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Plasmonic Nanohole Arrays for Label-Free Kinetic Biosensing in a Lipid Membrane Environment

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Abstract— **We integrate periodic nanohole arrays in a thin gold film with lipid membranes in a microfluidic channel. Surface plasmon-enhanced light transmission through the periodic nanohole arrays enables real-time label-free sensing of molecular binding on the lipid membrane surface. This membrane biosensor can potentially act as a natural platform for studying binding kinetics of proteins with their binding partners anchored in the lipid membrane. We also present the concept of using nanopore arrays for kinetic assays of transmembrane proteins in a free-standing lipid membrane.**

I. Introduction

Cell membranes and their associated proteins are crucial to the fundamental processes of life [1]. Indeed, more than 50% of currently marketed small drug molecules target membrane proteins [2]. For new drug development, therefore, it is crucial to understand the functional mechanisms of membrane proteins and to screen their ligands for high affinity and specificity.

Surface plasmon resonance (SPR) measurement techniques, *e.g.* as with BIAcore™, are currently the gold standard for measuring molecular binding kinetics and affinity [3]-[5]. In SPR instruments, molecular binding onto the gold surface changes the local refractive index, which, in turn, modulates the properties of surface plasmon (SP) waves—hybrid electromagnetic-plasma waves propagating right at the interface of a metallic film and a dielectric medium. The electromagnetic energy is confined to within ~200 nm of the gold interface, making this measurement technique more sensitive to surface-related processes than bulk measurement techniques. Existing SPR instruments that use a flat gold film as a substrate, however, are not suitable for working with lipid membranes or membrane proteins, since lipid layers supported by and in contact with the substrate cannot naturally incorporate transmembrane proteins. While tethered lipid bilayers can partially overcome this challenge by lifting the membrane a few nanometers above the substrate [6], it requires difficult chemistry and the membrane is still only accessible from one side.

While previous work has mainly used nanohole arrays as mechanical supports [7], [8] for lipid membranes, if these nanohole arrays are made in a thin metallic film, they can concurrently act as a SPR sensor due to the plasmon-mediated extraordinary optical transmission (EOT) effect [9]. When light is incident on a periodic array of nanoholes in a thin (~200 nm) gold film, the array itself scatters the incident light, generating SP waves on the gold film surface. Following an initial demonstration of using the EOT effect for biosensing [10], several groups have shown the potential of this platform for label-free kinetic SPR sensing [10]-[18]. We have also demonstrated the possibility of integrating nanohole array biosensors with optical imaging techniques to achieve high-throughput SPR imaging [14], [18]. Also, our recent developments have shown that using this technique can lead to a sub-micron resolution SPR imaging biosensor [16]. Although previous work [17] has demonstrated a supported phospholipid bilayer formed at the bottom of single nanoholes in a 20 nm-thick gold film on a glass substrate for localized surface plasmon biosensing, EOT sensing involving periodic nanohole arrays in gold films has not yet been developed for lipid membrane studies.

In this paper, we present lipid membrane formation and subsequent real-time sensing measurements from nanohole arrays in a thin gold film on a glass substrate. In the last section of this paper, we also present the design and preliminary device fabrication results of a substrate for studying free-standing lipid membranes over nanoholes milled through a suspended gold film.

II. Experimental Results

A. Lipid membrane formation on nanohole arrays

In Fig. 1(a), we present a scanning electron microscope (SEM) image of a nanohole array fabricated using focused ion beam (FIB) milling through a 200 nm thick gold film. The diameter of the nanoholes is 200 nm.

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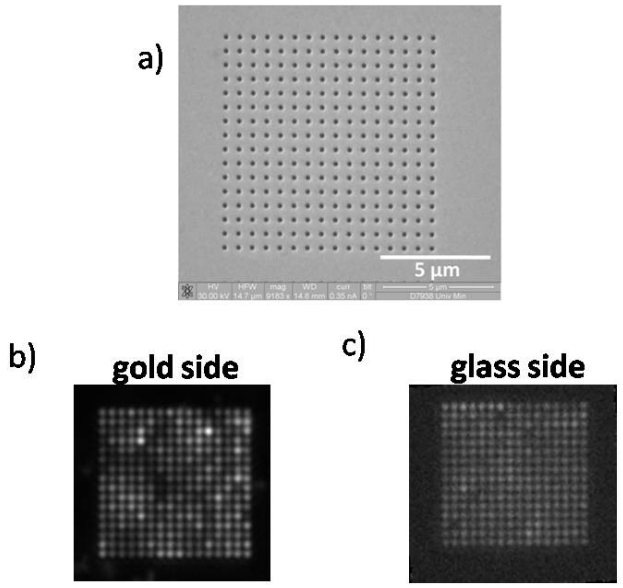

Figure 1: a) Scanning Electron Microscope image of a nanohole array with a periodicity of 600 nm and nanohole diameter of 200 nm. b) and c) are fluorescence images from an 800 nm periodicity array, from the gold and the glass side of the film, after phospholipid membrane formation. Lipids were formed on the gold surface and inside each nanohole.

Fig. 1(b) and 1(c) show the fluorescence images from both sides of the gold film of a nanohole array with a periodicity of 800 nm. Fluorescently labeled phospholipids were formed on the gold side of the nanohole array substrate. To form the lipid layer on the surface, a 10 µl lipid solution (50 µg/ml, dissolved in chloroform) was dropped onto the nanohole array substrate and then dried in a petri-dish. To remove residual solvent from the surface, the substrate was dried in vacuum. Fluorescent images were acquired using a Nikon Eclipse microscope with a 50× objective and a CCD camera. On the images, an array of 16×16 bright fluorescent spots can be observed corresponding to the array of 16×16 nanoholes. On the gold surface in between the nanoholes and around the array, the fluorescence signal is very low, suggesting that there is no significant lipid aggregate in this area. The brighter spots at the nanoholes can be observed from both sides of the gold film.

B. Measurement of biomolecular interactions in a lipid membrane environment

To demonstrate the capability of our platform to perform measurements in a lipid membrane environment, biotinylated membranes were formed on the nanohole array substrate as described in section A. A microfluidic chip with a single channel (7 mm long × 3.5 mm wide × 50 µm high) was attached over the gold film to deliver a streptavidin (SA) solution that binds specifically to the biotin.

Fig. 2 shows transmission spectra through a nanohole array before and after the lipid membrane formation, and after the streptavidin solution injection and washing with PBS buffer. Nanohole arrays present several transmission peaks mediated by SPs as described in the introduction.

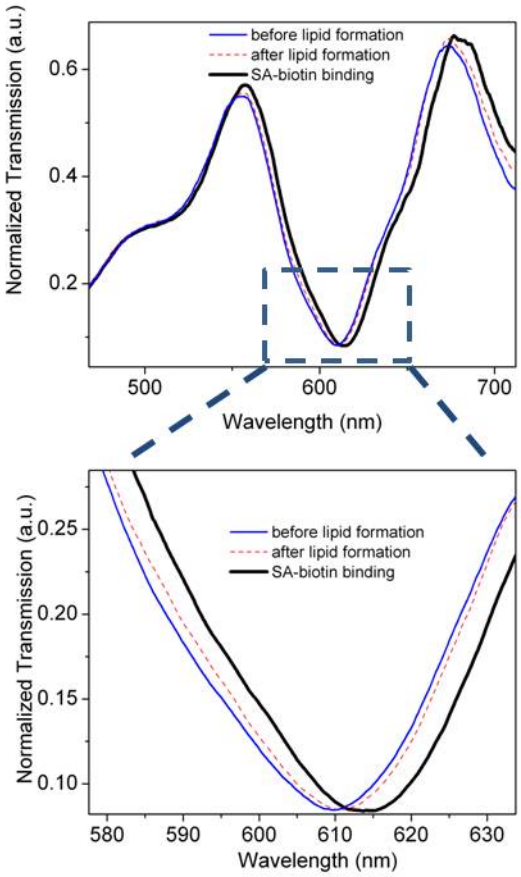

Figure 2: a) Transmission spectra before and after lipid membrane formation, and after streptavidin binding to biotinylated lipid. The nanohole array had a periodicity of 390 nm.

When lipid layers are formed on the nanohole array surface, the subsequent refractive index change at the surface induces a 1-2 nm shift of the transmission peaks. Then, when a 300 nM streptavidin solution is injected, an intermediate shift is recorded (not shown here). This shift is due to both the specific binding of the streptavidin with biotin-labeled lipid and to the bulk refractive index change of the surrounding buffer due to the presence of streptavidin. After washing with the buffer, the total spectral shift is reduced, but a 4 nm shift remains due to streptavidin-biotin specific binding (shown in figure 2). We also ran an experiment using phospholipids with no biotin labeling as a negative control (data not shown here). The resulting shift after injecting streptavidin was less than 1 nm, confirming that we were measuring specific streptavidin-biotin binding.

Fig. 3 presents the recorded transmitted intensity through the nanohole arrays using a HeNe laser at 633 nm and a cooled CCD for measuring several nanohole arrays at once in a real-time imaging manner as described in our previous publications [14], [16], [18]. Figure 3 shows the results obtained for two array periodicities (390 nm and 440 nm) and for biotinylated and non-biotinylated lipid membranes.

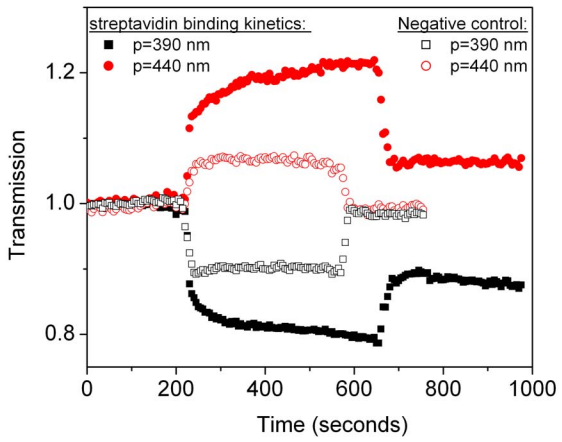

Figure 3: Kinetic measurements of the specific binding of streptavidin to biotin performed in a lipid membrane environment from arrays with two different periodicities: 390 nm and 440 nm. Negative controls were performed with lipid membranes without biotin.

There is either an increasing or decreasing intensity depending on the periodicity due to the slope of the transmission resonance peaks at the wavelength used [14], [16], [18]. If biotinylated lipids are used, an exponentially decaying profile is recorded after injection of the streptavidin solution, whereas only a sudden shift is measured from the non-biotinylated lipid membrane. This sudden shift comes from the bulk refractive index change due to the presence of streptavidin in the buffer.

Fig. 4 shows finite-difference time-domain (FDTD) calculations of the transmission spectra through the nanohole arrays for several different configurations involving the presence of a membrane above the nanoholes. The gold sensing surface is covered by a 5 nm thick film, whose refractive index is varied to simulate the formation of a lipid membrane or the presence of other molecules, both over the nanohole region, or just on the surface of the gold itself. The entire system is incubated in a buffer with a refractive index of 1.3. Initially, the thin film has a refractive index equal to that of the buffer, giving a baseline spectrum. When the refractive index of the film is changed, either over the nanohole region or over the gold surface region, the surface plasmon mediated transmission spectrum shifts. The spectrum red-shifts 5 nm when the refractive index of the entire film increases to 1.4. This calculated red-shift is comparable to that due to a thin monolayer of biomolecules, as reported in our previous work, substantiating the values of the various parameters used in the simulations. Then, if the refractive index of only the film directly on the gold surface increases to 1.5, an additional 2 nm shift is measured. Importantly, a similar shift is measured if only the part of the film suspended above the nanoholes (membrane) increases to 1.5. These FDTD calculations show that we can expect similar sensitivity when performing nanohole SPR biosensing from a suspended lipid membrane.

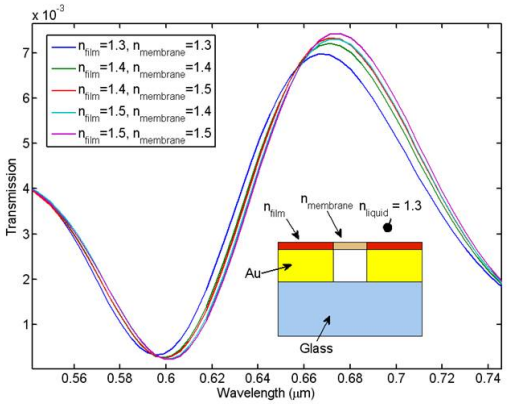

Figure 4: Numerical calculations (FDTD) involving several different configurations of a thin membrane covering the nanohole array.

C. Concept of the free-standing membrane nanopore SPR sensor

The concept of a free-standing lipid membrane nanohole array sensor is presented in fig. 5. The main advantage of such a setup is to access the lipid membrane from both sides with analyte or to insert transmembrane proteins. When the lipid membranes are formed on a gold surface or on nanohole arrays in gold films deposited on glass, the lipid membranes are accessible only from one side, limiting its utility for studying transmembrane signaling processes.

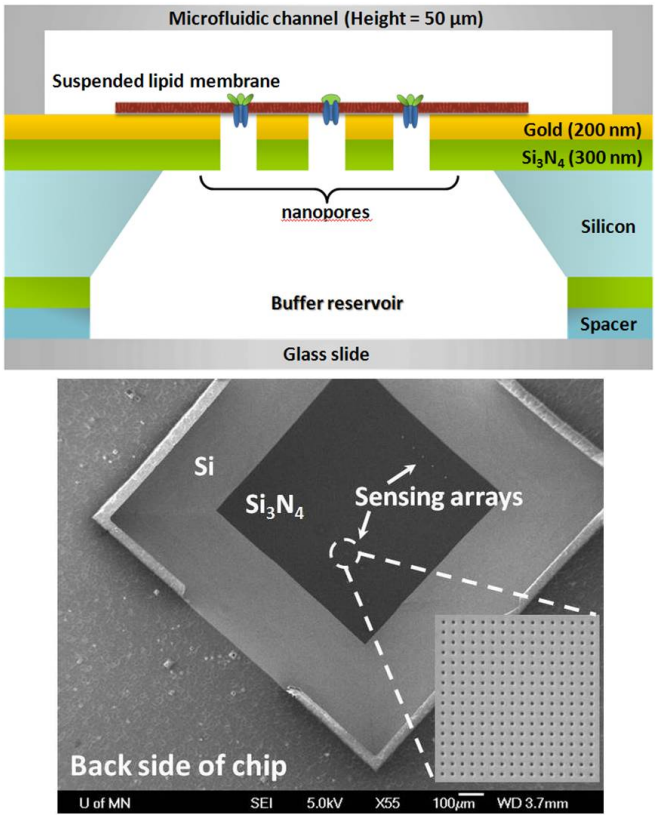

Figure 5: Schematic of the free-standing lipid membrane nanohole array sensor. SEM picture shows the suspended gold/Si_3N_4 film fabricating using KOH anisotropic etching.

Furthermore, transmembrane proteins in direct contact with a solid substrate often lose their functionality or denature, necessitating a new in vitro sensing platform that can present these important drug targets in an environment that can mimic biological cell membranes. To resolve this limitation, we propose to fabricate the nanohole arrays in a suspended gold film, making the lipid membranes formed over the nanoholes accessible from microfluidic reservoirs on both sides of the gold film. To fabricate such a platform, we use a suspended nitride film on which a thin gold film is evaporated. Fig. 5 presents a schematic of the proposed device with an SEM image of the fabricated samples integrated in a microfluidic platform. Tests with this new platform are currently underway.

III. CONCLUSION

We have demonstrated that the nanohole array sensor can work efficiently in a lipid membrane environment keeping the same advantages we outlined in our previous papers: high throughput, a simple optical configuration, a small footprint, and high resolution. Further work consists in developing a free-standing gold film and demonstrating the feasibility of the nanohole array sensor for studying the interactions of membrane proteins in a real-time, label-free manner. To achieve this goal, more sophisticated surface modification techniques will be used. Interactions of membrane proteins functioning specifically in the free-standing membrane region will be investigated.

IV. ACKNOWLEDGEMENT

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