Biophotonic studies on lipid membranes using Oblique Incidence Reflectivity Difference (OI-RD) ellipsometry

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ABSTRACT

[1]In this study we report new results on the changes in optical properties of glass surfaces induced during wet photolithography using Oblique Incidence Reflectivity Difference Ellipsometry (OI-RD). A novel wet UV-photolithographic method for patterning phospholipid bilayers into two-dimensional arrays of voids and patches on hydrophilic glass substrates is presented. Especially, this technique involves etching the glass substrate fused with lipid vesicle solution and subsequently illuminated with short-wavelength UV light through a photo mask thus creating voids in the irradiated regions. The effects of the chemical etching and subsequent UV irradiation on the surfaces of microscope glass slides are investigated using the OI-RD technique. In this study, we have observed that the UV irradiation after chemical etching further changes the properties of the surface, even in the absence of the lipid bilayer. As a result, irradiating the chemically etched surface before the UV photolithography step renders the whole surface homogenous. Furthermore, fluorescence recovery after photobleaching (FRAP) experiments have been conducted on such homogenized surfaces which reveal that the fluidic properties of the membranes are retained. The created patterns are suitable to study protein-DNA interactions in the lipid environment. Our long term goal is to utilize this technique as a new screening approach for testing drug interactions above and below the cell surface.

Keywords: Phospholipid bilayers, wet UV-photolithography, OI-RD ellipsometry.

1. INTRODUCTION

Biophotonics is an emerging science of generating and harnessing light (photons), to image, detect and manipulate biological materials. The objective for biophotonics research is to improve disease diagnosis, discover high throughput drug delivery and therapeutic methods. Investigators have used several optical techniques to improve biomedical imaging and sensing at the nanoscale level including microscopy, spectroscopy, and interferometry for quantitative detection of biomolecular interactions both *in vitro* and *in vivo*. Ellipsometry is another important optical technique used to characterize biological membranes like lipid bilayers.[1, 2]

This study is concerned with the discovery of the optical properties of lipid layers using different biophotonic techniques. Biological membranes consist of lipids, proteins, carbohydrates and many other functional components. Such membranes mediate and regulate the transport of metabolites, macromolecules and ions throughout the body. Moreover, important processes in the body like photosynthesis, oxidative phosphorylation, electron transport, muscle contraction etc. take place at the membrane above and below the cellular level.[3,4] It is of great importance to know the intricate properties and behavior of these biological processes at the membrane level which eventually will lead life scientists to develop new sophisticated cutting edge technologies for biomedical diagnostics, drug screening delivery systems and therapeutic applications such as cell management and cell rejuvenation.

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In order to better understand the vital processes that take place at the membrane level inside the animal and human body, it is a great advantage to start testing artificially fabricated lipid membranes and then introducing additional functional components like proteins and DNA to test the properties of these functional components and their interactions with the lipid membrane. Our research group at the Physics Department, University of Nevada Reno (UNR) and Nanolife Inc, Reno, NV collaborates with the University of California Davis (UCD) and the National Science Foundation (NSF) Center for Biophotonics Science and Technology (CBST) to develop new nano-biotechnology methods to unravel the functionality of these lipid membranes and their conformational components. Especially, using photolithographic techniques, we can study the physical, chemical and biological functions of lipid membranes which may lead to new high throughput approaches in sensor microarrays, genomics, drug screening and proteomics. Furthermore, this strategy could be used in wet environments in order to understand, emulate, pattern, and determine specific functional mechanisms of cell membranes.

A special kind of label-free ellipsometric technique called Oblique Incidence Reflectivity Difference (OI-RD) ellipsometry[1,5] is used for these studies. By means of OI-RD ellipsometry, an important optical change is observed, which is not detectable by the more conventional fluorescence microscopic techniques. This paper reports the details of the optical changes detected by the OI-RD method. Furthermore a novel cleaning approach is presented. Following this new technique, the surface is rendered homogenous which is used for exploration of further biomolecular interactions in the lipid environment.

2. WET UV-PHOTOLITHOGRAPHY OF LIPID BILAYERS

In this section, a wet photolithographic technique for micropatterning of fluid phospholipids bilayers is presented in which spatially directed illumination with deep-ultraviolet (UV) radiation is applied[2]. The process of using UV/Ozone light for the wet photolithography process to create voids is elucidated in Fig 1. These voids can be refilled with lipid solution of the same kind or with a different kind and with many functional components like proteins, carbohydrates, cholesterol, DNA etc, thereby providing a means for probing two dimensional (2D) reaction-diffusion processes, changing membrane compositions, and designing functional membrane arrays.

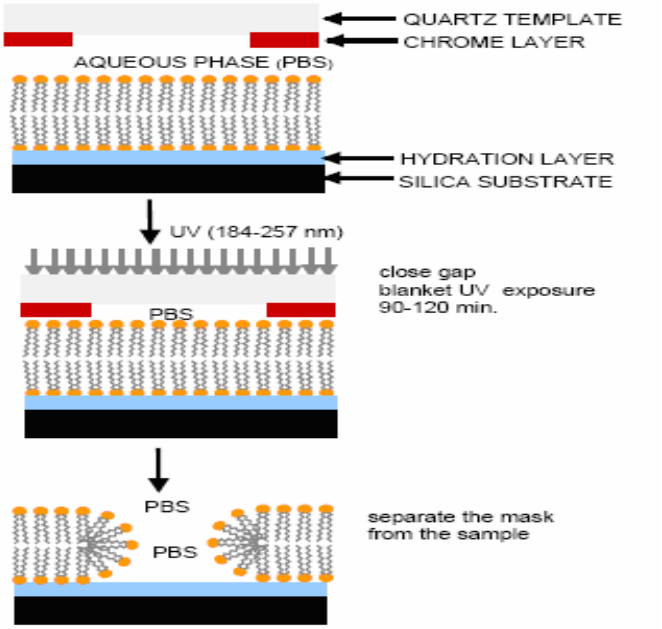
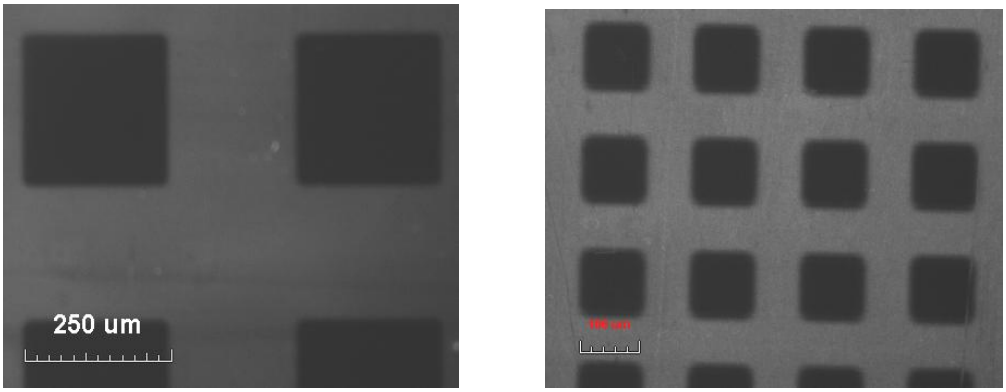


Fig. 1: The process of UV/Ozone degradation of the lipid bilayer creating voids.

As illustrated in Fig. 1, first the glass substrate is chemically etched by immersing the substrate in a mixture of 4 parts concentrated sulfuric acid (H_2SO_4) and 1 part hydrogen Peroxide (H_2O_2) at 100°C to remove organic contaminants and expose hydrophilic groups on the surface. Then, phospholipids are fused on the etched surface to form bilayers using

the method of unilamellar lipid vesicle fusion. The lipids used for these studies are Texas Red (TR) labeled P1-Palmitoyl-2-Oleoyl-Sn-Glycero-3-Phosphocholine (POPC) from Avanti Polar Lipids, Inc, Alabaster, AL.

When UV light is incident on the lipid bilayers at 184 nm wavelength, ozone gas is produced which penetrates through the quartz part of the photomask degrading the lipid bilayers thus creating voids. Exposure to UV light creates voids uniformly throughout the lipid membrane. Then the patterned sample is imaged under a fluorescence microscope and the fluidic properties of the lipid membrane are verified. Two typically patterned lipid bilayer samples imaged under a fluorescence microscope are shown in Fig 2.



(a) (b)

Fig. 2: (a) Shows the image of a TR labeled POPC lipid sample patterned using a photomask which has regular patterns of dimensions 250 nm. (b) Is the image of a TR labeled POPC sample patterned using a photomask with 100 nm patterns.

The grey background observed from Fig 2 corresponds to the fabricated lipid bilayer on glass substrate and the dark black squares seen are the voids created due to the exposure to UV/Ozone light. In order to monitor the biochemical interactions at the membrane level, we have employed a special type of ellipsometric technique called OI-RD.

3. OBLIQUE INCIDENCE REFLECTIVITY DIFFERENCE (OI-RD) ELLIPSOMETRIC TECHNIQUE

The OI-RD technique[1] measures the optical properties of a thin film on surfaces. The OI-RD ellipsometric method is a label-free technique which has the ability to detect biochemical interactions of protein molecules and DNA without the influence of fluorescent labeling agents. It can also be used to detect several changes in the surface properties of the substrate which are entailed in the results and discussions section of this report.

Zhu et al [5] studied the application of OI-RD ellipsometry for the detection and imaging of micro arrays on glass substrates. This work extends these studies using the OI-RD approach to image artificially fabricated lipid bilayers on solid glass slides. The experimental setup of the OI-RD microscope is shown in Fig 3. At oblique incidence, the reflectivities for *p*- and *s*- polarized light can change in response to the surface properties of the material. If r_{p0} and r_{s0} are the reflectivities from the bare substrate, and r_p and r_s the reflectivities from the surface covered with the lipid bilayer, respectively, then the differences Δ_p and Δ_s are defined in Eq (1) and Eq (2) as follows

$$\Delta_p = \frac{(r_p - r_{p0})}{r_{p0}} \qquad (1)$$

$$\Delta_s = \frac{(r_s - r_{s0})}{r_{s0}} \qquad (2)$$

Since the thickness *d* of the lipid membrane is much less than the wavelength λ of the incident light, the OI-RD technique enables direct measurements of the real and imaginary parts of $\{\Delta_p - \Delta_s\}$ [2]. The factor $\{\Delta_p - \Delta_s\}$ is called the reflectivity difference.

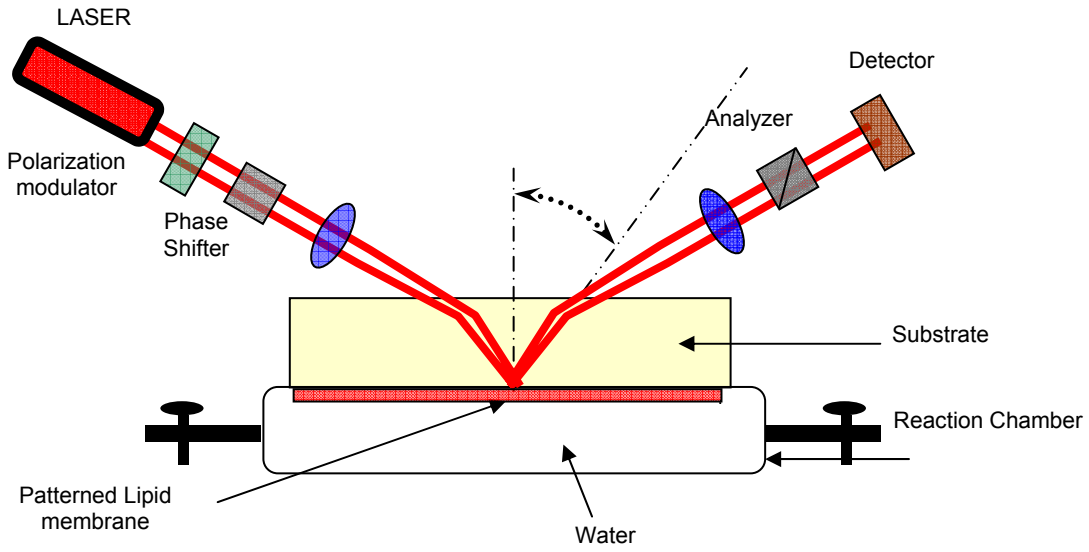


Fig. 3: Setup of an (OI-RD) ellipsometer

As shown in Fig. 3, a polarized He-Ne laser beam with λ = 632 nm passes through a polarization modulator, which, causes the output beam to oscillate between *p*- and *s*- polarization states. The polarization modulator is a photo elastic modulator with modulation frequency Ω = 50 kHz, maximum retardation of 180° with its modulation axis at 45° relative to p-polarization. The polarization-modulated beam is then passed through a phase shifter which introduces an adjustable phase Φ_0 between the s- and p- polarized components. A quarter wave plate (quartz) is used as a phase shifter for this setup. The principal axes (X and Y) of the quarter wave plate are rotated manually with respect to the 's' and 'p' polarization states to cause a shift in phase angle. The resultant beam is focused on the lipid membrane containing the surface at an angle of incidence θ (equal to 52°). After reflection and recollimation, the beam passes through an analyzer and the intensity of the transmitted beam $I_R(t)$ is detected with a photodiode and is Fourier analyzed with digital lock-in amplifiers. The first and second harmonic amplitudes of $I_R(t)$, $I(\Omega)$ and $I(2\Omega)$ are detected neglecting the higher harmonics.

Initially, the sample is positioned such that the laser beam reflects from the substrate without lipid bilayers. The angles of the analyzer and the quarter wave plate are manually adjusted such that $I(\Omega)$ and $I(2\Omega)$ are zeroed at this position. During the subsequent scan over regions of the surface with artificially fabricated lipid bilayers, the parameters $I(\Omega) \sim \mathrm{Im}\{\Delta_p - \Delta_s\}$ and $I(\Omega) \sim \{\Delta_p - \Delta_s\}$ are measured. In this present study, only $\mathrm{Im}\{\Delta_p - \Delta_s\}$ is recorded. The glass substrate was used as a window for the liquid cell and the laser beam is reflected from the back surface of the substrate. The substrate and the membrane are always immersed in water to maintain the diffusion properties of lipid membranes. Images were acquired by focusing the laser beam to a 3 μm spot size and subsequently scanned over an X-Y pattern underneath the fixed optics. It has been shown [6] that the $\{\Delta_p - \Delta_s\}$ is directly proportional to the thickness of the film (d) and is given by Eq.3:

$$\Delta_p - \Delta_s \approx -i\left[\frac{4\pi\varepsilon_s^{1/2}\varepsilon_0 \sin^2\theta\cos\theta}{(\varepsilon_0 - \varepsilon_s)(\varepsilon_0\cos^2\theta - \varepsilon_s\sin^2\theta)}\right]\frac{(\varepsilon_d - \varepsilon_s)(\varepsilon_d - \varepsilon_0)}{\varepsilon_d}\left(\frac{d}{\lambda}\right) \qquad (3)$$

Here θ is the angle of the back surface reflection, while ε_0, ε_s and ε_d are the optical dielectric constants for the ambient, substrate and lipid membrane respectively at the wavelength λ. At the He-Ne laser wavelength of 632 nm, ε_0 = 1.77 (water), ε_s = 2.28 (glass) and ε_d = 2.13 (lipid), and the reflectivity difference $\{\Delta_p - \Delta_s\}$ is determined as 5.27 $\times 10^{-3}$ where the thickness d is calculated to be 6 nm.

4. EXPERIMENTAL RESULTS AND DISCUSSIONS

In this section, first the OI-RD image of a Texas Red labeled POPC sample is presented in Fig 4. In addition, a substrate imaged without lipid fusion is shown for comparison in Fig. 5. Thus, the optical changes detected are described and a novel cleaning approach for glass surfaces is presented. Furthermore, the verification of the cleaning technique is provided by fluorescence recovery after photobleaching (FRAP) experiments.

4.1 OI-RD Image of a Lipid Sample

A Corning glass slide made of silica with dimensions 1 " (width) × 3 " (length) × 2.5 mm (thick) is etched and is fused with freshly extruded lipid vesicle solution. It takes approximately two minutes for the lipid bilayers to fuse onto the hydrophilic glass substrate. Then the lipid fused substrate is subjected to Ultraviolet/ozone (UV/O) light through a photomask to create two dimensional patterns on the fabricated lipid bilayer (as explained in Section 2 of this report). The resulting sample is carefully transferred to a Petri dish under water and imaged under a fluorescence microscope to check the quality of the patterns obtained and to verify the fluidic properties of the patterned lipid membrane. The same sample is transferred to the flow cell under water to be scanned with the OI-RD ellipsometer. Fig. 4 shows a typical OI-RD scan of a lipid fused sample.

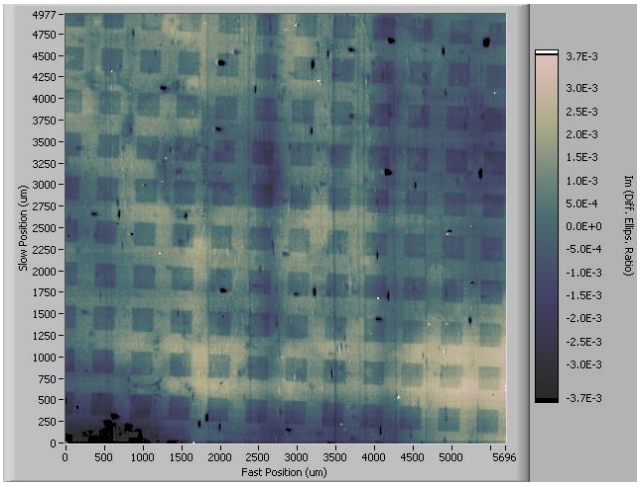


Fig. 4: OI-RD image of a lipid sample

4.2 Comparison of OI-RD Samples with and without lipid

The OI-RD image of a characteristic sample without lipid fusion is displayed in Fig. 5 (b). Moreover, the OI-RD image of a chemically etched glass substrate with lipid solution fused to it is indicated in Fig. 5 (a) for comparison. The glass substrate is etched and is directly illuminated by UV/Ozone light through a photo mask. Surprisingly, the OI-RD ellipsometer revealed the presence of patterns even in the absence of a lipid bilayer. These patterns were not detected so far by the conventional fluorescence microscopic technique.

Fig 5 elucidates a few important observations. Both (a) and (b) are samples that are cleaned using the same cleaning protocol under the same existing conditions. But, it is impossible to differentiate them since both reveal the

presence of regular patterns. Also, the reflectivity difference (Δ_p - Δ_s) measured for the sample in which lipid bilayers are involved is of comparable size when compared to the reflectivity difference of the sample without lipid.

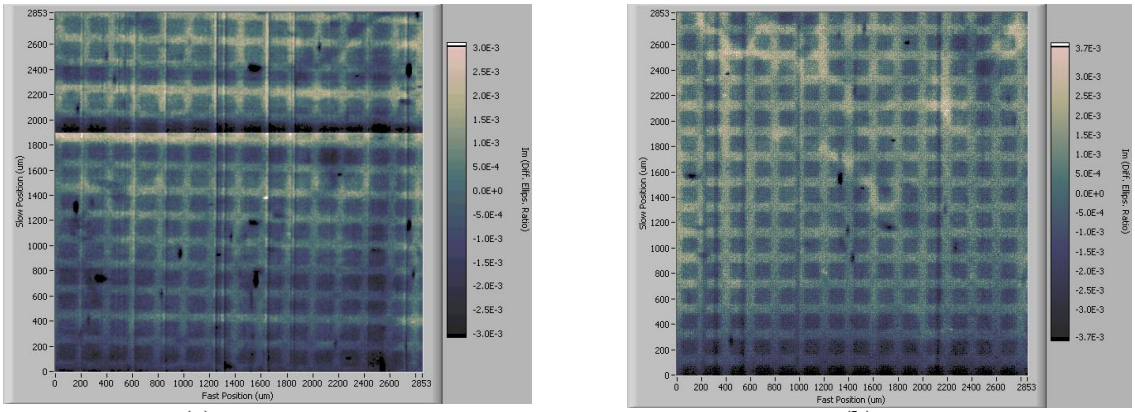


Fig. 5: OI-RD images of 2 different samples (a) with lipid and (b) without lipid

In this way, it is very difficult to quantitatively distinguish between lipid patterning (patterns caused due to lipid) and substrate patterning (patterns caused due to surface imperfections). Furthermore, if backfilling of the voids (see black dark squares on the image) with a different kind of lipid is desired, the changes obtained with the new lipid are different from the original membrane. Hence, further experiments were conducted in this direction to circumvent these problems and homogenize the surface.

4.3 Homogenizing the surface

In our optical studies of lipid membranes on glass substrates, it was inferred that surface contaminants and inhomogeneities may cause the unwanted patterns which complicate further experiments in the lipid environment. Therefore, a new technique to homogenize the glass surface was investigated and the method is described as follows.

A fresh glass substrate is chemically etched and is exposed to UV/Ozone light in water for 19 minutes. It is to note that the glass substrate is exposed to UV/Ozone light without fusing lipid vesicles on it. The idea here is to incident UV/Ozone light directly on the glass substrate to check whether it could remove the surface contaminants. The glass substrate is now placed on a photo mask and is subjected to UV/Ozone light in water once again for the same period of time.

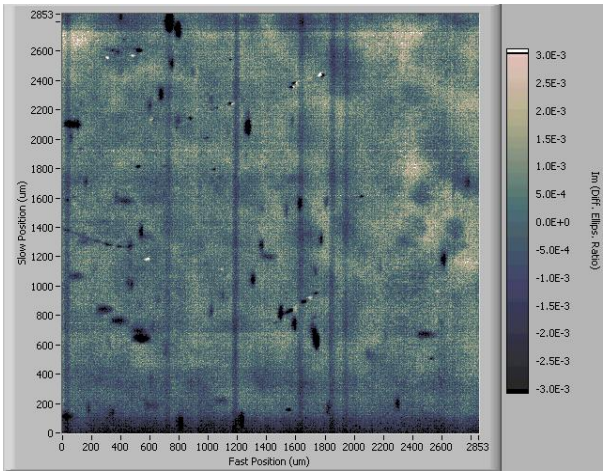


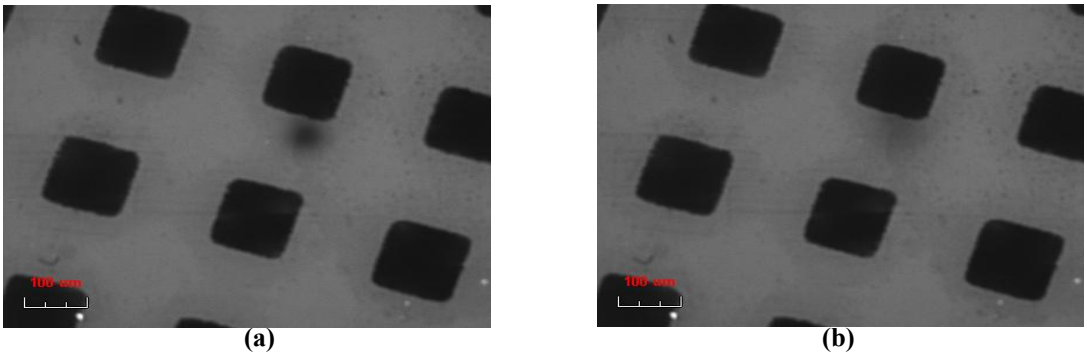
Fig. 6: Image of the homogenized substrate

In short, the etched glass substrate is exposed to UV/Ozone light two times, once without the photomask, and once with the photomask. After subsequent exposures to UV/O light, the substrate is transferred to the flowcell under water and OI-RD ellipsometric measurements are performed shown in Fig 6. The obtained image shown in Fig. 6 clearly shows no presence of any patterns. This indicates that when the etched substrate is subjected to UV/Ozone light for 19 minutes in a wet environment before the wet photolithography process, the surface becomes homogenous. The experiment was repeated three times to elucidate the consistency of the result obtained. The OI-RD image clearly exhibits that the surface properties of the irradiated squares remained the same as those of unirradiated regions. This is an indication that the surface has been rendered uniform.

4.4 Verification of the technique by Fluorescence experiments:

It has been previously shown that lipids fuse very well on chemically etched surfaces. Fluorescence recovery after photobleaching (FRAP) experiments were conducted on the homogenized surface cleaned by our new approach to verify that the properties of the lipid membranes are not affected.

Now, a new glass slide was homogenized using our novel cleaning procedure. The glass substrate was then fused with lipid vesicle solution and is subjected to UV/Ozone light through a photo mask. The patterned (array of voids and patches) lipid bilayer on the substrate is transferred to a clean Petri dish filled with water for fluorescence imaging. The fluorescence images shown in Fig.7 indicate important results mentioned as follows.



(a) **(b)**

Fig. 7: (a) Typical fluorescence image of a photo bleached spot on the lipid bilayer (b) image of the spot disappearing after 3 minutes

Firstly, the lipid vesicles fused perfectly on the substrate cleaned by our technique. Secondly, there were excellent patterns obtained after the wet photolithography process. These inferences proved that the cleaning approach followed did not hamper the quality of the patterns. Furthermore, FRAP experiments on this sample proved that the fluidic properties of the lipid membrane are retained. Here, the lipid membrane is photo bleached at a particular spot and allowed to recover. If the photo bleached spot disappears in due course of time (2 or 3 min), it indicates that the membrane still possesses its fluidic properties. If not, it can be inferred that the membrane has lost its diffusion capabilities. As shown in Fig. 7, the bleached spot disappeared after 3 minutes indicating that the lipid bilayer is fluid.

4.5 OI-RD results of lipids fused to a particular section of the substrate

In order to clearly differentiate between membrane patterning (patterning of the lipid membrane) and substrate patterning, a new strategy has been employed to quantitatively distinguish the surfaces with and without lipid. Lipids are fused to a certain portion of the entire glass slide on a homogenized glass substrate. A cover slip (1" × 1 " × 1 mm) is used to separate the bare substrate from the lipid fused substrate. The idea here is to fuse lipids only onto a particular region of the whole sample and track the edge of the lipid bilayer in a fluorescence microscope and the OI-RD ellipsometer to compare both results. Fig. 8 provides a comparison of fluorescence microscope and OI-RD ellipsometry images obtained for the same sample while tracking the edge of the lipid fused substrate.

Fig. 8 (a), (b), (c), (d), (e) represent the fluorescence microscope images of the edge and (P) represents the corresponding OI-RD results.

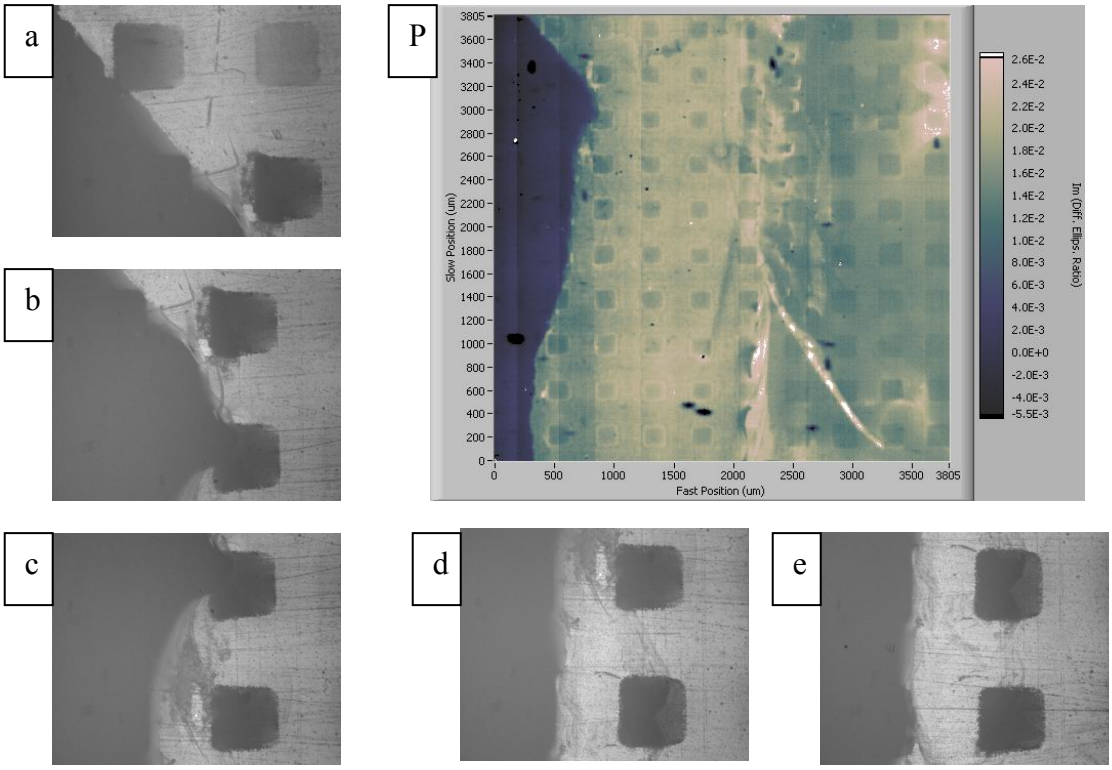


Fig. 8 (a),(b),(c),(d) are the fluorescence microscopy images of a TR-POPC lipid sample with track of its edge. (P) Shows the corresponding OI-RD image of the edge.

It is evident from Figure 8 (P), that there is no substrate patterning present thus confirming once again that the surface was rendered homogenous. This novel developed technique is very advantageous for studying the biomolecular interactions of different functional components with the lipid membrane at particular locations of the membrane. Since the voids (dark squares) can be tracked from edge to edge using a fluorescence microscope and OI-RD ellipsometer, the exact location where biomolecular interactions occur can be quantitatively monitored with precise control.

5. CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, we have applied the Oblique Incidence Reflectivity Difference (OI-RD) ellipsometric technique to monitor lipid bilayers on solid glass substrates. In particular, a new cleaning approach of the substrate was introduced and successfully applied for the creation of the lipid structures[7]. This new technique will allow us to study *in vivo* complex lipid molecular interactions on the nanoscale in future experiments. This approach is also suitable for backfilling experiments to investigate the protein-DNA interactions in the lipid environment[8]. We have also found that the ellipsometric optical technique provides more detailed information about liposome interactions when compared to the standard fluorescence microscopic techniques. Some of the future applications of this work are summarized in the following:

- Creations of biochips with molecular sensitivity in the nano range.
- Applications of biosensors, bioimaging devices, gene sequencing and other relevant diagnostic systems.
- Creation of sophisticated, portable, cost effective methods for biomedical diagnostics to enhance the quality of life.

Additional studies on liposome interactions are presently carried out using Near Infrared (NIR) and Middle Infrared (MIR) ellipsometry to elucidate further information on the molecular and cellular level.

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