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Multiphoton excitation fluorescence microscopy in planar membrane systems

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ABSTRACT

The feasibility of applying multiphoton excitation fluorescence microscopy-related techniques in planar membrane systems, such as lipid monolayers at the air–water interface (named Langmuir films), is presented and discussed in this paper. The non-linear fluorescence microscopy approach, allows obtaining spatially and temporally resolved information by exploiting the fluorescent properties of particular fluorescence probes. For instance, the use of environmental sensitive probes, such as LAURDAN, allows performing measurements using the LAURDAN generalized polarization function that in turn is sensitive to the local lipid packing in the membrane. The fact that LAURDAN exhibit homogeneous distribution in monolayers, particularly in systems displaying domain coexistence, overcomes a general problem observed when "classical" fluorescence probes are used to label Langmuir films, i.e. the inability to obtain simultaneous information from the two coexisting membrane regions. Also, the well described photoselection effect caused by excitation light on LAURDAN allows: (i) to qualitative infer tilting information of the monolayer when liquid condensed phases are present and (ii) to provide high contrast to visualize 3D membranous structures at the film's collapse pressure. In the last case, computation of the LAURDAN GP function provides information about lipid packing in these 3D structures. Additionally, LAURDAN GP values upon compression in monolayers were compared with those obtained in compositionally similar planar bilayer systems. At similar GP values we found, for both DOPC and DPPC, a correspondence between the molecular areas reported in monolayers and bilayers. This correspondence occurs when the lateral pressure of the monolayer is 26 ± 2 mN/m and 28 ± 3 mN/m for DOPC and DPPC, respectively.

1. Introduction

Lipid monolayers have a broad application in basic sciences (e.g. chemistry, biology, physical-chemistry, polymer science to mention a few). Even though this model represent "half of a bilayer" (one molecule thick), it is still extensively used to mimic basic molecular and supramolecular interactions among different types of lipids and proteins in membranes [1]. Examples of studies reported in monolayer systems at the air–water interface are (just to mention a few): (i) lateral structure of compositionally different lipid films [1–4], (ii) penetration studies of proteins, peptides or other drugs (anesthetics for example) into a monolayer [5–7], (iii) stability of peptide or protein monolayers [8,9], and (iv) enzymatic action of lipases [10–12]. Studies including rheological, topological, electrical and mechanical properties of monomolecular films are also accessible using Langmuir films [1,13,14]. For instance, studies of surface pressure and interfacial electrical potential as a function of average cross-sectional molecular area in Langmuir films provide insights into many interesting membrane-related parameters. Examples are lipid hydrocarbon chain ordering, lateral compressibility/elasticity, and dipole effects under various conditions including those that approximate one leaflet of a bilayer [1,13].

Importantly, Langmuir films have a concrete connection with relevant structures existing in biological systems. For example, it is well known that a proteo-lipid surfactant material exists in our body, e.g. in the respiratory airways, having important physiologically relevant functions [15]. Surfactant material is for example highly important during the breathing cycles in order to avoid lung collapse (by changing the surface tension in the surface of the alveoli) [15]. The organization of lung surfactant at the air–tissue interface has been modelled using monolayers (either Langmuir films or supported monolayers) composed of particular lipid mixtures (with or without proteins), surfactant lipid extracts and native surfactant material from different sources [16–21]. In most of the cases, the role of different components on the physical properties of the monolayer is evaluated under controlled environmental conditions [22,23].

At present, there is an array of different experimental techniques that can be used in order to study planar membrane model systems. Specifically, for Langmuir films the most classic measurements are the surface pressure (π), vs. molecular area isotherms and the surface potential-area isotherms [24]. However, additional structural information

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can be obtained using spectroscopic techniques such as Brewster angle and fluorescence microscopy, UV-Vis, IR, Raman, Second harmonic generation, X-ray diffraction and reflectivity [25,26]. These applications can be classified in two types: (i) those that measure main properties of the whole monomolecular film and (ii) those that provide a spatial distribution of a measurable property of the monolayer in an image, such as those measured in microscopy-based techniques. For example, different fluorescence microscopy experiments can provide the spatial correlation of several interesting parameters, such as rotation of molecules, extent of hydration, polarity, local pH, and lateral diffusion. Applications of these aforementioned fluorescence microscopy approaches have become popular in the last 10 years using lipid bilayer systems; particularly giant unilamelar vesicles [27–29].

The first applications of fluorescence microscopy on Langmuir films were reported in the 1980s [3,30]. This very popular and frequently used experimental approach is based in the acquisition of images reflecting the distribution of a fluorescent probe in the monolayer film upon compression. Although, as we mentioned above, fluorescent microscopy techniques offer the possibility of measuring of a variety of fluorescent parameters in the target system, this type of approach has not been fully exploited in Langmuir films except for a few exceptions [31,32].

This present report introduces and discusses for first time multiphoton excitation fluorescence microscopy applications in Langmuir films using the fluorescence probe LAURDAN. Additionally we describe some technical details about our systems, where a Langmuir trough has been incorporate on a custom built multiphoton excitation fluorescence microscopy.

2. Materials and methods

1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids and used without further purification. 6-dodecanoyl-2-dimethylamino naphthalene (LAURDAN) was obtained from Invitrogen (Denmark).

2.1. Preparation of Langmuir films

A solution of 1 mg/ml DPPC (or DOPC) dissolved in a chloroform mixed with 2 mol% of LAURDAN was prepared. 20 µl of the solution was carefully added to the air/water interface and the solvent was allowed to evaporate over 10 min. The trough has a maximum area of 312 cm^2 and a minimum area of 54 cm^2 and uses Teflon-coated ribbons as the barriers. The ribbons are moved so as to compress as a symmetrical double barrier. The pressure was measured using the Wilhelmy plate technique. We estimate the error between different isotherms to be on the order of ± 1 mN/m. The monolayer was compressed at a speed of 50 cm^2/min to the desired surface pressure, which was kept constant during the laser scanning experiments. The experiments were carried out on a MilliQ-water subphase at room temperature (room temperature, 21 °C). The data sets for the GP functions were based on 2 or 3 independent measurements of different monolayers of DOPC or DPPC, respectively.

2.2. Preparation of supported lipid membranes by spincoating

The preparation of supported membranes by hydration of spin-coated lipid films has been described previously [33,34]. To prepare the dry spin-coated lipid film on mica, we used a stock solution of 10 mM lipid containing 0.5 mol % LAURDAN in hexane/methanol (97:3 volume ratio). 30 µL of this lipid stock solution was then applied to freshly cleaved mica and immediately thereafter spun on a Chemat Technology, KW-4A spin-coater at 3000 rpm for 40 s. The sample was then placed under vacuum for 10–15 h to ensure complete removal of the organic solvents. The dry spincoated film was subsequently hydrated by immersing the sample in either pure water or phosphate buffer (10 mM phosphate, 128 mM NaCl, pH = 7) followed by heating to 55 °C for 1 h. The sample was then placed on the fluorescence microscope and flushed with 55 °C buffer/water using a pipette adjusted to 500 µL in order to remove excess lipid from the support. After the washing procedure, the liquid volume was gently exchanged 5–10 times to remove membranes in solution. Measurements of LAURDAN GP function in these membranes were performed using the same setup indicated in the next section. The GP experiments have been carried out twice and multiple images (up to 15) were collected from each individual samples. No substantial difference in the GP value was found between the samples made in pure water or those made in buffer.

2.3. LAURDAN GP function

The LAURDAN GP denotes the position of the probe's emission spectrum [35]. The fluorescence emission properties of LAURDAN are sensitive to the water dipolar relaxation process that occurs in the probe's environment. The energy of the excited singlet state progressively decreases when the extent of dipolar relaxation process is augmented. The extent of water dipolar relaxation observed in highly packed membrane regions (as the solid-ordered phase in bilayers) is very low compared to what it is observed in less packed regions (as the liquid-disordered phase in bilayers). For example when a solid-ordered/liquid-disordered phase transition occurs in the membrane, a prominent red shift in the fluorescence emission spectrum of the probe is observed (from blue to green; almost 50 nm shift) [35]. The GP function was defined analogously to the fluorescence polarization function as:

$$GP = \frac{I_B - I_R}{I_B + I_R} \quad (1)$$

where I_B and I_R correspond to the intensities at the blue and red edges of the emission spectrum (440 and 490 nm) using a given excitation wavelength [35–37]. In lipid bilayers high LAURDAN GP values (0.5–0.6) correspond to laterally ordered phases (e.g. solid-ordered or gel) whereas low LAURDAN GP values (below 0.1) correspond to liquid-disordered phases [35]. Coexistence of liquid-ordered and liquid-disordered lipid phases in bilayer systems have been characterized using LAURDAN GP images [38,39].

2.4. LAURDAN GP measurements

For LAURDAN GP measurements the fluorescence signals were collected in two different channels using bandpass filters of 438 ± 12 nm and 494 ± 10 nm. The fluorescence emission light was split between two PMTs (Hamamatsu H7422P-40) by a dichromatic Mirror splitting at 475 nm. The microscope is controlled by Globals for Images SimFCS. This software is developed by the Laboratory for Fluorescence Dynamics, University of California at Irvine, USA. It is important to notice that the GP values obtained from the GP images strongly depend on instrumental factors such as filter settings and gain of the PMTs used in the microscope. Therefore, the calculated GP images must be calibrated with a correcting factor G. As the GP function is based on the relative intensity of the blue and the green channel it is necessary to calibrate the relative intensity of the two channels to obtain an absolute measurement of the GP. Therefore the GP equation utilized to calculate the GP images contains a factor G (similar to the classical polarization equation), used in this case to calibrate the relative intensity of the two channels:

$$GP = \frac{I_B - (G \times I_R)}{I_B + (G \times I_R)} \quad (2)$$

In Eq. (2) the GP should be equal to that from a reference solution with a defined GP. The measurement of the factor G is performed by