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Unraveling complex nanoscale lipid dynamics in simple model biomembranes: Insights from fluorescence correlation spectroscopy in super-resolution stimulated emission depletion mode

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ABSTRACT

Dynamic heterogeneity (DH) at nanoscale due to lipid-lipid and/or lipid-protein interactions in cell membranes plays a crucial role in determining a broad range of important cell functions. In cell membranes, the dimensions of these nanodomains have been postulated to be in the order of 10's of nm and transient in nature. While the structural features of membranes have been studied in detail, little is known about their dynamical characteristics due to paucity of techniques which can probe nanoscale phenomena with simultaneous high temporal resolution. A combination of super-resolution stimulated emission depletion (STED) and fluorescence correlation spectroscopy (FCS) technique can overcome this limitation and provide information about the nanoscale dynamic heterogeneity in cell membranes. Using STED-FCS and FCS diffusion law, we provide an understanding of how nanoscale dynamically organizing lipid platforms can emerge in minimal system of model biomembranes. To illustrate the utility of the technique we have chosen cholesterol containing supported lipid bilayers and demonstrated the role of cholesterol concentration and/or added pore-forming protein, Listeriolysin O (LLO) in determining onset of lipid DH. In addition we have also looked at multi-component lipid bilayers with and without cholesterol to infer about the role of phospholipid and cholesterol composition on lipid dynamics. These results on simple biomimetic systems provide insights into fundamental pathways for the emergence of complex nanodomain substructures with implications for a wide variety of membrane mediated cellular events and depict the significant contribution that STED-FCS can make in resolving several outstanding issues in membrane biology.

1. Introduction

Cell membranes are widely believed to be dynamically rearranging at different length scales to facilitate and regulate variety of cellular mechanisms [1–3]. Existence of nanoscale membrane domains are reported to be essential for protein specificity and sorting [4–6] and are also believed to act as signaling pathway [7,8] as well as entry pathway for uptake mechanism of molecules [9–11] and impacts several other aspects of membrane biology [12–15]. These nanodomains in cell membranes are not static structures and they evolve temporally. Cell membrane is an active system and many factors can trigger such domain formation making it difficult to understand the origins of such structures. Instead, model membranes with varying degree of complexity are, often, the more convenient platforms of choice for performing systematic studies in this regard [3,16–22]. Length scales and timescales of these *dynamically heterogeneous nanoscale* regions can be resolved only using a combination of techniques with spatial resolution of tens of nanometer and μs – ms temporal resolution [1,23–25]. Due to small size of such nanodomains (<100 nm), it is extremely difficult to capture these domains by diffraction limited (~250 nm) optical techniques. Various methods like atomic force microscopy (AFM) [26], fluorescence resonance energy transfer (FRET) [27] and super resolution techniques [28–30] such as stimulated emission depletion (STED) microscopy [31,32], as photo activated localization microscopy (PALM) [33–35] and STORM etc. can provide spatial resolution down to ~10's of nm or better. However, techniques with only high spatial resolution are not sufficient in capturing dynamics studies and real-time monitoring measurements due to lack of appropriate temporal resolution. This shortcoming can be overcome by using different types of time resolved fluorescence based techniques like fluorescence recovery

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after photo bleaching (FRAP), fluorescence correlation spectroscopy (FCS), single particle tracking (SPT) that can provide information on lipid dynamics, molecular diffusion on the membrane, kinetics of interaction of molecules with membrane etc [36–42]. FCS is an ensemble averaged technique [43] and advantageous over other techniques with temporal resolution spanning from few micro to 100's of milliseconds and more. FCS can probe dynamics in bulk (3D) or in film (2D) with no technical difficulty and captures range of time scales. Yet, FCS is diffraction limited and comes with a best possible spatial resolution of $\geq$200 nm and is not sensitive to heterogeneous diffusion at nanoscale. Potential applications of the above spectroscopy tools have widened with the advent of super resolution techniques especially STED which helps in breaking the optical diffraction limit [31]. Combining STED and FCS, spatiotemporal heterogeneity of lipids in plasma membrane of live cells [44], transient trapping of molecules by cholesterol and cytoskeleton assisted nanoscale complexes [45], pore-forming toxin (PFT) induced nanoscale lipid re-organization [46] and detection of dynamical heterogeneity in simple model membrane [47,48] have been reported. Spot variation FCS (sv-FCS) coupled with FCS diffusion law has proven to be a robust tool in unraveling dynamically organization in biomembrane [49–52]. However the smallest length scale that can be probed by traditional sv-FCS [53,54] is also limited by the optical diffraction barrier. This probe length scale can be extended by performing sv-FCS in STED microscopy mode [32,55,56].

In this article, we explain the methodology of performing FCS experiments in both confocal and STED mode to study nanoscale lipid dynamics in model biomembranes. We first elaborate on the background of these techniques which have been employed in the work discussed here. We then discuss the emergence of nanoscale lipid dynamic heterogeneity induced by pore-forming protein, Listeriolysin O (LLO) interacting with membrane bound cholesterol. Next, we show that presence of cholesterol, above a threshold concentration, in a single component phospholipid membrane is sufficient to induce this heterogeneity even in the absence of proteins. Subsequently, we show that this heterogeneity can emerge even in absence of cholesterol in a two-component phospholipid biomembrane having a large content of a high melting lipid. Finally, we show that cholesterol added to this later two-component mixtures reduces the extent of such heterogeneity contrary to what was observed in the single phospholipid bilayers pointing to the subtle interplay of lipid-cholesterol organization and nanoscale dynamics in model membranes.

2. Experimental methods

2.1. Biomembrane (one-, two- and three-component system) method of preparation

Phospholipids such as 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, >99% purity), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, >99% purity), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC, >99% purity), 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC (or DL), >99% purity), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPPC (or DP), >99% purity) and cholesterol (Chl, >98% purity) was obtained from Avanti polar lipids. 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine labeled with Atto 488 was obtained from ATTO-TEC GmbH, Germany. Supported lipid bilayers are prepared onto a glass substrate (20 × 20 mm, Glaswarenfabrik Karl Hecht GmbH & Co KG, Germany); this planar configuration is ideally suited for observation using fluorescence microscopy [57].

Supported lipid bilayers (SLBs) of one, two and three-component lipids can be prepared using different protocols like vesicle fusion, spin coating, Langmuir-Blodgett (LB) technique etc [17–22]. Various reports suggest that the role of solid substrates and bilayer preparation methods influence the complex dynamics and phase behavior in bilayer membranes [58–61], and care must be taken for preparation of supported lipid bilayers. In this work, SLBs were formed upon controlled transfer of lipid interfacial monolayers onto pre-treated glass substrates by employing the LB technique as reported earlier [19,46,48,62]. In brief, during the preparation of bilayer using the LB method, multiple compression-expansion cycles were followed at a constant trough temperature of 15 ± 1 °C before the collapse surface pressure, and subsequently the bilayers were transferred at a highly condensed surface pressure to the hydrophilized glass slides by using layer-by-layer transfer. Prior to transfer, glass substrates (20 mm × 20 mm, Germany) were cleaned either using "piranha solution" (a 30:70 mixture of 30% hydrogen peroxide and concentrated sulfuric acid at 80 °C) or RCA (3:1 mixture of NH_4OH and H_2O_2) for 30 min and washed multiple times with MilliQ DI water (resistivity ~18.2 MΩ·cm). The first monolayer was transferred at a holding surface pressure of 33–35 mN/m by vertical withdrawal of the substrate at a speed of 5 mm/min with a transfer ratio of ~1.0 ± 0.1. The second monolayer transferred at the same surface pressure by a vertical down stroke at a speed of 3 mm/min yielded a centrosymmetric bilayer (Y-type) on the support. To make the bilayer luminescent, dye tagged lipid (Atto488-PE, 10^{-3} – 5×10^{-4} mol%) was mixed thoroughly with lipid mixtures before spreading at the air-water interface. After transfer, the bilayers were transferred to a container under water without exposing to air and stored at 25 °C for further use. All measurements were done on the prepared bilayers within 4–5 h of the LB transfer at 24 ± 2 °C. Several results reported here were also confirmed on vesicle fused bilayers.

2.2. Fluorescence correlation spectroscopy (FCS)

FCS is a conventional optical technique based on the statistical analysis of fluorescence intensity originated from the Brownian motion of single fluorophores through a tiny observation volume (typically in 1 μm^3). The size of the observation volume in confocal microscopy is reduced at single molecule level by the same objective lens serves as both spatially-selective illuminations by a focused laser, and detection through a pin hole (Fig. 1a). In the illumination volume, FCS analyses characteristic fluctuation (Fig. 1b) of the fluorescent particles in time t about an average value by calculating the second order auto-correlation function (Fig. 1c) according to Eq. (1) [63];

$$G(t_c) = \frac{\langle \partial F(t) \partial F(t + t_c) \rangle}{F^2} \quad (1)$$

where $\diamond$ denotes the time average, and $\partial F(t)$ and $\partial F(t + t_c)$ are the fluorescence intensity fluctuations around the mean value, F, at time t and $t + t_c$, respectively. In two dimensions (2D), the fluorescent particles (fluorescently-labelled lipids or proteins) embedded in bilayers the expression of autocorrelation function is given by Eq. (2):

$$G(t_c) = \frac{1}{N} \frac{1}{1 + \left(\frac{t_c}{\tau_D}\right)^{\alpha}} \quad (2)$$

where, τ_D is the transit time, N is the average particle number in the observation volume and α, is the anomaly coefficient. In all our FCS measurements, α is treated as fit parameter. For free Brownian diffusion, $\alpha = 1$ and $\alpha \neq 1$ corresponds to anomalous diffusion i.e. deviation from Brownian diffusion.

The diffusivity (D) in such a case can be evaluated using either Eqs. (3a) or (3b);