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High sensitivity plasmonic biosensor based on nanoimprinted quasi 3D nanosquares for cell detection

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Abstract
Quasi three-dimensional (3D) plasmonic nanostructures consisting of Au nanosquares on top of SU-8 nanopillars and Au nanoholes on the bottom were developed and fabricated using nanoimprint lithography with simultaneous thermal and UV exposure. These 3D plasmonic nanostructures were used to detect cell concentration of lung cancer A549 cells, retinal pigment epithelial (RPE) cells, and breast cancer MCF-7 cells. Nanoimprint technology has the advantage of producing high uniformity plasmonic nanostructures for such biosensors. Multiple resonance modes were observed in these quasi 3D plasmonic nanostructures. The hybrid coupling of localized surface plasmon resonances and Fabry–Perot cavity modes in the quasi 3D nanostructures resulted in high sensitivity of 496 nm/refractive index unit. The plasmonic resonance peak wavelength and sensitivity could be tuned by varying the Au thickness. Resonance peak shifts for different cells at the same concentration were distinct due to their different cell area and confluence. The cell concentration detection limit covered a large range of $5 \times 10^2$ to $1 \times 10^3$ cells ml$^{-1}$ with these new plasmonic nanostructures. They also provide a large resonance peak shift of 51 nm for as little as 0.08 cells mm$^{-2}$ of RPE cells for high sensitivity cell detection.

Keywords: localized surface plasmon resonance, Fabry–Perot cavity mode, nanoimprint lithography, cell concentration detection, quasi 3D plasmonic nanostructure

(Some figures may appear in colour only in the online journal)

1. Introduction
Measuring cell concentration, especially for cancer cells, is important for disease diagnostics. It is desirable for diagnostic techniques to be highly sensitive to allow detection of very low cell concentration. Although many methods including flow cytometry [1], optical density measurement [2], photo-electrochemical [3], electrocatalytic [4], and surface plasmon resonance [5] techniques have been developed for cell detection, they have limitations such as long detection time, insufficient sensitivity, needs for enzymatic or fluorescent labeling, and high cost related to complex set-up. Localized surface plasmon resonance (LSPR) based biosensors have the advantages of being fast, highly sensitive, label-free, and simple to set up [6, 7].

Localized surface plasmon resonances are collective oscillations of free electrons confined in metallic nanoparticles (NPs) which are excited by an incident light wave, resulting in strong enhancement of local electromagnetic (EM) fields [8–10]. Their LSPR spectra depend on the size, geometry, distribution, and material of the NPs, as well as the
refractive index (RI) of their surrounding medium (i.e. solvent and analyte cells). The cell concentration can then be distinguished through resonance peak shifts ($\Delta \lambda_{sp}$, nm) of the LSPR spectra [6, 11]. Typically, the refractive index sensitivity (RIS) of a LSPR biosensor is defined as peak shift per refractive index unit (RIU), and therefore high RIS is desirable. Previous studies showed that maximum peak shift of 6.8 nm was achieved for $1 \times 10^9$ colony forming unit (CFU) ml$^{-1}$ of *Bacillus thuringiensis* with triangular shaped silver (Ag) NP biosensor based on LSPR and antibody attachment [12]. Thrombin with a concentration of $10 \mu$g ml$^{-1}$ could be detected with a peak shift of 15 nm for antibody attached gold (Au) LSPR biosensor [13]. Integrated LSPR biosensor with microfluidic platform could detect low concentration of 124.4 cell mm$^{-2}$ human leukemia monocytic THP-1 with a peak shift of 0.18 nm [14]. The number of cells per area was defined as $\rho \times V/A$, where $\rho$ is the cell concentration in cells ml$^{-1}$, V is the sample volume, and A is the surface area of the substrate.

Our group has first demonstrated the effects of Au nanodisk size and cell type on cell concentration detection without antibody attached on nanodisks. Cell concentration in the range of $10^2$–$10^6$ cells ml$^{-1}$ was detected for retinal pigment epithelial (RPE) and MCF-7 breast cancer cells with sample volume of 120 $\mu$l over 96 mm$^2$ area. For low cell concentration of $10^4$ cells ml$^{-1}$, Au nanodisks could detect 12.5 cells mm$^{-2}$ (based on 120 $\mu$l sample volume over 96 mm$^2$ sample area) of RPE and MCF-7 cells with peak shifts of 34 and 12 nm, respectively [15]. Beside the single layer Au nanodisk LSPR biosensors, our group have also developed multiple layer Au/SiO$_2$/Au nanodisks, which provided double resonance modes and extended the cell detection range down to $10^3$ cells ml$^{-1}$, larger peak shift of 35 nm was achieved for detecting 1.25 cells mm$^{-2}$ (based on 60 $\mu$l sample volume over 48 mm$^2$ sample area) of MCF-7 cells using 120 nm diameter (dia.) Au/SiO$_2$/Au nanodisks.

To increase the sensitivity of the LSPR biosensors, NPs with different shapes such as dots [17], holes [18], rings [19], squares [20, 21], cubes [22], and asymmetrical structures [23–25] have been investigated. Most of these studies focused on in-plane, two-dimensional (2D) plasmonic nanostructures in which the plasmon mode only originates from the coupling between adjacent nanodisks with limited sensitivity and LSPR mode. A large sample volume was often needed for the measurements, otherwise the peak shift would be too small to distinguish. To overcome this limitation, we have investigated a quasi three-dimensional (3D) plasmonic nanostructure in this study, which consists of plasmonic nanostructures separated by an insulator to provide additional plasmonic modes. Previously, 100 nm deep nanohole arrays with Au nanoholes on top and isolated Au nanodisks on the bottom to generate LSPR and Bloch wave surface plasmon polaritons (BW-SPPs) modes had been demonstrated [26]. An additional Fabry–Perot cavity mode was achieved by increasing the depth of nanoholes arrays and allows coupling between two plasmonic layers and the Fabry–Perot cavity mode [27, 28]. Besides, quasi 3D nanostructures with Au-capped mushroom arrays have been fabricated using double exposure interference lithography [29], thermal imprint lithography with porous anodic alumina molds [30], and detachment process based on silk film [31]. However, these techniques have the shortcomings of being time consuming, nonuniform, and difficult to reproduce.

In this work, quasi 3D plasmonic nanostructures composed of Au nanosquares on top of polymer-based pillars and Au nanoholes on the bottom between the pillars were fabricated by nanoimprint lithography (NIL) with simultaneous thermal and UV (STU) exposure. The quasi 3D nanostructures show higher sensitivity than previously reported quasi 3D nanodisks because the electronic charges around the sharp corners of the nanosquares provide higher EM field intensity around the corners of the nanosquares compared to the nanodisks [21]. The NIL with STU exposure is fast, uniform over large area, and provides high throughput for quasi 3D nanostructures, which makes it suitable for producing biosensors with nanostructures. To the best of our knowledge, this is the first study on quasi 3D plasmonic nanosquares generated by NIL with STU exposure for cell concentration detection without antibody attachment.

The effects of Au thickness on the sensitivity of quasi 3D plasmonic nanostructures were investigated. For cell concentration detection, a previous study showed *Escherichia coli* (*E. coli*) detection in range of $10^3$ to $10^6$ CFU ml$^{-1}$ was obtained using LSPR split aptasensor to measure adenosine triphosphate (ATP) in *E. coli* cells with peak shift from 1 to 3 nm [32]. Human cervical cancer HeLa cells with 100 $\mu$l $5 \times 10^2$ cells ml$^{-1}$ concentration could be detected by folic acid-conjugated Au nanorods with peak shift of 10 nm [33]. Our previous work using single and multiple layer nanodisks showed a low cell concentration detection limit of 12.5 and 1.25 cells mm$^{-2}$ for RPE and MCF-7 cells with peak shift of 34 and 35 nm respectively. In this study, three different cell types including human lung cancer A549 cells, RPE cells, and MCF-7 cells were detected using the quasi 3D plasmonic nanostructures. We have expanded the detection limit to a range of $5 \times 10^3$ to $1 \times 10^4$ cells ml$^{-1}$ with low sample volume of 20 $\mu$l. The high cell detection sensitivity is related to the high RIS of the quasi 3D plasmonic nanostructures with a combination of nanosquares and nanoholes. Resonance peak shifts of A549, RPE, and MCF-7 cells with low cell concentration of 0.08 cells mm$^{-2}$ (based on $5 \times 10^2$ cells ml$^{-1}$ with 20 $\mu$l sample volume over 120 mm$^2$ sample area) were 18, 51, and 40 nm respectively. This is the first demonstration of such high sensitivity and large resonance peak shifts of over 10 nm for very low cell concentration of 0.08 cells mm$^{-2}$. Therefore, the square shaped quasi 3D nanostructures fabricated by NIL are useful biosensors for detecting cell concentration over a large range, especially for very low cell concentration.

2. Experiment and methods

2.1. Fabrication of quasi 3D plasmonic nanostructures

Figure 1 shows the fabrication process of quasi 3D plasmonic nanostructures. A 2-inch nanoimprint stamp with nanosquare
pillars that were 280 nm wide, 535 nm pitch, and 500 nm deep was used for the patterning of nanosquares. An Eitre 6 NIL system (Obducat Technologies AB, Sweden) with temperature, pressure, and alignment control, as well as UV (365 nm) exposure capability was used. As shown in figure 1(a), the nanoimprint stamp was first transferred to a transparent intermediate polymer stamp (IPS) film [34] through thermal NIL at 150 °C and 40 bar for 120 s. The use of this flexible IPS stamp could prevent damage to the master stamp, allow UV photons to pass through, and provide better

Figure 1. Fabrication process of quasi 3D plasmonic nanostructures. (a) Transfer patterns from master nickel stamp onto intermediate polymer stamp (IPS) soft stamp. (b) Imprint SU-8 polymer layer with simultaneous thermal and UV (STU) exposure, followed by Au thermal evaporation.

Figure 2. (a) Micrograph of IPS stamp with 280 nm wide, 535 nm pitch, and 500 nm deep square nanoholes across 3.8 cm diameter. (b) Top and (c) side views of nanopillars in SU-8 polymer fabricated by STU nanoimprint process with IPS soft stamp.
contact between the stamp and the sample. The glass transition temperature of the IPS film is 140 °C. After imprinting, the IPS stamp was demolded at 20 °C and treated with trichloro(1H, 1H, 2H, 2H-perfluorooctyl)silane (FOTS) (Sigma-Aldrich, 97%) as shown in figure 1(b). The micrograph of the IPS stamp is shown in figure 2(a).

To prepare the plasmonic biosensors, glass substrates (10 × 12 mm²) were first rinsed with deionized (DI) water and cleaned in a boiling piranha solution (3:1 H₂SO₄ : H₂O₂) for 30 min at 90 °C, followed by rinsing with acetone, isopropanol, and DI water for 20 min. After drying by N₂, the glasses were dehydrated on a hot plate at 120 °C for 5 min to remove residual moisture. Subsequently, an O₂ plasma in a reactive ion etching (RIE) system (PlasmaTherm 790, USA) with 20 sccm O₂, 80 W rf power, and 20 mTorr pressure was used to clean the glass surface and enhance adhesion between the glass and the coated polymer. The cleaned glass substrates were spin-coated with 160 nm photoresist (LOR-3A, MicroChem, MA, USA) as an adhesion promoter, followed by 490 nm SU-8 polymer (MicroChem, MA, USA) coating. The photoresist was baked at 180 °C for 5 min and the SU-8 polymer was baked at 65 and 95 °C for 2 min each. The SU-8 polymer was imprinted using the silane treated IPS soft stamp by the STU process at 90 °C, 30 bar, and 365 nm UV exposure for 60 s. Unexposed SU-8 polymer has a glass transition temperature of ~55 °C [35]. After demolding the IPS stamp at 20 °C, nanosquare shaped SU-8 pillars were formed and hard baked at 150 °C for 5 min to achieve good mechanical properties. Figures 2(b) and (c) show the micrographs of the top and side views of nanosquare pillars in SU-8 fabricated by NIL using the IPS soft stamp. Typically, 2 nm chromium (Cr) and 20 nm Au films were thermally evaporated on the top and bottom of the SU-8 nanosquare pillars to form quasi 3D plasmonic nanosquares and nanoholes.

2.2. Measurement of refractive index sensitivity

A UV-visible-near infrared spectrophotometer (PE Lambda 750, PerkinElmer, USA) was used to measure the normalized extinction spectra. The quasi 3D plasmonic nanostructures were illuminated by a normal incidence light source through a scanning spectrometer and transmission spectra over an area of 12.6 mm² confined by an optical aperture were obtained. The RIS of quasi 3D plasmonic nanostructures was measured by immersing the same sample in the following media: air (RI = 1.000), water (RI = 1.330), and certified refractive index liquids (Cargille Laboratories, NJ, USA) with RI of 1.464 and 1.640. The resonance peak shifts of different cells at different concentrations were calculated by comparing the extinction spectra with and without cells.

2.3. Cell culture and fixation

The samples with quasi 3D plasmonic nanostructures were first washed with Milli-Q water and then air-dried in a tissue culture hood before being put into 35 mm sterile tissue culture dishes. Different cell concentrations of A549, RPE, and MCF-7 cells were maintained in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, CA, USA), in a humidified incubator at 37 °C and 5% CO₂. 20 µl of this medium with cells at different cell concentrations were loaded onto the surface of the samples over an area of 120 mm² with quasi 3D plasmonic nanosquares and nanoholes. After 24 h, pipettes were used to remove the culture medium. The attached cells were washed 2 times with phosphate-buffered saline (PBS) solution, followed by adding 20 µl 4% sterilized paraformaldehyde (PFA) solution to the samples for 15 min to fix the cells. The fixed cells were stained with 20 µl 2 μg ml⁻¹ propidium iodide (Sigma, MO, USA) for 20 min, and washed with 20 µl Milli-Q water twice before being air-dried in a tissue culture hood.

2.4. Imaging using scanning electron and confocal microscopes

An environment scanning electron microscope (SEM) (XL30 ESEM-FEG, Philips Electronics, Netherlands) was used to capture the top and side view of the quasi 3D plasmonic nanostructures as shown in figures 2(b) and (c). Fluorescence signals from the cells with different cell concentrations were detected using a confocal laser microscope (TCO-SPE, Leica Microsystems, Wetzlar, Germany). ImageJ software (NIH, MD, USA) was used to compute the average cell area and confluency from the fluorescent confocal micrographs.

2.5. Numerical calculations by finite difference time domain (FDTD) method

The FDTD method (Lumerical Solutions, Vancouver, Canada) was used to simulate the extinction spectra and EM field distribution of the quasi 3D plasmonic nanostructures. Five square particles with normal incident plane wave were used for typical simulations [36]. Periodic boundary conditions were applied to both x and y directions. Perfectly matched layers were assumed for absorption of light waves to minimize reflection errors in the z direction. The size and distribution of the nanosquares and nanoholes were obtained from the SEM measurements and a mesh size of 2 nm was used. Palik’s model was used to define the dielectric function of Au [37]. The RI of the photoresist and SU-8 polymer used in the simulations were 1.54 [38] and 1.61 [39], respectively.

3. Results and discussion

3.1. Multiple resonance modes of quasi 3D plasmonic nanostructures

Figure 3(a) shows the schematic of quasi 3D plasmonic nanostructures with 2/20 nm Cr/Au nanosquares on top of the square pillars (280 nm wide, 535 nm pitch, and 500 nm deep) and on the bottom of the pillars to form nanoholes for simulations. The normalized simulated and experimental extinction spectra are shown in figure 3(b). The black curve in figure 3(b) was the simulation result, which matched well with the experimental result in red. Three main resonance
peaks were observed at 592, 826, and 1115 nm. The simulated EM field intensity distributions are shown in figures 3(c)–(e). As shown in figure 3(c), the EM field intensity distribution for P1 (592 nm) was enhanced between the bottom Au layer and the polymer underneath. This implies that P1 could be attributed to the BW-SPPs involving diffracted light propagating parallel to the bottom of the Au layer–polymer interface. This BW-SPPs could only be observed in a quasi 3D nanostructure with a high degree of spatial uniformity [28]. The peak wavelength (λ) of P1 could be approximated by [40, 41]:

$$\lambda = \frac{P}{\sqrt{i^2 + j^2}} \sqrt{\varepsilon_d \varepsilon_m}$$  \hspace{1cm} (1)

where $P$ is the lattice periodicity, $i$ and $j$ are the grating orders of the reciprocal lattice, and $\varepsilon_d$ and $\varepsilon_m$ are the wavelength dependent relative dielectric constant of medium and metal, respectively.

The EM field distribution for P2 (826 nm) was localized around the edge of Au nanoholes as shown in figure 3(d). This P2 peak was caused by the LSPR of the Au film that formed the nanoholes [42]. The EM field intensity distribution for P3 (1115 nm) was found to concentrate at the corners of the top Au nanosquares and the top of Au nanosquares. As the EM field of Fabry–Perot resonance was confined in the insulator cavity instead of the metallic surface, this enabled hybrid coupling of the Fabry–Perot mode with the LSPR modes [40]. The peak wavelength of Fabry–Perot mode ($\lambda_{FP}$) could be approximated by:

$$\lambda_{FP} = 2dn_{eff}$$  \hspace{1cm} (2)

where $d$ is the depth of cavity and $n_{eff}$ is the effective RI of the cavity consisting of air and insulator. The concentrated EM field distributions at the corners of the top Au nanosquares and the bottom Au film that formed the nanoholes were due to the LSPR of the Au nanosquares on top and nanoholes on bottom. Therefore, P3 came from the hybrid coupling of the Fabry–Perot cavity mode and LSPR modes of Au nanosquares and Au nanoholes. The combination of the Fabry–Perot cavity mode and the LSPR modes made P3 more sensitive to the surrounding medium and an ideal candidate for low cell concentration detection.

3.2. Refractive index sensitivity of quasi 3D plasmonic nanostructures with different Au thicknesses

The quasi 3D plasmonic nanostructures’ resonance modes and peaks were controlled by the thickness of the Au film and the dimensions of the pillars, nanosquares, and nanoholes. The coupling efficiency of LSPR modes of Au nanosquares and Au nanoholes could be tuned by varying the

Figure 3. (a) Schematic of quasi 3D plasmonic nanostructures with 2/20 nm Cr/Au nanosquares on SU-8 pillars and nanodisks on the bottom. Nanosquares were 280 nm wide, 535 nm pitch, and 500 nm deep. (b) Normalized extinction spectra of simulated (black) and experimental (red) responses. (c)–(e) Simulated electromagnetic field intensity distributions for P1 (592 nm), P2 (826 nm), and P3 (1115 nm).
Au thickness. The effect of Au thickness on the sensitivity of quasi 3D plasmonic nanostructures was investigated. Figure 4 shows the normalized extinction spectra and resonance positions of quasi 3D plasmonic nanostructures with different Au thicknesses of 20, 35, and 40 nm in different surrounding media. As shown in figure 4(a), the quasi 3D plasmonic nanostructures with 20 nm thick Au on top of the SU-8 nanopillars and on the bottom that formed the nanoholes had three main resonance peaks, P1, P2, and P3, located at 592, 787, and 1078 nm respectively. Different RI media including air, water, and certified RI liquids were used to measure their extinction spectra. The results show that plasmonic resonance peaks or valleys were highly sensitive to the surrounding medium. With increasing RI from 1 to 1.640, the resonance peaks red-shifted. Figure 4(b) shows the resonance peak position as a function of RI of the media. The measured sensitivity of the 787 and 1078 nm resonance peaks were 167 and 496 nm RIU\(^{-1}\) respectively.

The full width at half maximum (FWHM) of the resonance peaks increased with increasing RI of the surrounding medium. This is because the speed of light will reduce when it propagates in a higher RI medium, resulting in energy loss and increased radiative and non-radiative damping, which contribute to the increased FWHM of the resonance peaks [43]. The normalized extinction spectra of 35 nm thick Au plasmonic nanostructures are shown in figure 4(c). Compared with 20 nm Au thick devices, the resonance peaks were blue-shifted. This result showed that the plasmon resonance wavelength of quasi 3D nanostructures could be tuned by changing the Au thickness, similar to the trend of peak shifts in previous study [44]. The sensitivity of the 751 and 1068 nm resonance peaks for the quasi 3D plasmonic nanostructures with 35 nm thick Au were 76 and 272 nm RIU\(^{-1}\) respectively. Figure 4(e) shows the normalized extinction spectra of quasi 3D plasmonic nanostructures with 40 nm thick Au. Compared with 20 and 35 nm thick Au nanostructures, the resonance positions were further blue-shifted. The sensitivity of the 751 and 1064 nm peaks were 104 and 162 nm RIU\(^{-1}\) respectively, as shown in figure 4(f). All these results show that resonance peaks at longer wavelength provide higher sensitivity. The effect of Au thickness on the sensitivity of quasi 3D plasmonic nanostructures is shown in figure 4(g). 20 nm thick Au nanostructures showed the highest sensitivity at 496 nm RIU\(^{-1}\) and the sensitivity decreased with increasing Au thickness from 20 to 40 nm. This could be related to the reduced effective depth of the Fabry–Perot cavity with increasing Au thickness and the blue-shifted Fabry–Perot resonance peak [40]. Since the quasi 3D plasmonic nanostructures with 20 nm thick Au showed the highest sensitivity and multiple resonance peaks, all the subsequent cell concentration detections were carried out with 20 nm thick Au. The detection sensitivity also depended on the depth of the quasi 3D plasmonic nanostructures. Maximum sensitivity will be obtained when there is coherent interference due to the hybrid coupling of the Fabry–Perot cavity mode with the LSPR mode. With the resonance peak at the longer wavelength of 1078 nm, where the refractive index sensitivity was the highest, a cavity depth of 500 nm satisfied the condition for constructive interference as indicated in equation (2). The desired depth of the plasmonic nanostructures to provide the maximum detection sensitivity should be adjusted based on the material and size of the nanostructures, as well as the resonance peak wavelength.

### 3.3. Resonance peak shift as a function of cell concentration

Quasi 3D plasmonic nanostructures showed multiple resonance peaks which could provide more information for cell detection. Our design of the nanosquares on top of the SU-8 pillars and nanoholes on the bottom provided hybrid coupling of the LSPR and Fabry–Perot cavity modes with a high sensitivity of 496 nm RIU\(^{-1}\). To investigate the performance of these quasi 3D plasmonic nanostructures as a biosensor, three different cell types—A549, RPE, and MCF-7 cells—were studied. These cells have different cell area and confluence, and they have different responses to the quasi 3D plasmonic biosensor. Confluence is defined as the percentage of areas covered by the cells. The average cell area was calculated using the imageJ software. At lower cell concentration, cells were more spread out with large average cell area. With higher cell concentration, due to cell–cell contact with cells in close proximity of one another, the average cell area became smaller with less spreading.

#### 3.3.1. Lung cancer A549 cells

Figure 5(a) shows the fluorescent confocal micrographs of lung cancer A549 cells immobilized on the quasi 3D plasmonic nanostructures with cell concentration varying from 5 × 10\(^2\) to 1 × 10\(^7\) cells ml\(^{-1}\). As shown in figure 6, the A549 cell area was 145 μm\(^2\) at cell concentration of 5 × 10\(^2\) cells ml\(^{-1}\), and increased slightly to 153 μm\(^2\) at 5 × 10\(^6\) cells ml\(^{-1}\). The cells remained as a monolayer on the surface of the nanostructures, while the confluency increased from 0.4% to 79%. As A549 cell concentration was increased further to 1 × 10\(^7\) cells ml\(^{-1}\), the confluency reached 100% and the cells started to form double layers. The cell area reduced to 67 μm\(^2\) as there was less room for cells to spread when the cells reached 100% confluency [45]. Figures 7(a) and (b) show the extinction spectra and resonance peak shifts as a function of A549 cell concentration. Three resonance peaks were found at 591, 783, and 1077 nm in air without any cells. The normalized extinction spectra were red shifted with increased cell concentration, and the 1077 nm resonance peak showed the largest resonance peak shifts due to its higher sensitivity. The shifts of the 783 and 1077 nm peaks with cell concentration of 5 × 10\(^2\) cells ml\(^{-1}\) were 10 and 18 nm, respectively. With cell concentration increased to 1 × 10\(^7\) cells ml\(^{-1}\), the 783 nm peak shifted 55 nm and the 1077 nm peak shifted 287 nm. The shift of the 1077 nm resonance peak was about five times larger than the 783 nm peak as shown in figure 7(b). However, as the cell concentration increased to 2 × 10\(^7\) cells ml\(^{-1}\), its resonance peak shift did not shift further, implying that the detection range for A549 cells is 5 × 10\(^2\) to...
Figure 4. Extinction spectra of quasi 3D plasmonic nanostructures with Au thicknesses of (a) 20 nm, (c) 35 nm, and (e) 40 nm. (b), (d), and (f) Resonance peak position as a function of surrounding media for corresponding Au thicknesses. (g) Refractive index sensitivity as function of Au thickness.
1 × 10^7 cells ml^-1. The resonance peak shift was the response of effective RI changes due to the presence of cells on the plasmonic nanostructures. The amount of resonance peak shift (Δλ_R) could be described as [46]:

\[
\Delta \lambda_R = m(n_{\text{adsorbate}} - n_{\text{medium}}) \left(1 - e^{-\frac{2z}{l_d}}\right)
\]

where \( m \) is the bulk RI sensitivity, \( n_{\text{adsorbate}} \) and \( n_{\text{medium}} \) are the RI of the adsorbate and the surrounding medium respectively, \( t \) is the effective adsorbate thickness, and \( l_d \) is the decay length. The \( l_d \) of 2D nanostructures is ∼52 nm, and it depends on the size and shape of the Au NPs [47]. This small \( l_d \) of 2D nanostructures limits their performance in cell concentration biosensors because the EM field decays exponentially with \( l_d \). However, the hybrid LSPR and Fabry–Perot cavity modes obtained in our design could effectively increase \( l_d \) to over 100 nm [28] and make the quasi 3D plasmonic nanostructures more efficient in detecting cells at the low concentration of 0.08 cells ml^-1.

Figure 5. Fluorescent confocal micrographs of (a) lung cancer A549 cells, (b) retinal pigment epithelial RPE cells, and (c) breast cancer MCF-7 cells on quasi 3D plasmonic nanostructures at different concentrations. A549 cell concentrations were 5 × 10^2, 5 × 10^5, 5 × 10^6, and 1 × 10^7 cells ml^-1. RPE and MCF-7 cell concentrations were up to 5 × 10^6 cells ml^-1.

Figure 6. Cell area and confluency of A549, RPE, MCF-7 cells at different concentrations.
3.3.2. Retinal pigment epithelium cells. Figure 5(b) shows the fluorescent confocal micrographs of RPE cells immobilized on quasi 3D plasmonic nanostructures. As shown in figure 6, as RPE cell concentration increased from $5 \times 10^2$ to $5 \times 10^6$ cells ml$^{-1}$, the cell area was first increased from 217 to 374 $\mu$m$^2$, then dropped to 134 $\mu$m$^2$. The confluence increased from 0.6% to 100% and double layers were formed for cell concentration of $5 \times 10^6$ cells ml$^{-1}$. Compared with A549 cells, RPE cells contain more proteins and have larger cell area, indicating that the resonance peak shifts of RPE cells should be larger than A549 cells because the effective RI is mainly determined by the cell membrane protein concentration [48]. As shown in figures 7(c) and (d), the peak shifts of the 783 and 1077 nm resonance peaks at cell concentration of $5 \times 10^2$ cells ml$^{-1}$ were 50 and 51 nm respectively. For A549 cells, the peak shift at $5 \times 10^2$ cells ml$^{-1}$ for the 1077 nm peak was only 18 nm. With RPE cell concentration up to $5 \times 10^6$ cells ml$^{-1}$, the 1077 nm peak showed larger resonance peak shift of 295 nm compared to the 783 nm peak shift of 61 nm due to its higher sensitivity. However, when the RPE cell concentration reached $5 \times 10^6$ cells ml$^{-1}$, double layers were formed and its resonance peak shift did not change further, implying that the detection range for RPE cells is $5 \times 10^2$ to $5 \times 10^6$ cells ml$^{-1}$. Compared to
our previous work, a larger peak shift of 51 nm was obtained for the lower RPE cell concentration of 0.08 cells mm$^{-2}$ while the sample volume was reduced from 120 to 20 μl [15]. With the new quasi 3D plasmonic nanostructures, the biosensor provides higher sensitivity and larger resonance peak shift for low cell concentration detection due to its high RIS.

### 3.3.3. Breast cancer MCF-7 cells

The fluorescent confocal micrographs of breast cancer MCF-7 cells with different densities immobilized on quasi 3D plasmonic nanostructures are shown in figure 5(c). As shown in figure 6, the MCF-7 cell area with concentration of $5 \times 10^5$ cells ml$^{-1}$ was 242 μm$^2$ cell area and the confluency was 0.7%. With cell concentration up to $5 \times 10^6$ cells ml$^{-1}$, MCF-7 cells were still monolayer with 236 μm$^2$ cell area and 82.9% confluency. When the MCF-7 cells formed double layer with concentration of $5 \times 10^6$ cells ml$^{-1}$, their cell area decreased to 106 μm$^2$ and the confluency was 100%. Figure 7(e) shows the normalized extinction spectra of the quasi 3D plasmonic nanostructures with and without MCF-7 cells. A low cell concentration down to $5 \times 10^2$ cells ml$^{-1}$ could be detected due to the high sensitivity of these quasi 3D plasmonic nanostructures and the resonance peak shifts of the 783 and 1077 nm peaks were 15 and 40 nm respectively. With cell concentration up to $5 \times 10^6$ cells ml$^{-1}$, the resonance peaks were red shifted and the 1077 nm peak showed larger resonance peak shift of 280 nm compared to the 783 nm peak due to its higher sensitivity. The peak shift of MCF-7 cells was smaller than RPE cells with the same concentration due to their cell area and confluency because a smaller cell area and confluency will result in a smaller adsorbate thickness $t$, therefore a smaller peak shift for the MCF-7 cells. In our previous work, detection limits of 12.5 and 1.25 cells mm$^{-2}$ MCF-7 cells were achieved by single and multiple layer nanodisks with resonance peak shift of 12 and 35 nm, respectively [15, 16]. Using the quasi 3D plasmonic nanostructures with a combination of nanosquares and nanoholes, the detection limit was further extended down to 0.08 cells mm$^{-2}$ with a large resonance peak shift of 40 nm due to high RIS.

Figure 8 shows the comparison of resonance peak shifts for A549, RPE, and MCF-7 cells at different cell concentrations. The results show that resonance peak shifts of RPE and MCF-7 cells were larger than A549 cells due to their larger cell area and confluency, as shown in figure 6. The RI of cells is mainly determined by their nucleus and membrane protein concentration. Different cells show different RI, e.g. cancer cells have larger RI than normal cells because they contain more proteins. For the quasi 3D plasmonic nanostructure with nanosquares on top of the nanopillars and nanoholes on the bottom, they have the advantage of the additional hybrid coupling of the LSPR and Fabry–Perot cavity modes. These result in higher sensitivity, longer decay length, and larger resonance peak shift, which enhance the detection limit of cell concentration down to $5 \times 10^2$ cells ml$^{-1}$ with low sample volume of 20 μl and a low cell concentration of only 0.08 cells mm$^{-2}$.

### 4. Conclusions

Quasi 3D plasmonic nanostructures with Au nanosquares on top of the SU-8 nanopillars and Au nanoholes on the bottom were fabricated by NIL with the STU process. With the Au thickness increased from 20 to 40 nm, the plasmonic resonance wavelength was blue shifted and the RIS was reduced. These plasmonic nanostructures show multiple resonance modes and high sensitivity of 496 nm RIU$^{-1}$ with 20 nm thick Au due to the hybrid coupling of the LSPR and Fabry–Perot cavity modes. For resonance peaks at longer wavelength, the peak shifts of 20 μl of A549, RPE, and MCF-7 cells at $5 \times 10^6$ cells ml$^{-1}$, which represented a very low cell concentration of 0.08 cells mm$^{-2}$, were 18, 51, and 40 nm respectively. While the maximum cell detection concentration for RPE and MCF-7 cells was $5 \times 10^6$ cells ml$^{-1}$, it was $1 \times 10^7$ cells ml$^{-1}$ for A549 cells due to their smaller cell area and lower confluency. RPE and MCF-7 cells have larger surface area than A549 cells, and this resulted in larger resonance peak shifts. With multiple resonance peaks and different spectral responses, the quasi 3D plasmonic nanostructures could be used to detect A549, RPE, and MCF-7 cells with high sensitivity.

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