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Review

Taking another look with fluorescence microscopy: Image processing techniques in Langmuir monolayers for the twenty-first century

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ABSTRACT

Fluorescence microscopy has become a powerful and standard complementary technique in the study of amphiphilic films at the air–water interface. For nearly three decades the coupling of traditional thermodynamic measurements with direct visualization has provided a better understanding of self-assembled Langmuir monolayers and their application in the study of the physical properties of membranes and interfaces. As an introduction we provide a brief overview of this established technique and demonstrate its continued utility in the recent observation of novel phase behavior in monolayers of 25-hydroxycholesterol (25-OH) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC). We then focus our review on new analysis techniques which take advantage of the ability to store, process, and analyze large sets of images. We pay particular attention to efforts measuring the line tension between coexisting two dimensional fluid phases in the Langmuir monolayer. Using non-perturbative methods, we can measure fundamental mechanical properties of these two dimensional systems. Finally, we highlight the use of Model Convolution Microscopy as a new tool to provide insight on the experimental limits in these studies.

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For the past three decades, surface scientists utilizing Langmuir monolayers as model systems have complemented traditional thermodynamic measurements with fluorescence microscopy [1]. One of the most powerful uses of this tool has been to identify phase transitions and map out phase diagrams for single and multi-component systems by correlating visual observations of the monolayer with quantitative readings from surface pressure or surface potential sensors. However, researchers have also sought to extract additional information from monolayer images through image processing techniques and direct spatial measurements in the images themselves. This work has enriched our understanding of phase transitions in two dimensions and membrane biophysics. We believe that the increasing availability of data storage, computing power, and image processing routines will stimulate the development of new tools to capitalize on these visualizations for an even greater physical understanding.

The direct visualization provided by fluorescence microscopy has revealed a rich behavior of pattern formation and varied

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morphologies in two dimensional self-assembled systems. Materials scientists, physical chemists, cell biologists, and biophysicists have formed a strong foundation for our current understanding of Langmuir films through measurements of phase diagrams [2,3], diffusion rates [1,4–6], Brownian motion and surface rheology [7–10], molecular orientation and chirality [11,12], and critical behavior [13–15] through this experimental setup. The rapidly increasing reach of this technique motivates many researchers previously unfamiliar with these tools to now interpret the relevant experiments of others from this perspective. Here, we provide a brief review on fluorescence microscopy as a technique to characterize Langmuir monolayers as well as highlight some of the important long standing contributions of the technique. Readers already familiar with the basic principles and experimental techniques may wish to skip this section of the manuscript; others may wish to read several expert reviews on the subject [16–19], as well as more contemporary overviews of related topics [2,20,21]. The review by Kaganer et al. will serve as an excellent starting point to the field for the novice reader [21].

In the remainder of the manuscript, we review our own work and that of others to measure the line tension between coexisting phases in phospholipid/cholesterol systems. This work is inspired by the need to better understand the formation and properties of lateral inhomogeneities within model lipid membranes. We also discuss the use of image simulation or *Model Convolution Microscopy* to assess the quality and effectiveness of image processing routines and potential sources of error. Finally, we provide an overview of recent work combining the direct visualization of Langmuir monolayers with the extraction of quantitative data through software. The cumulative effect of these advances offers a response to the once lamented lack of quantitative analysis in spite of rich examples of pattern formation and domain morphologies [22]. We believe that techniques highlighted here will enable researchers to shed light on remaining questions relevant to both studies of fundamental two dimensional phenomena as well as specific to biological interfaces. Many of these questions revolve around the thermodynamic behavior of multi-component Langmuir monolayers. For example, are domains within cholesterol/phospholipid monolayers truly a two-phase system [23]? As recently noted by Andelman and Rosensweig, it is often difficult to assess if the domains observed within Langmuir monolayers are the result of a kinetically trapped system or equilibrium behavior [24]. A related question is what factors determine domain morphology in multi-component monolayers. For example, our own preliminarily observations indicate that the rate of monolayer expansion can strongly influence the size distribution of coexisting liquid phases within cholesterol/phospholipid monolayers.

1. Introduction

An early and illustrative example of the power of fluorescence microscopy came from the direct observation of Langmuir monolayers of dipalmitoylphosphatidylcholine (DPPC), the major component of lung surfactant. By itself, the plateau of DPPC's pressure-area isotherm near 7 mN/m demonstrates the existence of a transition between liquid-expanded and condensed phases. However, in monolayers the exact order of phase transitions like this, the role of impurities, and experimental techniques proved to be debatable [16,25–27]. Resolution ultimately came through imaging the monolayer directly before, during, and after the liquid-expanded to condensed transition [6,11,28], see Fig. 1. In addition to observing the formation of a condensed phase, fluorescence microscopy also revealed a striking asymmetry to the shape of the domains and suggested long range order within the condensed phase [11,29]. This was ultimately explained by the chiral nature of the DPPC molecule [11]. Additionally, the influence of repulsive forces was made evident as the domains avoided contact [30], and the Brownian motion of condensed phase domains was used to verify theoretical descriptions of fluid dynamics

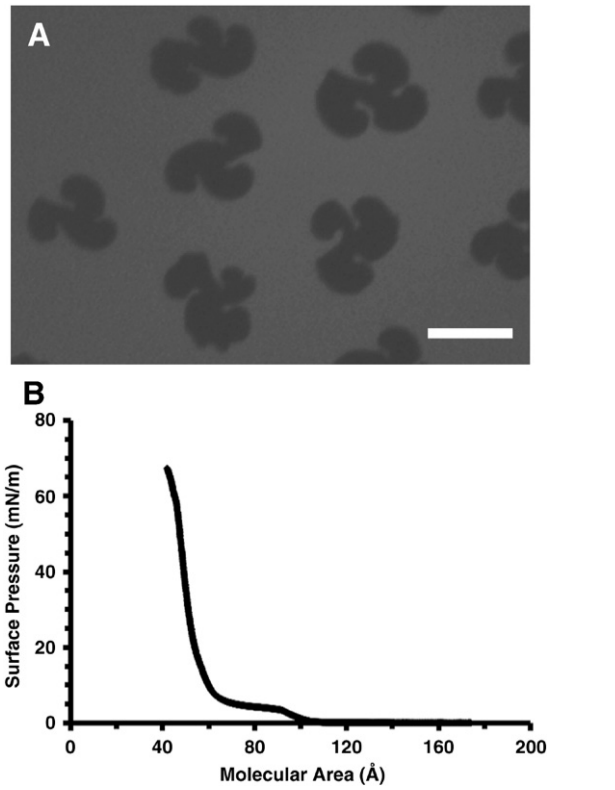

Fig. 1. Solid–liquid existence in DPPC monolayers. A) A striking asymmetry is observed in the condensed phase of a DPPC monolayer. This feature is ultimately the result of the chiral nature of DPPC itself, but had not been previously identified. Texas Red DPPE was added at 0.5 mol% to provide the contrast. B) The pressure-area isotherm indicating a transition between liquid-expanded and condensed phases. Scale bar is 50 μm.

[10]. Expanding on these studies, the addition of cholesterol to the monolayers was observed to decrease the line tension and stabilize the interface between the condensed and liquid-expanded phase [31].

Recently we enjoyed a similar example of the utility of these combined experimental tools in the system of DPPC and 25-hydroxycholesterol (25-OH). A small but detectable kink in the pressure-area isotherm was observed to correlate well with a liquid–liquid miscibility transition [32]. A puzzling but long standing question for lipid monolayer researchers is the failure to observe similar deflections in the pressure-area isotherms corresponding to miscibility phase transitions of more commonly studied cholesterol and phospholipid raft forming mixtures [33,34]. The work of Smaby et al. illustrates how a careful analysis of pressure-area isotherms can identify deflections [35]. Unfortunately for the liquid–liquid miscibility transition no similar general correlations have been found [36]. At pressures below 1.5 mN/m, monolayers of the saturated phospholipid DPPC containing 25-OH also exhibit a surprising molecular area expansion for concentrations between 10 and 20 mol% [32]. This finding is consistent with the previous observation that 25-OH and 27-hydroxycholesterol both cause an expansion in the monolayer's molecular area when mixed with the unsaturated phospholipids palmitoyloleoylphosphatidylcholine (POPC) and dioleoylphosphatidylcholine (DOPC) [37,38]. A potential explanation for the novel behavior of 25-OH is that the two hydroxyl groups at the opposite ends of the steroid ring structure anchor the sterol flat at the air–water interface for low pressures. During compression these molecules orient themselves upright in the monolayer resulting in the observed pressure-area isotherm discontinuity. The phase behavior of cholesterol analogs offers a tool to probe the relationship between sterol structure and mixing/demixing transitions. Additionally, sterols are of biophysical interest in their own right. For example, oxysterols have been linked to apoptosis in aortic smooth