Multifunctional-layered materials for creating membrane-restricted nanodomains and nanoscale imaging

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Experimental platform that allows precise spatial positioning of biomolecules with an exquisite control at nanometer length scales is a valuable tool to study the molecular mechanisms of membrane bound signaling. Using micromachined thin film gold (Au) in layered architecture, it is possible to add both optical and biochemical functionalities in *in vitro*. Towards this goal, here, I show that docking of complementary DNA tethered giant phospholiposomes on Au surface can create membrane-restricted nanodomains. These nanodomains are critical features to dissect molecular choreography of membrane signaling complexes. The excited surface plasmon resonance modes of Au allow label-free imaging at diffraction-limited resolution of stably docked DNA tethered phospholiposomes, and lipid-detergent bicelle structures. Such multifunctional building block enables realizing rigorously controlled *in vitro* set-up to model membrane anchored biological signaling, besides serving as an optical tool for nanoscale imaging. © 2016 AIP Publishing LLC.
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Many important biological processes, such as cholesterol and lipid transfer,¹ cellular signaling interactions at ER (Endoplasmic Reticulum) and PM (Plasma Membrane)² junctions, ER and Mitochondria³ junctions, viral entry into host cells,⁴ and vesicle fusion at post synaptic neurons,⁵ involve molecular interaction at restricted junctions between two membranes. Conventional biochemical method of solubilising membrane proteins using detergents cannot fully restore the functional detergent-micelle-protein complex of most membrane proteins involved in physiological signaling. On the other hand, commonly employed biological approach of over expressing proteins of interest result in cellular stress perturbing the physiological environment. Consequently, there is no robust experimental tool to conduct mechanistic studies by varying key experimental variables (concentration of salts and proteins, lipid composition, etc.) at native protein expression levels. Such a tool is valuable for biologists to re-engineer biological signals within membrane-restricted nanodomains. For instance, Ca²⁺ signaling in T-cells and mast cells is strongly dictated by the gating of PM resident Ca²⁺ selective channel protein, ORAI1 by activated ER resident Ca²⁺ sensor, STIM1. Many important mechanisms that modulate STIM1-ORAI1 interaction within ER-PM junction of ~7–30 nm have remain incomprehensible,⁶ but their implications on Ca²⁺ signaling in various cells⁷ have a profound biological significance. Realizing tightly regulated membrane-restricted nanoscale domains with spatial control of reconstituted (or membrane localized) functional proteins demand engineered multifunctional systems at the interface of biology and materials physics. A scalable platform comprising an array of membrane-restricted nanodomains will allow visualizing key events under rigorously controlled environment that is technically not possible in cells. Furthermore, such an engineered *in vitro* setup allows to

confirm and amend several hypotheses on cellular signaling, which are now bolstered by cellular and biochemical assays at high expression levels of proteins.

Technologies borrowed from traditional semiconductor processes will immensely benefit biology to realise scalable *in vitro* platforms to model nanoscale environment between membrane junctions. A range of different classes of materials with varied functionalities inducted into semiconductor processes over the last two decades manifest the opportunities to design multifunctional tools for studying the membrane signalling complexes. This paper focuses on the application of micro/nanofabrication technology for creation of membrane-restricted nanodomains and sub-micron label-free imaging of macromolecular structures at diffraction-limited resolution.

Docking phospholiposomes on a planar surface using molecular tethers is an attractive method for modeling membrane-restricted nanodomains. A variant of such a system⁸ was already engineered but lacks imaging capability at diffraction-limited resolution. This work demonstrates the potential of micromachined thin film gold (Au) evaporated on a glass slide to meet optical and biochemical requirements. This serves as a template for docking 2D array of liposomes besides being an evanescent light source at nanoscales under surface plasmon resonance (SPR) effect. Figure S1 shows a schematic of phospholiposome docked on thin film Au surface using molecular tethers. Propagation of evanescent light wave due to SPR modes of Au allows imaging membrane-restricted nanodomains at diffraction-limited resolution.

Photoluminescence of Au nanodots/rods due to SPR is an established phenomenon dealt by several earlier works.⁹ However, imaging of sub-micron features at diffraction-limited resolution using planar thin film Au (~10 nm thick) was not known before. Figures 1(a)–1(d) show optical micrographs (Zeiss Axiovert 200 M) of different conditions,

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FIG. 1. Positive and negative controls demonstrating the conditions for docking of unilamellar phospholiposomes on a Ti/Au glass slide. Optical micrographs shown above are inverted bright field images processed by ImageJ software. (a) Stably docked phospholiposomes observed due to SPR of Au when both DNA1 and cDNA1 were used for tethers. (b) and (c) Neither liposome docking nor its suspension was observed when either DNA1 or cDNA1 was absent. Large macromolecular complexes of phospholipid-DNA1 (c) and lipid detergent micelles (d) were observed only when cDNA1 was not bound to Au surface. All scale bars correspond to a distance of 5 μm .

which confirms the docking of phospholiposomes by DNA tethers on Au surface (see supplementary material).²⁸ The excitation/emission filters chosen were at far-red Cy5 (608–648 nm/672–712 nm), and the samples were exposed for 132 ms through 63 $\times$ oil objective. Figure 1(a) shows stable docking of phospholiposomes on Au achieved by hybridization of DNA1 and cDNA1 strands. Several liposomes were docked within chosen field of view but were not visibly apparent in Figure 1(a). This was evident from Figure S3 which is Figure 1(a) processed by ImageJ software by applying 25% threshold below the minimum gray scale intensity. The variation in pixel intensities of docked liposomes can be attributed to differences in the length scale and orientation of exposed nanoscale Au at the vicinity of the docked sites. This was confirmed from Figures 1(c) and 1(d), which show macromolecular complexes only when cDNA1 was absent so that evanescent waves near Au surface were not attenuated by cDNA1 as in Figure 1(b). Spatial patterning of Au by UV lithography will offer greater control on liposome docking to preferential sites. Another striking feature observed in Figure 1(d) was the ability to visualize sub-micron lipid-detergent bicelles using optical microscopy. The SPR effect allows visualizing putative elliptic lipid-detergent bicelle structures (<1.6 μm in major axis and $\ll 1 \mu\text{m}$ in minor axis) besides resolving submicron features with a sharp contrast from the background. Formation of bicelle structures at such length scales¹⁰ attributes to the q-ratio of the phospholipids mixture and relative concentration of detergent.^{10,11} This is surprising because hitherto the photoluminescent properties of Au nanorods in transverse ($\lambda_t = 540 \text{ nm}$) and longitudinal directions ($\lambda_{L1} = 661 \text{ nm}$, $\lambda_{L2} = 765\text{--}780 \text{ nm}$, and $\lambda_{L3} = 840 \text{ nm}$) were found to be suitable for nanoscale imaging^{8,9} using prism based set-up. The ability of a planar thin film Au surface to detect/image

nanoscale objects under SPR opens avenue for label-free reporting, which was utilized for imaging Ca^{2+} signalling in cells.¹²

Figure 2(a) shows concentric Newton's rings created by differential path length of evanescent wave between liposome and Au surface at site 1. The grayscale intensity profile across a section AA' was shown in Figure 2(b). As expected, the maximum intensity occurs at the centre, which corresponds to zeroth order bright ring. Alternate dark and bright rings of increasing order appear concentrically with increasing ring radius. Assuming photoluminescent emission wavelength of nanoscale Au film to be equal to the upper cut-off wavelength of the emission filter, $\lambda_{\text{em}} = 712 \text{ nm}$ and the refractive index of water, $n = 1.33$, the diameter of the liposome, $D = 2n(r_3^2 - r_2^2)/\lambda_{\text{em}} \sim 6.53 \mu\text{m}$. The parameters $r_2 = 1.122 \mu\text{m}$ and $r_3 = 1.734 \mu\text{m}$ are spatial locations corresponding to the peak intensities of second (middle) and third (exterior) order bright fringes obtained from Figure 2(b) and these are shown in detail in Figure S4(a). The optical path length for the first order fringe at $r_1 = 0.357 \mu\text{m}$ ($\sim \lambda_{\text{em}}/2$) is $\sim 53 \text{ nm}$, which suggests the possibility to create nanodomains of few tens of nanometres in the vicinity of site 1. The number of hybridized complementary tethers, N_h , within a restricted nanodomain was estimated to be between $1 \ll N_h < 2698$ for the experimental conditions considered (see Figure S4(b) and supplementary Table T2).²⁸

The ability to image and analyse the morphology of lipid-detergent bicelle complexes shown in Figure 1(d) manifest the potential of SPR imaging technique. A set of nine randomly oriented bicelle particles within the chosen field of view considered for image analysis was indicated in Figure S5(a). The gray scale intensity profiles in longitudinal direction (along major axis) of particles # 1–8 at diffraction-

FIG. 2. (a) Concentric rings of alternate bright and dark bands observed at the docked site 1. (b) Gray scale intensity profile across the section AA' shown in (a). The maximum intensity value corresponds to the centre of the liposome.

limited resolution (51 nm) were plotted in Figure S5(b). Spatial locations of several peaks identified from each of the eight intensity profiles were listed in the supplementary Table T1. It is evident from the tabulated data that most of the spatial locations at which peaks occur within all particles considered are integer multiples of wavelengths of three longitudinal SPR modes (λ_{L1} , λ_{L2} , and λ_{L3}) of Au nanorods. This confirms the fact that localised SPR modes interfered by nanoscaled objects on thin film Au will have a similar spectral response as that of Au nanorods. The spatial locations of the peak intensities obtained from the profiles were approximately close to quantized integer multiples of λ_{L1} , λ_{L2} , and λ_{L3} within the resolving ability of 51 nm. It is evident from the intensity profiles in Figure S5(b) that peak intensities at spatial locations of $1.0 \lambda_{L1}$, $1.0 \lambda_{L2}$, and $1.0 \lambda_{L3}$ were higher and more pronounced as opposed to the peaks associated with other higher/lower SPR modes. In particular, the peaks associated with $1.0 \lambda_{L1}$ are comparatively more pronounced than those of the other modes due to its proximity to lower cut-off wavelength of emission filter. Spatial locations of certain peaks could not be assigned to a particular quantized level due to limitations of 51 nm spatial resolution, and these were assigned to one of the probable quantized levels. Furthermore, the variability in the intensity values can be attributed to the aspect ratio and orientation of the bicelles as similar to such behaviours was previously observed in Au nanorods.¹³

Figure 3 shows iso-intensity contours of particle #9 obtained from discrete intensity values at diffraction-limited resolution of 51 nm using a MATLAB code. The SPR modes of Au enable to image nanoscaled structures with a sharp contrast creating a “hot spot.” The spatial locations of maximum intensity were close to λ_L (600–700 nm) and $(\lambda_L \text{ or } \lambda_L)/2$ (255–306 nm) in the longitudinal and transverse directions. The minor intensity peaks at those locations which correspond to non-integer ($1/2$, $3/2$, $5/2$, ...) diffraction orders of SPR modes were very small against the background signal compared to that of the dominant first order mode (see also Figure S5(b)). The contour patterns of particles vary with their orientation, which dictates the interplay between the principal SPR modes undergoing diffraction. Consequently, the ability to image a nanoscaled structure using this setup is governed by the spatial intensity, which indirectly characterizes the particle thickness at various sections.¹⁴ I speculate

that the true size of the particle could be less than the actual size of the intensity map because the intensity at the junction between Au and lipid-detergent bicelle complexes decays within a length of about ~ 200 nm.¹⁵

Figure 4(a) shows average intensity along the longitudinal direction across a mid-transverse plane (Figure S5(b)). The notation “BG” refers to background signal. The error bars denote standard error of mean of the intensity profile. The signal intensities of the chosen particles were at least five times the background intensity. Another critical performance metric of nanoscale imaging is the signal-to-noise ratio (SNR) and this is defined here as a ratio of the mean to the standard deviation of a signal. Figure 4(b) shows the SNR obtained using both signal (SNR_{SN}) and background (SNR_{BN}) noises. Complementing the visual perception of the image shown in Figure S5(a), the SNR_{BN} of all the chosen particles were at least greater than that of the BG by a factor of 4.58 except for particle #6 ($\text{SNR}_{\text{BN}} = 4.37$). The highest $\text{SNR}_{\text{BN}} \sim 7.14$ was observed for particle #3 in the chosen set of particles. As per the Curie-Rose criteria,¹⁶ $\text{SNR} \geq 4.653$ is required in order to achieve 95% confidence in discerning the true-positive and true-negative signals in an image

FIG. 3. Iso-intensity contours of particle #9 ($1.6 \mu\text{m} \times 0.9 \mu\text{m}$) shown in Figure S5(a) plotted by MATLAB programming using discrete gray values processed by ImageJ. The spatial coordinates at which maximum intensity occur were close to the wavelengths of transverse and longitudinal SPR modes.

FIG. 4. (a) Average gray scale intensity values of several lipid-detergent micelle complex particles using their gray scale intensity profiles in Figure S5(b). BG denotes background signal. Error bars represent standard error of mean associated with each signal profile. (b) Signal-to-noise ratio (SNR) obtained based on signal noise, SNR_{SN} (black bars) and background noise, SNR_{BN} (grey bars).

detection system. Since the SNR_{BN} for all particles were close to or greater than 4.653, most nanoscaled particles within the chosen field of view were detected with 95% confidence using this setup.

Unlike SNR_{BN} , the achievable values of SNR_{SN} were less than the threshold value for a few chosen particles (3, 4, and 7). The highest and lowest values of SNR_{SN} were 9.5 and 2.5 and these correspond to particles #1 and 3, respectively. Note that SNR_{SN} for background signal was 28.5 and it can be resolved spatially with a greater confidence than any of the true signals associated with the particles. The nanoscaled domains within particles #3, 4, and 7, however, may not be resolvable to a greater extent than other chosen particles and may not be suitable for detailed morphological studies.

Unlike the traditional Kretschmann prism set-up,^{17,18} the current work demonstrated the SPR effect using micro-machined Ti/Au on glass slide. The proposed method enables detection of nanoscaled features (with higher SNR_{BN}), but the resolving ability within nanoscaled domains (characterized by SNR_{SN}) also depends on morphological and optical properties of particles. Reducing the working distance so that Au surface is in a tighter focus within the optical plane could enhance SNR_{SN} . One approach to meet this

is by depositing Au on thin coverslip instead of a glass slide as shown here. This will allow membrane restricted nano-domains between liposomes and Au surface are within the optical plane of focus, thereby increasing the signal intensity. Second, an optimal thickness of Au allows exciting greater number of plasmons¹⁹ and this is reported as five times the Au layer thickness used in the present set-up (~ 10 nm). The background noise can be greatly reduced in confocal setup, which allows focusing the optical plane near membrane-restricted nanodomains within 200 nm. Furthermore, engineering the point spread functions of the microscope enable to tease out 3D spatial information at nanometer resolutions.²⁰

The ability to visualize sub-micron structures will open avenues to probe many critical biological mechanisms applying single molecule imaging methods *in vitro*. Recently reported methods such as optical signal enhancement from fluorescent “hotspots” on metallic films and nanoclusters²¹ and amplification of Raman signatures by Surface Enhanced Raman Scattering²² were indeed promising for single molecule studies. In contrast, the method described here enables label-free reporting of sub-micron structures at diffraction-limited resolution of 51 nm. Hence, issues associated with fluorescent probes like bleaching, blinking, and phototoxicity could be avoided with the proposed imaging technique.

Dissecting the geometry and topology of particles has a profound impact in many biophysical and biochemical studies. Investigating the morphology of bicelles allows identifying suitable conditions that produces stable and soluble protein-detergent complexes²³ and lipid-detergent bicelle complexes²⁴ for membrane proteins crystallography. Small Angle X-ray Scattering (SAXS) of micelles produced from commonly used detergents at concentrations exceeding critical micelle concentration (CMC) revealed that micelles tend to form spherical or elliptic (prolate or oblate) shapes at nanometre length scales.²³ In particular, 94 mM of Dodecyl-D-Maltopyranoside (DDM) forms oblate shaped micelles (major dimension, $b = 2.79\text{--}2.83$ nm; minor dimension, $a = 1.57\text{--}1.61$ nm; thickness, $t = 0.54\text{--}0.58$ nm; and aspect ratio, $(a/b) = 0.56\text{--}0.58$) in phosphate buffer. The lipid-detergent bicelles shown in Figure 1(d) are apparently elliptic in sub-micron dimensions (average major dimension, $b \sim 1.761 \pm 0.189$ μm ; average minor dimension, $a \sim 0.834 \pm 0.084$ μm ; aspect ratio $(a/b) \sim 0.476 \pm 0.049$) with an aspect ratio close to that of the DDM micelles reported previously. Although final shape of a micelle structure depends on detergent concentration, temperature, length of the carbon chain, hydrophobicity of the environment, etc., the aspect ratio of the structure seems to be almost conserved.

From a biological standpoint, visualizing spatially resolved protein complexes is critical to improve our understanding on molecular structures involved in various cellular functions. Several opportunities exist in field of cell biology where nanoscale-imaging techniques could be potential tool for answering important biological questions.²⁵ The ability to visualize up to second order bright fringe of micron-sized liposomes by SPR modes (Figure 1(a)) confirms that it is possible to discern nanoscaled architecture of macromolecular complexes between apposed membranes. Platforms that enable juxtaposition of giant phospholiposomes decorated

with proteins of interest within an approachable limit of few nanometres can be realised by creating Ti/Au islands of a couple of microns separated by about the size of phospholiposomes using microfabrication methods. This requires optimization of the pitch of Ti/Au islands besides the number of DNA1/cDNA1 tethers, which governs the maximum intensity achievable by SPR modes. Current biological methods are not matured enough to reverse engineer the key steps of even simple cellular signalling pathway in *in vitro*. Leveraging microfabrication processes will enable to dissect the molecular choreography of cellular signals. For instance, protein-protein interaction at low affinities competing with binding partners, drive signalling cascade events in many crucial biological pathways such as RAf-Ras,²⁶ Atk-PI(4,5)P₂.²⁷ A robust platform that allows modeling spatially confined interactions at native concentrations within membrane-membrane contacts would pave the way for interrogating confounding cellular signalling pathways.

In summary, micromachined 2D array of Au surfaces created on thin glass substrate could serve as a platform for docking giant phospholiposomes using protein or DNA tethers. Such a stably docked phospholiposomes when reconstituted with membrane proteins would allow modeling the interactions of membrane signalling complexes at apposed membrane junctions. Towards this goal, phospholiposomes ($D \sim 6.53 \mu\text{m}$) were docked by complementary DNA strands and imaged using SPR modes. The intensity of SPR modes at different docked sites characterizes the number of complementary tethers that bolster the phospholiposomes. Furthermore, the potential of SPR imaging to discern sub-micron structures was demonstrated by imaging lipid-detergent bicelles formed in a control experiment. The intensity profiles and maps of a few chosen lipid-detergent bicelle complex particles confirmed the interplay between several SPR modes, which were previously known to occur in Au nanorods. The methods and data presented here underscore the emergence of a radically different approach to study biological mechanisms by exploiting multifunctional properties of materials.

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