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Multiplexed plasmonic sensing based on small-dimension nanohole arrays and intensity interrogation

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Abstract

We performed multiplexed sensing on nanohole array devices to simultaneously obtain information on molecular absorption, scattering, and refractive-index change, which were distinguished by using different array structures with distinct optical behavior. Up to 25 arrays were fabricated within a 65 $\mu\text{m} \times 50 \mu\text{m}$ area to provide real-time information of the local surface environment. The performance of multiplexed sensing was examined by flowing NaCl, coomassie blue, bovine serum albumin, and liposome solutions that exhibit different visible light absorption / scattering properties and different refractive indices. Experimental artifacts from light source fluctuation, sample injections, and light scattering induced by aggregates in solutions were detected by monitoring superwavelength holes or nanohole arrays with different periodicity and hole diameters.

Keywords

surface plasmons; nanoholes; subwavelength holes; SPR; liposomes

1. Introduction

Surface plasmons (SPs) are light-excited electron density oscillations confined at the metal-dielectric boundary (Pitarke et al., 2007). The incident light resonantly interacts with free electrons in the conduction band of metals and generates collective charge oscillations. The light-electron coupling process is typically performed with prisms (Kretschmann geometry), waveguides, or grating couplers, which increase the momentum of light to satisfy a special dispersion relation of surface plasmon waves (Raether, 1988). Because of the nature of collective charge oscillations, surface plasmons are very sensitive to any change on the refractive index around the interface of metallic and dielectric materials, such as the adsorption of biomolecules to the metal surface. This phenomenon has been extensively adopted to probe chemical and biochemical interactions. Receptive biomolecules are normally immobilized on

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a continuous metal film and the angular distribution, reflected spectra, or reflected intensity are measured (Homola et al., 1999). Plasmonic sensing techniques (also known as surface plasmon resonance, SPR), especially those based on prism-coupling, have been successful in performing binding affinity analysis of proteins or small molecules, and commercial instruments are available (Cooper, 2002).

The greatest attraction of plasmonic sensing is that it does not require external fluorescent labels to report the binding of a ligand to its receptor. Due to the size and the hydrophobic nature of the fluorescent compounds, the labeling step could interfere with the molecular interaction, change the binding properties, and cause serious problems like background binding and autofluorescence (Cooper, 2002). The SP phenomenon transduces chemical binding directly into an optical change, which can be measured in the far-field. In many life science fields ranging from drug discovery to systems biology, high-throughput protein microarray techniques provide new and rapidly growing platforms for analyzing a large number of proteins in parallel. Most of current high-throughput techniques are based on fluorescence readout (Sauer et al., 2005; Templin et al., 2002).

In the last decade, considerable effort has been devoted to performing high throughput assays with prism-coupled SPR devices for label-free sensing. SPR imaging provides an alternative and potentially very powerful solution for multiplexed plasmonic sensing. It is generally an intensity interrogation technique that uses a CCD camera to monitor spatial distribution of the refractive index at the metal surface in real time (Brockman et al., 2000; Campbell and Kim, 2007). Biomolecules are normally immobilized into an array by commercial robotic spotters and simultaneous monitoring at the rate of ~1000 interactions per run has been demonstrated. The feature size of protein microarray is reported as 100~200 μm for commercially available SPR microscopes, that typically requires ~ 1 nL droplets for each spot (Campbell and Kim, 2007).

Miniaturization has been a strong trend in the development of high-throughput techniques since it significantly reduces the sample volume and increases the density of assays (Sauer et al., 2005). This is particularly important for surface-sensitive techniques like SPR, as the surface to volume ratio becomes higher through miniaturization. Although prism-coupled SPR imaging has been demonstrated to be promising on multiplexed equilibrium and kinetics measurements, it encounters a major obstacle when shrinking the array feature size down to 1 μm . The utilization of prisms limits the numerical aperture (NA) and magnification of an imaging system, resulting in difficulty in achieving the spatial resolution needed for high density multiplexed sensing. The improvement of imaging optics, including using different prism geometry (Shumaker-Parry and Campbell, 2004) and adopting the configuration of total internal reflection fluorescence (TIRF) microscopy (Huang et al., 2007), is in progress to overcome this limitation.

The discovery of extraordinary optical transmission through metallic subwavelength-hole arrays (Ebbesen et al., 1998), with an efficiency that is orders of magnitude greater than that predicted by classic optical theory, has generated many exciting opportunities for plasmonic sensing. Surface plasmon polaritons (SPPs) are generally proposed to explain this phenomenon observed on periodically arrayed nanoholes: the surface nanostructure couples the incident light to SPs, which transmit through the holes to the second surface, and then re-emission from the second surface (Genet and Ebbesen, 2007). In this plasmon mode, the SP wave propagation on the metal film with nanoholes is similar to that on Kretschmann configuration. Several studies exploiting periodically arrayed nanoholes fabricated by ebeam lithography (Ji et al., 2008), soft lithography (Henzie et al., 2007; Stewart et al., 2006), and focused ion beam (Brolo et al., 2004; De Leebeeck et al., 2007; Lesuffleur et al., 2007; Stark et al., 2005; Tetz et al., 2006), have demonstrated the unique advantages when used as label-free chemical sensing

devices. Compared with conventional coupling techniques, the nanohole geometry has a much smaller probing area and can be fabricated with extremely high spatial density. The transmission optical configuration allows the use of high NA optics and provides better opportunities to design compact devices integrated with microfluidics.

Although the advantages of multiplexed sensing have been well recognized, most of the recent nanohole sensing studies used polychromatic light sources and spectrometers for single-channel measurements. In order to achieve multiplex and real-time sensing capability, we used intensity interrogation with monochromatic light instead of commonly used wavelength (spectroscopic) interrogation. The change of transmission intensity of monochromatic light is defined as the sensing signal. We have shown that 30 nanohole arrays fabricated in a $48\text{ }\mu\text{m} \times 58\text{ }\mu\text{m}$ area could provide independent sensing signals from GST/Anti-GST binding (Yang et al., 2008). Each nanohole array has a feature size less than $5\text{ }\mu\text{m}$ by $5\text{ }\mu\text{m}$, corresponding to possible $\sim 150\text{ fL}$ (femtoliter) droplets of protein solutions required for each spot. Compared with wavelength interrogation, measuring the intensity change of a single wavelength leads to a much simpler optical configuration. The spatial resolution of multiplexed measurement is essentially diffraction-limited and modern high NA optics could be used for imaging. It also enables kinetic measurements with high temporal resolution by using high-quality CCD cameras. On the other hand, since the incident light goes through the running buffer solution and the adsorbate layer before arriving the nanohole surface, any phenomenon that can change transmission intensity, including molecular absorption and Rayleigh / Mie scattering, would impose a significant disturbance on the refractive-index measurement. In this paper, we demonstrate that the advantage of multiplexed sensing is not only on increasing the throughput, namely that the simultaneous acquisition of signal from nanostructures that have resonance peaks at different wavelengths can be used to separate the contribution from light extinction (absorption + scattering) and surface refractive-index change. Small-dimension periodic nanohole arrays ($< 16\text{ }\mu\text{m}^2$) are used to achieve high array density. Several solutions that exhibit different visible light absorption / scattering properties and different refractive indices were used to characterize the properties of these arrays and optimize their detection performance.

2. Materials and methods

2.1. Nanohole array fabrication

Glass slides were first cleaned with acetone, IPA, and methanol thoroughly and blown-dry with nitrogen. A 75-nm layer of Cr was thermally evaporated ($0.8\text{ }\text{\AA}/\text{s}$) on the glass as the adhesive layer for gold and as the blocking layer of direct transmitted light. 100 nm Au was then deposited on the Cr layer with a rate of $0.5\text{ }\text{\AA}/\text{s}$. An FEI DB235 focused ion beam system with 30 kV acceleration voltage and 10 pA ion current was applied to mill nanohole arrays on the gold film (Fig. 1a). Ion image magnification was calibrated before each milling. Before the sensing test, the nanohole array films were cleaned in a hot Piranha solution (3:1 H_2SO_4 :30% H_2O_2 , $90\text{ }^\circ\text{C}$) for 3 mins. A PDMS block with a microfluidic channel (volume $\sim 3\text{ }\mu\text{L}$) was mounted on the nanohole film. A syringe pump was used to deliver solutions through this channel at a $30\text{ }\mu\text{L}/\text{min}$ flow rate. The running solution was degassed with an on-line vacuum degasser (Degasser AF, Water Corporation, MA).

2.2. Chemicals and assays

Several solutions were prepared to examine the performance of multiplexed imaging. NaCl solutions (EMD Chemicals, 99.5%) were prepared in different concentrations (0.5%, 4%, 10%, 21%) and their refractive indices in the visible range at room temperatures were calculated (1.3339, 1.3400, 1.3505, 1.3702, respectively). Bovine serum albumin (BSA), and coomassie blue were both obtained from Sigma-Aldrich.

To prepare liposomes, phosphatidylethanolamine, phosphatidylcholine, sphingomyelin, cholesterol, and phosphatidic acid (supplied in chloroform, Avanti Polar Lipids) were mixed in molar ratios of 1:1:1:1.5:0.3, respectively (Tuthill et al., 2006). Chloroform was evaporated with nitrogen and the lipid film was dried overnight under vacuum. 50 mM HEPES (pH 7.3) / 50 mM NaCl solution was used to rehydrate dried lipid at a concentration of 1 mg/ml. After thoroughly mixed by vortexing, the hydrated lipid was extruded through a 100 nm-pore-size membrane (Avanti Polar Lipids) to form liposomes.

2.3. Optical setups

Transmission spectra and real-time sensing responses were both collected using an inverted microscope with common bright-field accessories (Nikon eclipse TE300). A multispectral illumination source and an intensity imaging CCD were used instead of a polychromatic spectrometer for multiplexed spectral acquisition. Non-polarized white light from a 75W Xenon lamp was passed through a double pass grating monochromator (Jobin-Yvon SPEX 1680B) and a condenser lens. The NA of condenser lens was set as 0.34 because that provides better signal/noise (S/N) ratio than using a collimated light (Yang et al., 2008). As shown in Fig. 1a, the light coming out from the nanohole arrays was collected with a 20 $\times$ objective (NA = 0.5) and then focused on an uncooled CCD (QImage, Intensified Retiga). The spectra were corrected by the lamp transmission profile through the glass substrate. The bandpass of the monochromator was set to 1 nm for acquiring transmission spectra, and 4 nm for single-wavelength real-time measurements. An image processing program was created to extract the intensity value of the individual nanohole arrays from CCD images. For every nanohole array of interest, the reading of each CCD pixel inside a square containing the image of the nanohole array was added up as the sensing signal. The area of the square was set as 3 ~ 4 times of the nanohole array image size to reduce the error from imaging drift and defocus. The background (stray light and CCD dark current) was subtracted by the overall pixel reading in another square with the same area, where no nanohole was located.

3. Results and discussion

SEM micrographs in Fig. 1b were obtained from a 9 $\times$ 9 nanohole array perforated on the gold surface. The Au and Cr layers can be distinguished easily from the Fig. 1b insert. The FIB etching rate of Au is faster than Cr so the hole diameter on the Au layer is larger than that on the Cr layer. The thick Cr layer was used because its high absorption coefficient in the visible range can be used to suppress directly transmitted light that does not contribute to refractive-index sensing. The intensity of direct transmitted light of 100 nm Au/75 nm Cr was measured as 3.3% of 100 nm Au only.

Hole diameters (d), periodicity (P, hole-to-hole distance), and numbers of nanoholes in arrays perforated on gold substrates have been reported to have profound effect on transmission spectral features (Ebbesen et al., 1998; Thio et al., 1999; van der Molen et al., 2004; Wang et al., 2003; Yang et al., 2008). One of the attractions of multiplexed nanoplasmonic sensing is that different nanostructures, which have distinct optical behavior, can be fabricated on the same platform to provide surface information simultaneously. For the transmission configuration with intensity interrogation, the incident light goes through a thin layer (~ 250 μ m) of flowing solution before reaching the nanohole surface. Therefore, it is necessary to have a reference source providing information of light source fluctuation and absorption / scattering from the solution. The reference source needs to respond to any change in the system except the change of the local surface refractive index. In order to examine this concept, 20 nanohole arrays with different structures and 5 superwavelength holes were perforated on the same gold film and the CCD image is shown in Fig. 2a. Columns A and B contain single superwavelength holes (diameter = 1.6 μ m) milled with different hole depths. The hole

diameters for 9×9 nanohole arrays in Columns C to F are 180, 170, 160, and 150 nm, respectively. The row index 1 to 5 represents different periodicity/hole diameter ratio ranging from 1.67, 2, 2.33, 2.67, and 3, respectively. Since SPP propagation length on gold along the X-Y direction is less than $3 \mu\text{m}$ for excitation wavelengths shorter than 630 nm (Homola et al., 1999), $12 \mu\text{m}$ is a safe distance to prevent interference between each array. For real-time sensing measurements, the excitation wavelength was typically selected from which the best S/N ratio was found. Fig. 2b shows the sensing response from nanohole array D3 (the codes indicate the column and row according to Fig. 2a) upon introducing 4% NaCl. Some instrumental noise around $t = 200$ and 600 sec can be observed. In Fig. 2c, a superwavelength hole was used as a reference source to correct the signal from Array D3 since it did not respond to the change of the surface refractive index. The signal from Array D3 was divided by the intensity ratio of the superwavelength hole without using scaling factors. This resulted in a nearly complete elimination of instrumental shot noise (Fig. 2c). The concept of using nanohole arrays as the signal channel and superwavelength holes as the reference channel is similar to regular spectrometers with a two-beam configuration, where the reference beam monitors the influence from light source and solvent, providing real-time compensation. In order to explore this concept on solutions with strong molecular absorption, Coomassie blue, which has a strong absorption at 635 nm, was used to examine the interference to the nanohole arrays when the measuring wavelength was 635 nm. As can be seen in Fig. 3, both nanohole array and superwavelength hole have similar transmittance when introducing 0.2 mM coomassie blue in HEPES solution at $\text{pH} = 7.3$. The response after removing the Coomassie blue absorption is shown as Plot (c) in Fig. 3. The optical setup functioned as a visible absorption spectrometer with a very short optical path ($< 250 \mu\text{m}$) in this case.

In addition to removing instrumental noise and background absorption from running buffer solutions, this concept also can be used to monitor the scattering/absorption from biological species adsorbing on the nanohole arrays. In this case, refractive-index-insensitive nanohole arrays are more suitable than superwavelength holes since the superwavelength holes have very different local surface roughness from sensing nanohole arrays. Fig. 4a shows the transmission intensity change monitored from three nanohole arrays (D4, E3, and F2) with different resonance peak positions, when switching flow solutions from DI water to 21% NaCl. Each peak in this figure represents a red shift of a resonance peak contributed from the gold/solution interface when increasing the refractive index of flowing solutions. Peaks from other interfaces will not appear in Fig. 4a since they will not move with the refractive index. If the probing wavelength is set to 545 nm, the transmission intensity change will be positive for Array D4, negative for Array E3, and nearly zero for Array F2, when increasing the surface refractive index. Figs. 4b and 4c show the real-time response from these three arrays when changing the concentration of NaCl solutions. The direction of signal change is consistent with Fig. 4a. It is obvious that the spike at $t = 582 \text{ s}$ was from the sample injection since it was on the same direction from all three arrays. The response of Array D4 after correcting by Array F2 is shown in Fig. 4d.

Fig. 5 shows the difference in the adsorption of BSA (bovine serum albumin) and liposomes on clean gold surfaces. BSA is an abundant and stable protein that can adsorb on clean or modified gold surfaces by electrostatic interactions (Silin et al., 1997). From Fig. 5a, BSA stayed on the array surfaces very well after PBS buffer ($\text{pH} = 7.4$) washing. There was no significant signal shift from Array F2 (Fig. 5b), suggesting that the intensity change observed from Arrays D4 and E3 was purely from the refractive index change. This also implies that in a $\text{pH} = 7.4$ buffer and room temperatures, adsorbed BSA does not form aggregates that are large enough to scatter visible light (Bagger et al., 2007). On the other hand, liposomes are optically heterogeneous particles and their light scattering properties are extensively used to characterize vesicle sizes and concentrations (Edwards and Baeumner, 2006). In Figs. 5d, 5e, and 5f, 0.5 mg/ml liposome suspension was introduced to the microfluidic channel. Extrusion

of the liposomes 10 ~ 20 times though the pores is generally sufficient to obtain suspensions of predominantly unilamellar liposomes with a narrow size distribution (Bubeck et al., 2005). A big spike at $t = 88$ s was due to the noise of the illumination system. We observed a 2.1% decrement (compared with $t = 0$) in intensity from the reference array F2 after removing the liposome solution and washing by HEPES buffer at $t = 210$ s, implying a large amount of liposomes adsorbed on the surface of nanoholes and scattered part of the incident light. Unruptured / fused liposomes (Richter et al., 2006) are the causes of this large scattering loss. This is consistent with some reports describing that unmodified gold is not an ideal substrate for supported lipid bilayer formation (Keller and Kasemo, 1998; Richter et al., 2006; Sofou and Thomas, 2003). The liposome adsorption process has been of great interest since it is the first step of forming supported lipid bilayers, which have been exploited extensively to study membrane-mediated bio-recognition events (Castellana and Cremer, 2006; Sackmann, 1996). The multiplexed imaging with nanostructures of distinct optical behavior provides valuable information of the quality of lipid bilayers supported on the substrate.

It should be addressed that the optical setup used in this work was not designed for low-detection-limit measurements. The wavelength sensitivity observed from Array D4 is 350 nm/RIU and the intensity sensitivity at 545 nm is 750 %/RIU (from Fig. 4a). With 0.1% noise level, the noise-limited refractive index resolution (2σ) is around 2×10^{-4} RIU. Although this figure is not close to state-of-art prism-coupled SPR instrument (10^{-7} RIU), the noise level can be improved significantly by replacing Xenon arc lamps with high power filament lamps regulated by highly stable power supplies. Additional signal filters designed for specific noise frequencies can also substantially decrease the noise level. We believe that with an optimal optical setup and proper surface chemistry, the nanohole sensing technique based on intensity interrogations is capable of detecting biomolecules with low concentrations.

4. Conclusions

Multiplexed sensing was used on nanohole array devices based on intensity interrogation to increase throughput and improve experimental reliability. Different array structures with distinct optical behavior were fabricated on the same platform to provide information of the local surface environment simultaneously. We examined the performance of multiplexed sensing with different solutions and biomolecules. The contributions from molecular absorption, scattering, and refractive-index change can be distinguished by using different nanostructures. Experimental artifacts from light source fluctuation, sample injections, and light extinction can also be determined.

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Figure 1.
 (a) Schematic of the nanohole device and experimental setup (b) SEM micrographs of a 9×9 nanohole array

Figure 2.

(a) CCD image of superwavelength holes (diameter = 1.6 μm) and nanohole arrays at $\lambda = 540$ nm. (b) Sensing response of Array D3 ($d = 170$ nm, $P = 340$ nm) toward 4% NaCl solutions. Measuring wavelength = 630 nm (c) Corrected sensing response from Fig. 4b using a superwavelength hole (A4) as the reference source. No noise reduction or smoothing algorithms were applied to Figs. 2b and 2c.

Figure 3.

Plot (a): sensing response from a nanohole array F5 ($d = 150$ nm, $P = 350$ nm) upon introduction of $100\ \mu\text{L}$ 0.2 mM coomassie blue in the HEPES solution ($\text{pH} = 7.3$). Flow rate = $24\ \mu\text{L}/\text{min}$, excitation $\lambda = 635$ nm. Plot (b): sensing response from a superwavelength hole (diameter = $1.6\ \mu\text{m}$). Plot (c): after correcting Plot (a) with Plot (b). The responses were smoothed with 7-point box average by the acquisition software automatically.

Figure 4.

(a) Intensity change ($\Delta T/T$ in percentage) for $\Delta n = 0.0371$ (DI water to 21% NaCl) from three nanohole arrays. (D4: $d = 170$ nm, $P = 454$ nm; E3: $d = 160$ nm, $P = 373$ nm; F2: $d = 150$ nm, $P = 300$ nm) (b) Sensing response from Arrays D4 and E3 toward three different NaCl concentrations. Measurement was performed at $\lambda = 545$ nm. (c) Sensing response from Array F2. (d) Sensing response of Array D4 corrected by Array F2. No noise reduction or smoothing algorithms were applied to Figs. 4b, 4c, and 4d.

Figure 5.

(a, b, c) Sensing response from Arrays D4, E3, and F2 upon BSA adsorption. Fig. 5c is the response of Array D4 corrected by Array F2. (d, e, f) Sensing response upon liposome adsorption. Fig. 5f is the response of Array D4 corrected by Array F2. Measurements were all performed at $\lambda = 545$ nm. No noise reduction or smoothing algorithms were applied to all plots in this figure.