (nm ml/mg) for the spectral SPR and $d\theta/dc$ (ml/mg) for the angular SPR.¹⁵ However, the sensitivity of SPR devices is greatly affected by the analytes, the receptors on the surface, the schemes to immobilize the receptors on the surface, and

other experimental conditions. Therefore, further studies are required to systematically compare the sensitivity of the device presented in this letter with other types of SPR devices.

In summary, we have demonstrated that nanometric holes in optically thin Au films on glass slides can be used as biochemical sensors based on changes in the extinction spectrum of visible light passing through these holes. Binding and adsorption of biomolecules to the surfaces of Au and glass result in a redshift of the LSPR peak in the extinction spectrum of the Au holes. Selective sensing of analytes such as tumor markers can be realized using this type of device by functionalization of the Au and glass surfaces with bioreceptors such as antibodies. Because of the simplicity of the fabrication method, and the *small* probing area in the transmission configuration, we expect that this technique could be developed into a simple, robust, and inexpensive platform for fabrication of a variety of label-free and array-based biosensors.

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