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Fluorescence of Supported Phospholipid Bilayers Recorded in a Conventional Horizontal-Beam Spectrofluorometer

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1. Abstract

Supported phospholipid bilayers are a convenient model of cellular membranes in studies of membrane biophysics and protein-lipid interactions. Traditionally, supported lipid bilayers are formed on a flat surface of a glass slide to be observed through fluorescence microscopes. This paper describes a method to enable fluorescence detection from the supported lipid bilayers using standard horizontal-beam spectrofluorometers instead of the microscopes. In the proposed approach, the supported lipid bilayers are formed on the inner optical surfaces of the standard fluorescence microcell. To enable observation of the bilayer absorbed on the cell wall, the microcell is

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placed in a standard fluorometer cell holder and specifically oriented to expose the inner cell walls to both excitation and emission channels with a help of the custom cell adaptor. The signal intensity from supported bilayers doped with 1% (mol) of rhodamine-labeled lipid in the standard 3-mm optical microcell was equivalent to fluorescence of the 70-80 nM reference solution of rhodamine recorded in a commercial microcell adaptor. Because no modifications to the instruments are required in this method, a variety of steady-state and time-domain fluorescence measurements of the supported phospholipid bilayers may be performed with the spectral resolution using standard horizontal-beam spectrofluorometers.

Keywords: supported lipid bilayers, fluorescence, planar membranes, phospholipids, microcell

2. Introduction

Supported phospholipid bilayers were long established as a model of cellular phospholipid membranes.[1] The phospholipid bilayers attached to the glass surface (with and without crosslinking to the glass) are physically very similar to other membrane mimics in their biophysical properties.[2] The main approach to recording fluorescence signals from such supported bilayers is through the microscopes with the bilayer deposited on the horizontal surface of the glass slide and covered by a layer of the aqueous buffer.[3,4] Fluorescence microscopy of supported bilayers allows analysis of spontaneous phase separation[5,6] as well as detection of molecular events at the bilayer with a single-molecule resolution.[7,8] One highly useful feature of the supported lipid bilayer (relatively to liposomes) is that it is *immobilized* allowing for dynamic replacement of the solution in contact with the bilayer. Supported lipid bilayers are routinely studied in the flow-cell mode with the microscopy setups to reveal interactions of membrane proteins with lipids and with soluble ligands.[9,10] However, if one is particularly interested in *spectral* properties of the lipid-associated fluorophores and their changes due to ligand interactions, the microscopes must use multi-wavelength lasers and include detectors with spectral resolution, which are expensive and not generally available.[11] The best practical approach to record fluorescence of lipid bilayers in standard spectrofluorometers was to utilize solutions (suspensions) of liposomes or protein-liposome complexes.[12-15] Yet, liposomes are not very stable and do not allow for easy replacement of the surrounding buffer as they are freely diffusing particles. Supported lipid bilayers represent a convenient alternative because they are

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easily prepared by spontaneous fusion of lipid vesicles with the glass surfaces yet require use of sophisticated fluorescence microscopes for their analysis.[1,2] This paper reports a proof-of-principle experiment where a supported lipid bilayer is created in a standard fluorometer microcell and fluorescence measurements are performed in a typical horizontal-beam spectrofluorometer. We believe that this inexpensive approach may serve a number of needs in studies membrane protein interactions with lipids and soluble ligands.

3. Materials and Methods

Preparation of liposomes

Lipid mixtures contained 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoylsn-glycero-3-phospho-1'-rac-glycerol (DOPG) in the molar ratio of 89:10:1 (DOPC:DOPG:fluorophore). The 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine labeled with lissamine rhodamine B (Rhod-DPPE) was used as a fluorescent marker of the lipid bilayer. All lipids were obtained from Avanti Polar Lipids Inc. and used without further purification. Large unilamellar vesicles (LUV) were prepared by extrusion through 0.2 μm polycarbonate membrane using Avanti Mini-Extruder. The total concentration of lipids during extrusion was 1 mM. The lipids were diluted to 0.1 mM for deposition of the supported lipid bilayers. Use of LUV versus multi-lamellar vesicles (MLV) or small unilamellar vesicles (SUV) does not appear to be a strict requirement in this protocol; Brian and McConnell[1] successfully utilized MLV in their pioneer work while SUV were employed by Cremer and Boxer[16] for the same purpose.

Preparation of supported lipid bilayers

Supported lipid bilayers were formed on the inner surface of a rectangular 3-mm fluorometer microcell (Starna #3-3.45-Q-3) by completely filling the cell with the LUV solution of 0.1 mM total lipid (a minimum concentration allowing for the stable bilayer formation[1,5,16]). Bilayers with negatively charged lipids were reported to form very fast at physiological pH and ionic strength.[16] To keep the bilayer adhered to the walls, the cell must stay filled at all times because air-water

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boundary destroys the attached bilayers[16]). Therefore, to remove the initial LUV solution, the cell was flushed with a continuous flow of the phosphate saline buffer. To perform the flush, the cell was positioned horizontally and the buffer was introduced towards the bottom of the cell with the needle; the displaced solution was allowed to exit the cell and drip into the waste container. Capillary forces were sufficient to keep cells completely filled during the entire procedure. Relatively wide spacing between walls in the microcell (3 mm) made the buffer exchange process somewhat inefficient— 50 to 100 ml of buffer at a flow rate (approx.) 2 ml/min was required to ensure complete removal of residual LUV from the bulk solution inside the microcell. Quality of the LUV removal was checked by residual fluorescence of the bulk solution in the microcell.

Confocal fluorescence microscopy

Images of supported lipid bilayers were taken using Nikon Perfect Focus Ti-E inverted research microscope. To support the 3 mm cell in horizontal position in front of the objective, the custom cell holder was utilized.

Fluorescence spectroscopy

Photon Technologies International QuantaMaster 40 spectrofluorometer (Horiba) was used for recording emission spectra from supported lipid bilayers. The fluorometer was equipped with a standard 1 cm sample holder, emission and excitation monochromators, and Xenon steady-state excitation source. A custom cell adaptor was designed to hold the standard 3 mm microcell from Starna (Cat# 3-3.45-Q-3). The adaptor included internal slits to trim the excitation beam and to expose the illuminated cell surface to the emission channel. Details of the adaptor design are described in the International Patent Application No. PCT/US2015/060658 filed on November 13, 2015.

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4. Results

Visualization of the supported lipid bilayers

Prior to spectroscopic investigation, formation of supported lipid bilayers on the inner surfaces of the fluorometer cell may be confirmed using fluorescence microscopy. Figure 1 shows representative rhodamine fluorescence images of the inner volume of the 3-mm rectangular microcell. The sample platform was adjusted such that left-hand-side of the image corresponds to interior of the optical cell, while the right-hand-side is always inside the material of the vertical cell wall (Panels a and b). To demonstrate that the fluorescent lipids are found exclusively on the inner walls for the optical cell and not in the solution, the confocal planes were imaged at three vertical coordinates indicated in Panels b and c as (I), (II), and (III). The position III is 200 μm below the inner wall and shows no fluorescence as the confocal plane is entirely localized inside the horizontal cell wall. The position II is aligned with the inner surface of the horizontal cell wall and reveals bright fluorescence (left of the cell wall junction) corresponding to rhodamine in the phospholipid bilayer coating the inner cell surface. The uppermost position I is 25 μm above the inner surface of horizontal cell wall intersecting the vertical inner cell surface in the middle of the image. Left-hand-side of the image revealed no significant fluorescence from solution inside the cell indicating that all suspended LUV were successfully removed by the flushing procedure. The bright straight line in the middle originates from fluorescence of the supported lipid bilayer formed on the vertical inner cell wall. Real thickness of the lipid bilayer cannot be estimated from these images due to inherent resolution limit of optical spectroscopy of about 400 nm at this wavelength. However, the lipids were previously reported to absorb at the glass surfaces as a bilayer with the thickness of about 5 nm.[5,16] Black area on the right of the image corresponds to the non-fluorescent interior of the vertical cell wall.

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Supported lipid bilayers in a horizontal-beam spectrofluorometer

For observation of fluorescence from phospholipid bilayers the microcell was set inside the standard 1-cm cell holder to expose the surface with the bilayer to both excitation and emission channels. Figure 2.a shows one of the possible positions of the microcell for bilayer observation compared to the standard centered position (Panel b). Panel c gives emission spectra recorded from the supported lipid bilayers and the diluted reference LUV solution. In this measurement, the excitation monochromator was set at 560 nm; the identical 5-nm slits were used in the excitation and emission channels. Illumination of the surface of the cell (Panel a) generates both the useful fluorescence from the surface and scattered light from the corners of the microcell; therefore, one has to use additional adjustable slits trimming the excitation beam as well as limiting exposure of the emission channel to cell edges. Solid red line in Panel c shows fluorescence signal from the most optimal combination of cell position and adjustable slits. This optimal combination is likely to vary slightly from one instrument to another due to differences in tuning of the focusing optics.

To confirm that the recorded fluorescence comes from the supported bilayer rather than from any residual LUV in the bulk solution, the solution fluorescence from the same sample was recorded using a standard microcell adaptor with a centered cell location (FCA3, Starna) shown in Panel b. The FCA3 adaptor is manufactured to enable observation of fluorescence from internal volume of the solution; its narrow windows shield corners of the cell from exposure to excitation and emission channels. In preparation of the supported bilayers the microcell was flushed with a buffer in several stages; every time the residual LUV fluorescence from the bulk solution in the microcell was recorded using the FCA3 adaptor. After passing about 50 ml of buffer through the cell, the rhodamine fluorescence reduced to a minimum value shown as a dotted blue line in Figure 2.c. Further flushing with another 50 ml did not reduce it any more indicating that this residual fluorescence signal likely comes from the supported bilayers on the front and back walls (facing the emission channel) excited indirectly by the light scattered in solution.

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Relative sensitivity of measurements from the supported lipid bilayer sample may be evaluated by comparison of its fluorescence to the signal intensity of a reference LUV solution in the centered adaptor FCA3. The dashed black line in Figure 2.c is a spectrum of the initial LUV solution used for preparation of supported lipid bilayers diluted to 5 μM total lipid concentration prior to the measurement (resulting in 50 nM concentration of rhodamine). Thus, the signal from supported lipid bilayers with 1% (mol) rhodamine in a 3-mm microcell was equivalent to 70-80 nM of rhodamine in LUV solution (recorded with 5 nm excitation and emission slits in the 3-mm cell).

5. Discussion

Typical applications of supported lipid bilayers utilized glass slides or flow-cells positioned horizontally to observe the bilayers from below or above in some kind of a microscopy setup. Supported lipid bilayers formed on the inner surfaces of the fluorometer cells may be investigated while oriented vertically because van der Waals forces holding the bilayers in place are stronger than gravity while the optical cell ensures that the bilayer is covered with solution at all times. In this report, the supported lipid bilayer samples were stable for more than a week at 4°C. Vertical orientation of lipid bilayers in the proposed method has another advantage over the horizontal orientation: biomolecular aggregates (or dust) sediment to the bottom of the microcell, thus leaving the observation area. With the supported lipid bilayers located horizontally in the microscopy setups, all aggregates settle directly onto the bilayer under study thus potentially biasing measurement results.

Observation of fluorescence from the solution near the cell surface dates back as far as to Sir Frederick William Herschel who described fluorescence of optically dense quinine solutions.[17] Since then the solutions of high optical density or/and significant turbidity were routinely studied with illumination of the cell surface at an angle such that the incident light penetrates a thin layer of solution inside the cell, which fluoresces. These applications typically utilize triangular cells with the cell surface oriented at the 45° angle to both emission and excitation channels. This arrangement, however, is not suitable for fluorescence detection from supported lipid bilayers because the weak

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fluorescence signal is swamped with the excitation light reflected into the monochromator thus destroying the signal/noise ratio. Lakowicz recommended smaller 20-30° angles to reduce scattered light with such triangular cells [18]—this is approximately the angle in Figure 2.a. However, the optical cells that would expose the front cell surfaces to the excitation beam at these sharp angles are not generally available. Utilizing the commercial microcell that is smaller than the size of the standard 1-cm cell holder allowed us to explore multiple positions and orientations of the cell achieving illumination of the cell surfaces at different angles.

Another requirement in biochemical applications is a small size of the sample. The triangular 45° cells manufactured by Starna or Hellma require 1.7 ml of solutions to fill. This report utilizes a 3-mm Starna microcell, which nominally holds 315 μL of a sample bringing it to the volume range of flow-cells in microscopy applications. By attaching a cell cap with the needle and inletoutlet tubing one might construct a flow cell to be able to replace solution in the cell directly in the fluorometer (in this study, for the sake of simplicity, the cell was flushed outside of the instrument).

It has been demonstrated that proteoliposomes may be used to create supported lipid bilayers incorporating membrane proteins.[1,2] Using the method described in this report one may perform titration studies of receptor-ligand interactions by adding the ligand to the bulk solution in the cell. Subsequent flushing out the ligand regenerates the unbound form of the membrane-associated receptor thus allowing for multiple repeated experiments with the same bilayer sample.

Standard spectrofluorometers are often equipped with temperature-controlled cell holders opening an avenue for studies of temperature-dependent thermodynamic and kinetic properties of the supported bilayer samples. One may note that a potential alternative way of exposing supported bilayers at the cell surfaces to the excitation and the emission channels at different angles could be to utilize the solid sample holder with a microcell adaptor. However, the solid sample holders cannot be thermostatted due to their open design thus precluding studies of membrane proteins, which require accurate temperature control. The described method with placement of

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the optical microcell inside the standard enclosed 1-cm cell holder allows for effective heat transfer between the cell holder and the sample enabling experiments at different temperatures.

The proposed approach to observation of the supported bilayer fluorescence is also agnostic to the type of the excitation light source, therefore time-domain studies are equally possible on the same samples used for spectral recording. In summary, a combination of a straightforward bilayer-forming technique, commercial optical cells coupled with the simple custom adaptors, and the standard spectrofluorometer hardware allows for an inexpensive way of recording fluorescence from supported lipid bilayers thus facilitating detailed studies of lipid membranes, membrane proteins, and their interactions with the time-domain and spectral resolution.

6. Acknowledgements

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7. Figures

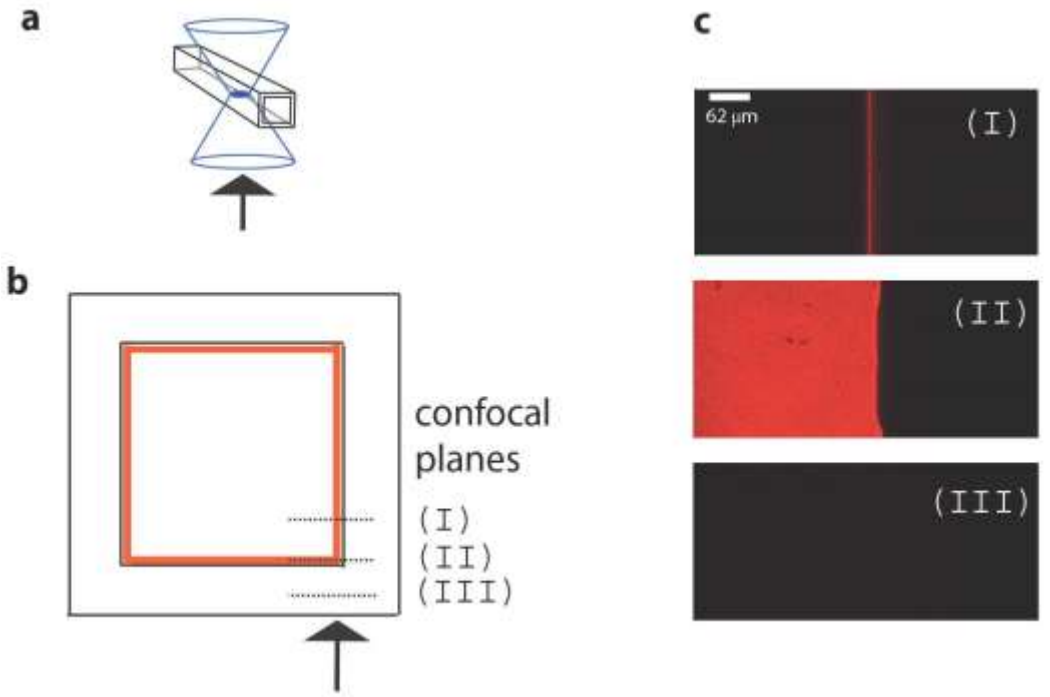

Figure 1. Confocal microscopy imaging of supported lipid bilayers formed on the interior surface of the fluorometer cell. (a) The rectangular quartz cell (3 mm path) on a sample platform to observe the inner cell wall facing the objective. (b) Vertical cross-section of the microcell. The microscope was focused at three different vertical coordinates to image the slices above, at, and below the horizontal inner cell wall (positions I, II, and III). The cross section of the cell is schematically shown as a black outline. The lipid bilayer location is indicated by a red line. The objective was centered at the vertical inner wall boundary to create strong contrast between the fluorescent cell content on the left and non-fluorescent optical material of the cell wall on the right of the image. The drawing is not to scale. (c) Images of the 3 mm cell containing supported lipid bilayer including 1% (mol) of Rhodamine-DPPE taken with the 40x objective. The slices were imaged at three vertical positions (I, II, and III) of the confocal plane corresponding to the relative Z-coordinate of 25 µm, 0 µm, and –200 µm, respectively. Thickness of the optical section was 4.3 µm.

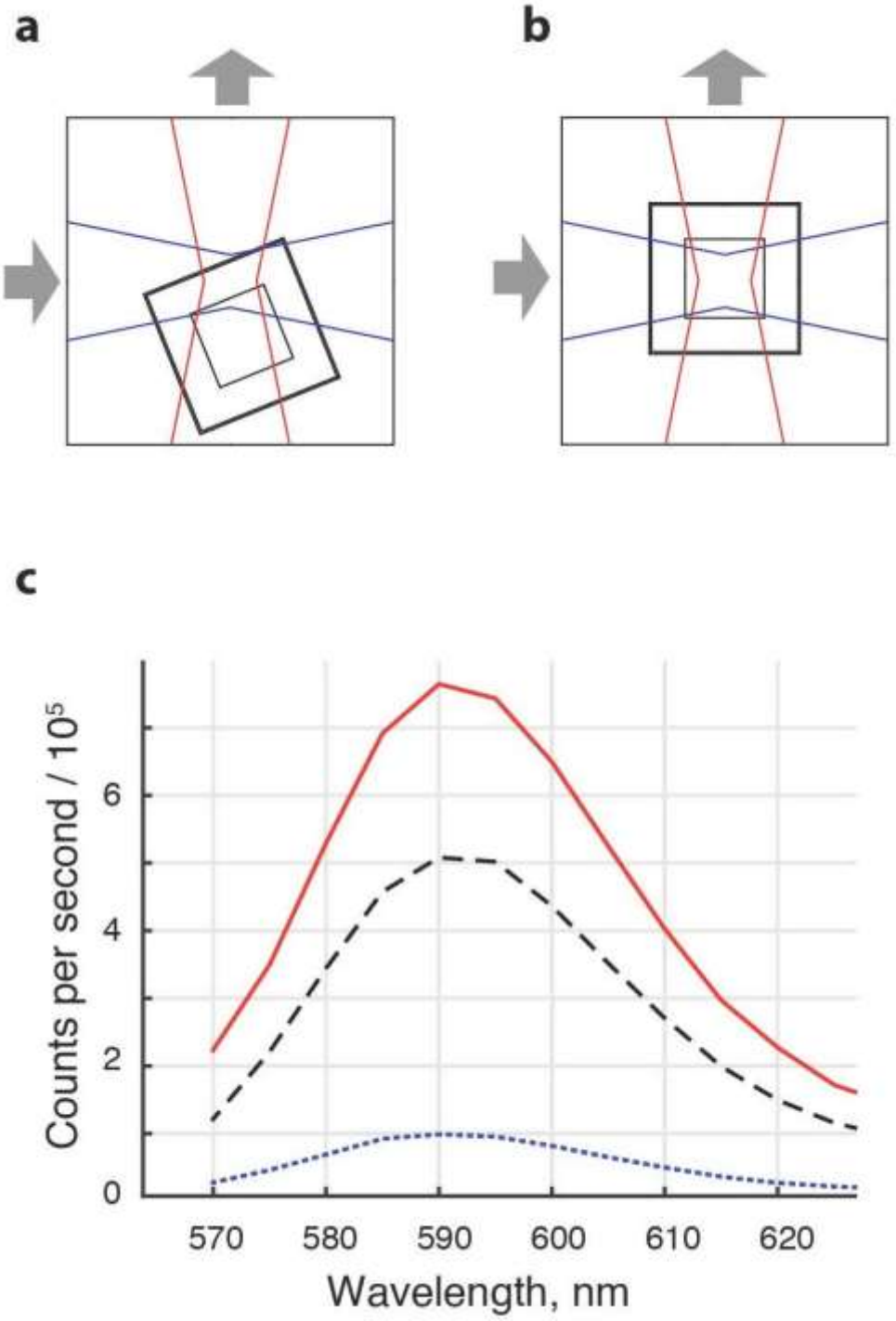

Figure 2. Detection of fluorescence from supported lipid bilayers and evaluation of sensitivity of the method. (a) Positioning of the rectangular 3 mm microcell inside the custom-designed cell adaptor. The excitation and emission channels are indicated by arrows, the light paths focused by optics and trimmed by custom slits are schematically depicted by blue and red lines. (b) For comparison, schematic of the microcell placement in the standard 3 mm cell adaptor (Starna, Cat# FCA3) avoiding

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direct illumination of the inner cell walls. (c) Solid red line, the rhodamine emission spectrum of the supported lipid bilayer in the 3 mm microcell recorded using the custom adaptor shown in Panel a. The bilayer was deposited utilizing 100 μM LUV containing 1% rhodamine-DPPE. Dotted blue line, the same sample was placed in the centered position of the FCA3 adaptor (Panel b) showing bilayer fluorescence excited by the light scattered in solution. Dashed black line, a reference 5 μM LUV solution with 1% rhodamine recorded in
FCA3 adaptor (Panel b).

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