Nanoscale dynamics of phospholipids reveals an optimal assembly mechanism of pore-forming proteins in bilayer membranes†

Nirod Kumar Sarangi,a K. G. Ayappa,*b,c Sandhya. S. Visweswariah cd and Jaydeep Kumar Basua,a

Cell membranes are believed to be highly complex dynamical systems having compositional heterogeneity involving several types of lipids and proteins as the major constituents. This dynamical and compositional heterogeneity is suggested to be critical to the maintenance of active functionality and response to chemical, mechanical, electrical and thermal stresses. However, delineating the various factors responsible for the spatio-temporal response of actual cell membranes to stresses can be quite challenging. In this work we show how biomimetic phospholipid bilayer membranes with variable lipid fluidity determine the optimal assembly mechanism of the pore-forming protein, listeriolysin O (LLO), belonging to the class of cholesterol dependent cytolysins (CDCs). By combining atomic force microscopy (AFM) and super-resolution stimulated emission depletion (STED) microscopy imaging on model membranes, we show that pores formed by LLO in supported lipid bilayers can have variable conformation and morphology depending on the fluidity of the bilayer. At a fixed cholesterol concentration, pores formed in 1,2-dioleoylsn-glycero-3-phosphocholine (DOPC) membranes were larger, flexible and more prone to coalescence when compared with the smaller and more compact pores formed in the lower fluidity 1-palmitoyl-2-oleoylsn-glycero-3-phosphocholine (POPC) membranes. In contrast, 1,2-dimyristoylsn-glycero-3-phosphocholine (DMPC) membranes did not show any evidence of pore formation. Fluorescence correlation spectroscopy (FCS) in STED mode revealed the appearance of a length scale, ξ, below which lipid dynamics, under the influence of LLO protein binding and assembly, becomes anomalous. Interestingly, the magnitude of ξ is found to correlate with both lipid fluidity and pore dimensions (and flexibility) in DOPC and POPC bilayers. However this length scale dependent crossover, signalling the onset of anomalous diffusion, was not observed in DMPC bilayers. Our study highlights the subtle interplay of lipid membrane mediated protein assembly and lipid fluidity in determining proteolipidic complexes formed in biomembranes and the significant insight that STED microscopy provides in unraveling critical aspects of nanoscale membrane biophysics.

1 Introduction

Lipid–protein interactions are fundamental to the organization and function of biomembranes.1,2 However, the mechanisms by which proteins can modify membrane structure,3 or the dependence of membrane lipid composition on the nature of interactions4 between intrinsic membrane proteins, are still poorly understood. Aggregation of proteins into larger proteolipidic complexes can occur through a process of self-assembly, which follows from an initial step of protein binding to the cellular membrane. This phenomenon of membrane mediated self-assembly has widespread implications, ranging from acting as the primary virulent pathway for pore forming toxins (PFTs) to the formation of protein aggregates in Alzheimer’s and Parkinson’s disease.5,6,7,8,9 Pore-forming toxins are expressed by several strains of bacteria such as Staphylococcus aureus, Escherichia coli, and Listeria monocytogenes to name a few.6,7,8,9 Monomeric proteins are expressed in a water soluble form which seek out the target membrane. Upon encountering the target cell membrane, rapid binding and oligomerization lead to the formation of a...
transmembrane pore which eventually causes cell lysis.\(^7\) Oligomerization is unregulated, limited only by the concentration of the bound toxin, and binding is usually receptor mediated. The process of binding and oligomerization in the membrane leads to disruption in the surrounding lipid structure, alters lipid dynamics and induces local compositional heterogeneity around the transmembrane pore complex.\(^{10,11}\) Pore aggregation can further extend the spatial influence of disruption in the lipid environment. Since transmembrane pores typically range from 10 to 30 nm in diameter,\(^{12,13}\) capturing the induced perturbation in lipid structure and dynamics at these length scales has been a challenge. Unraveling these nanoscopic changes in the membrane environment has fundamental implications on our understanding of transmembrane mediated signaling and other biophysical cellular events.

Cholesterol dependent cytolysins (CDCs) are a large class of cholesterol dependent PFTs which have been widely studied in terms of the pore formation efficiency, kinetics and morphology.\(^{14,15}\) LLO expressed as a water-soluble monomer binds to cholesterol which acts as a receptor in the host cell membrane. Subsequent oligomerization leads to the formation of prepore complexes (30–50 subunits), which undergo a conformational change forming transmembrane pores.\(^{16,17}\) While it is desirable to study pore formation in plasma membranes of cells, the chemical complexity of typical plasma membranes makes identification of the dominant processes determining pore formation difficult. Hence, the use of artificial lipid bilayer membranes with controlled complexity and organization is more useful as a bottom-up platform on which protein–lipid interactions, in general, and PFT–lipid interactions, in particular, can be conveniently studied. Model biological membranes have been widely used to understand the organizational and dynamical aspects of real cell membranes as well as their interactions with proteins, polymers and nanoparticles.\(^{18–28}\) In this regard, several recent studies with supported lipid bilayer (SLB) membranes and LLO PFTs have revealed the subtle dependence of the nature of protein assemblies and pores formed on the composition of lipid membranes.\(^{29,30}\) Obtaining microscopic insight into the causal connection between lipid composition and pore formation efficacy has proven to be difficult, largely due to both the small length scale involved for both pore formation (10–100 nm) as well as the dynamic and co-operative nature of the potential interaction and response.

The advent of super-resolution microscopy techniques, especially the stimulated emission depletion based technique (STED)\(^{31,32}\) in combination with well established fluorescence correlation spectroscopy (STED-FCS),\(^{33–38}\) has paved the way for exploration of fundamental biological and especially protein–membrane interactions with the high spatio-temporal resolution required to probe the phenomenon at the nanoscale.\(^{39}\) Here, using supported phospholipid bilayer platforms with variable lipid composition/fluidity and STED-FCS, we have studied the process of lipid membrane bound assembly and pore formation of LLO and their connection to the nanoscale lipid dynamical heterogeneities (DH) induced upon LLO binding to these model biological membranes. Cholesterol (25%) was included in all lipid mixtures such as DOPC, POPC and DMPC, since it serves as the receptor for LLO in biological membranes. By employing STED-FCS, we show how the fluidity of the membrane lipids is critical for the membrane-bound oligomerization process of LLO. While pore formation is associated with the onset of nanoscale lipid DH for the fluid phase lipid membranes composed of DOPC and POPC, neither pore formation nor DH is observed for DMPC membranes. Interestingly, POPC with intermediate fluidity seems to form more homogeneous and smaller, compact pores, compared to DOPC which seems to form less compact and larger as well as more heterogeneous pores. This also seems to be consistent with the relatively larger length scale for the onset of anomalous lipid diffusion for DOPC when compared with POPC as revealed in STED-FCS measurements. Our results shed light not only on the mechanistic pathway for induced nanosized proteolipid domains directed by the host cell membrane lipid composition and fluidity but also opens a new methodology to obtain insight into various biological and bio-membrane mediated processes occurring at the nanoscale.

2 Experimental

2.1 Materials and methods

Phospholipids such as DOPC (> 99% purity), POPC (> 99% purity) and DMPC (> 99% purity) and cholesterol (Chl, > 98% purity) were 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 by the self-assembly of lipids into bilayers onto a glass substrate (20 \(\times\) 20 mm, Glaswarenfabrik Karl Hecht GmbH & Co KG, Germany); this planar configuration is ideally suited for observation using fluorescence microscopy.\(^{40}\) The SLBs stained with Atto 488-PE (0.001 mol%) dye were prepared on pre-treated glass slides by using the Langmuir–Blodgett (LB) method. The lipid solutions were prepared in chloroform (HPLC grade, Sigma Aldrich) and sprayed on the water subphase for the formation of interfacial monolayers. Ultrapure water with a resistivity of 18.2 M\(\Omega\) cm was used as the subphase for all monolayer studies and was produced using a two-stage Elix-3 and Milli-Q (Millipore Academic) system. Prior to the experiment, the mini trough was cleaned with ethanol (extra pure AR grade, Fine Chemicals, India) several times and finally rinsed with ultra pure water. In addition, bilayers were also prepared using the vesicle (~100 nm) fusion method. The detailed description of the preparation of the SLBs can be found in the ESL.\(^+\) Listeriolysin O (LLO) was expressed in E. coli and purified by Ni-NTA affinity chromatography as described earlier.\(^{41}\) LLO (0.08 \(\mu\)M) was incubated with the SLBs for 30 min at 37 °C and at pH 5.4. The plasmid encoding LLO was obtained from Dr Gregor Anderlüh.

2.2 Atomic force microscopy

Atomic force microscopy (AFM) measurements were carried out using a Park System (NX 100, Korea) AFM under buffer using contact mode with a Pyrex-Nitride-Probes–Silicon Nitride (PNP–SiN) cantilever with force constant 0.08 N m\(^{-1}\). The SLBs of
pristine bilayers and LLO incubated bilayers are transferred into the liquid cell. The images were acquired at the scanning rates of 0.65 Hz, operated at room temperature 22–23 °C using a 50 μm scan head. Images obtained were processed and analyzed using the NX 100 XEI 1.8.0 program.

2.3 STED-FCS nanoscope

For imaging and FCS, we applied STED-FCS nanoscopy using a commercial CW-STED setup (SP5x, Leica Microsystems GmbH, Mannheim, Germany) with a 592 nm fiber laser for STED. The APD signal intensity as well as fluorescence correlation spectroscopy (FCS) from the SLBs was recorded using the microscope software SymPhoTime, PicoQuant coupled with Leica LAS AF software, which communicates with the PicoQuant SymPhoTime software as an already integrated FCS package in LAS AF. The detailed description for the measurement parameters is presented in the ESI.†

2.3.1 STED-FCS measurements and analysis. The SLBs (for the preparation method, see the ESI†) were placed on top of the microscope objective. The optimal z-position in the center of the SLB was chosen by maximizing the fluorescence intensity counts in the plane of the bilayer. During the measurement, the adaptive focus control was enabled to avoid a drift in the z-direction between measurements. FCS data acquisition was set for a total duration of 10 s for both confocal- and STED-FCS recordings. For each optimized bilayer position, a fixed master power of the Ar laser (25%) and a full series of STED power (with an increment of 10% from 0 to 100%) were obtained. The STED power values in mW were measured using a power meter from the top of the 10× (air) objective. The detailed descriptions of the analysis are given in the ESI.† At least 30 to 50 independent measurements were made for each sample at different positions and the FCS protocol was repeated for three independent sample sets to confirm reproducibility of the data.

3 Results

3.1 Visualization of pore assemblies

Pores formed on model supported lipid membranes were initially visualized by AFM at room temperature. Typical bilayers, composed of different lipids such as DOPC (T_m = −17 °C), POPC (T_m = −2 °C) and DMPC (T_m = 24 °C), with a fixed cholesterol (25%) were prepared using the Langmuir–Blodgett (LB) technique at highly liquid condensed (LC) surface pressures (see Fig. S1, ESI†). The topographic images of the pristine bilayers do not show any evidence of phase separation before LLO incubation (Fig. S2a–c and e, ESI†) indicative of the bilayers being, mostly, homogeneous in nature. After LLO incubation, the induced heterogeneities and pore-like features are observed for DOPC– and POPC–cholesterol bilayers as shown in AFM images in Fig. 1a and b respectively. In contrast, for DMPC:Chl bilayers no such features were observed as evidenced from Fig. 1c. It is further interesting to note that the pore–pore assembly, possibly driven by pore coalescence, is more pronounced for the DOPC:Chl bilayer leading to a heterogeneous distribution of arcs, individual pores and coalesced pores (features highlighted in blue in Fig. 1a) with pore sizes ranging from ~40 to 100 nm. The height profile analyses (bottom panel, Fig. 1a) of the corresponding green lines (as shown in Fig. 1a–c) reveal that the protrusion of LLO domains of ~3.6 ± 0.2 nm are not perpendicular to the plane of the bilayer surface as expected from earlier observations,30 but slightly splayed. This suggests that the pores are flexible and hence,

Fig. 1 Top panel shows the atomic force microscopy images of LLO incubated supported lipid bilayers of (a) DOPC:Chl, (b) POPC:Chl and (c) DMPC:Chl. In the DOPC:Chl (a) bilayers we observed arcs, fully formed pores and coalesced pores marked in blue as a, p and c respectively. Left and right panels in (b) illustrate isolated and colonies of assembled pores, respectively, in two different regions in the POPC:Chl bilayer. No evidence of pore formation was observed for DMPC:Chl bilayers (c). Bottom panel illustrates the height variation corresponding to the green line in the AFM topographical images (top).
could possibly, trigger the coalescence of oligomerized LLO pores in the DOPC:Chl host membrane (cf. Fig. S2d, ESI†). However, in the case of POPC:Chl individual pores (left panel in Fig. 1b) and intact pore colonies (right panel in Fig. 1b) could be seen, similar to earlier observations.30 Further, the protrusion above and the insertion below the membrane surface are larger than that in DOPC membranes as evidenced from the height profile shown in Fig. 1b (bottom panel). The ring shaped structure of the pores along with the absence of lipids at their center strongly suggests that these could be fully inserted pores and are likely to be functional. In addition to the pore-like features, we also observe arc-shaped aggregates along with the ring-like structures for DOPC:Chl bilayers (cf. Fig. 1a and Fig. S2d, ESI†), similar to the earlier observations for LLO.29

The wide variety of structures observed in our case are also in line with the previous reports, where the membrane reorganization upon protein/toxin incubation are directed by the lipidic host cell membranes.43–45

In contrast to DOPC:Chl and POPC:Chl bilayer membranes, incubation of DMPC:Chl bilayers with LLO did not yield such oligomerized pores or coalesced pore-like structures. The voids that are seen in Fig. 1c are possibly due to ejection of lipids induced by LLO binding without oligomerisation or pore formation. This is also clear from the corresponding typical height profile around such voids which indicate a depth of ~4 nm without any significant protrusion or rim formation above the membrane as would be expected for pore-like structures formed by the incorporation of LLO into the lipid bilayer membrane. The absence of such structures could be due to hydrophobic mismatch or to the lower fluidity and greater ordering of the saturated alkyl chains of DMPC lipid.4 We will discuss these aspects further. While the AFM images and height profile analyses of SLBs reveal the morphological perturbation induced in the bilayer membranes due to LLO, no information about the corresponding changes in lipid dynamics is revealed. Fluorescence correlation spectroscopy is the ideal technique to reveal such microscopic information on membrane lipid dynamics.46 However, before we proceed to discuss lipid dynamics, we first demonstrate the morphological features that are visible in confocal and STED super-resolution microscopy, and correlate these features with the AFM images. To visualize the LLO induced perturbation, SLBs were stained initially with Atto 488 PE (0.001 mol%).

The confocal microscopy images of pristine bilayers of DOPC:Chl, POPC:Chl and DMPC:Chl appear largely homogeneous (cf. Fig. 2a–c). In contrast, confocal microscopy images of DOPC:Chl and POPC:Chl SLBs, after LLO incubation, show significant inhomogeneities due to the redistribution of lipids as shown in Fig. 2d and e respectively. While, large scale (1–2 μm) structures are dominant in DOPC:Chl SLBs they are much less prominent in confocal images of LLO incubated POPC:Chl bilayers. Such large scale structures, which are also seen in our AFM images (Fig. 1), probably occur due to pore coalescence during LLO incubation.40 In STED mode images (at maximum power) for the same SLBs, we could clearly identify several nanoscale domains with ~100 nm resolution for both DOPC:Chl (Fig. 2g) and POPC:Chl (Fig. 2h) which is otherwise not clearly visible in their respective confocal images. The intensity line profiles from the region of interest (as marked in white lines) for both confocal and STED images for DOPC:Chl and POPC:Chl are shown in Fig. 2j and k respectively. In contrast to DOPC and POPC lipids, we did not observe such nanoscale domains and/or membrane perturbations for DMPC:Chl bilayers (Fig. 2f, i and l) after LLO incubation. The absence of such structures is consistent with our AFM images indicating inefficient pore formation for DMPC:Chl bilayers after LLO incubation. Our microscopy imaging results compare well with other toxin–membrane interaction scenarios with, for instance, α-synuclein (AS) which when interacting with small unilamellar vesicles composed of DOPC and POPC showed moderate binding affinity but did not significantly bind to DMPC.47 Although the images reveal the manifestation of LLO induced bilayer perturbation, the modulation of lipid dynamics around such proteolipid complexes can only be obtained from spatially resolved FCS data.

3.2 Spatially resolved lipid mobility around pore complexes using confocal FCS

To probe membrane lipid dynamics, we first performed spatially resolved FCS in confocal mode48–51 by carefully sampling distinctly different regions of the membranes (for representative spatially resolved points, see Fig. S3, ESI†). Fig. 3a–c shows the intensity–intensity autocorrelation function \(G(t_c)\) data for the cholesterol containing bilayers for DOPC, POPC and DMPC, respectively, before and after LLO incubation. The spatiotemporal correlation data (Fig. 3a–c) were fitted using eqn (1),33,52,53

\[
G(t_c) = \left(\frac{1}{N}\right) \frac{1}{1 + \left(\frac{t_c}{\tau_D}\right)^a} \quad (1)
\]

where \(\tau_D\) is the transit time, \(N\) is the particle number and \(a\) is the anomaly coefficient. In all the confocal FCS correlation functions \(a \sim 1\), indicating the presence of free Brownian diffusion at confocal spatial resolution. The diffusivity \(D\) in such a case can be evaluated using eqn (2):33

\[
\tau_D = \frac{d^2}{D8\ln 2} \quad (2)
\]

where \(d\) is the diameter of the confocal spot \((d = 200 \text{ nm})\). eqn (2) has a slightly different form from what is typically used in confocal FCS measurements;33,38 however, we have used eqn (2) to be consistent with STED-FCS measurements to be discussed later.

The diffusivity values for cholesterol containing SLBs made up of DOPC, POPC and DMPC were found to follow a unimodal distribution with mean values of \(3.9 \pm 0.23\), \(2.8 \pm 0.2\) and \(1.7 \pm 0.21 \text{ μm}^2 \text{s}^{-1}\), respectively, consistent with earlier results on similar membranes.54 However, upon incubating DOPC:Chl bilayers with LLO, a bimodal distribution of diffusivities (Fig. 3d) with equal populations of slower and faster (but lower in magnitude when compared with bilayers before LLO incubation) diffusivities are obtained. The lowest diffusivity component \((D = 1.1 \pm 0.21 \text{ μm}^2 \text{s}^{-1}\)) is significantly smaller than the
...diffusivities obtained for the corresponding LLO free bilayer. This lowered lipid diffusivity occurs mainly in the proximity of low fluorescence intensity regions (region I, Fig. 2d) which we attribute to the boundaries of the pore aggregates ranging from nanometer-to-micrometer in size. Lipids with diffusivity, \( D = 2.5 \pm 0.21 \, \mu \text{m}^2 \, \text{s}^{-1} \), correspond to regions away from the pore aggregates (region II in Fig. 2d).

The mean \( D \), in these regions, is also slightly smaller than that of the corresponding pristine DOPC:Chl SLBs and this reduction could be due to the presence of isolated pores which are not resolved in our diffraction-limited confocal microscopy measurements. The equal probability of the smaller and larger values of observed diffusivities could be correlated with the different types and sizes of LLO pore structures including coalescence pores and pre-pore aggregates, which exist in our AFM images, as discussed earlier. Interestingly, in the case of POPC:Chl bilayers upon LLO exposure, although we also observe a bimodal distribution of diffusivities (Fig. 3e), the distribution is strongly skewed towards smaller diffusivities (\( D = 1.2 \pm 0.22 \, \mu \text{m}^2 \, \text{s}^{-1} \)). The contribution to the smaller diffusivity component in DOPC:Chl SLBs could arise from tightly bound lipids in the vicinity of fully inserted pores and hence the nature of the diffusivity distribution could be related to a larger population of fully inserted pores when compared with a preponderance of pre-pore and arc-like aggregates observed in POPC:Chl SLBs.

In contrast to the more fluidic lipids such as DOPC and POPC, diffusivities in the DMPC bilayers are smaller and did not alter significantly after LLO incubation. After LLO incubation, the diffusivity values were found to be \( 0.8 \pm 0.14 \, \mu \text{m}^2 \, \text{s}^{-1} \) showing a unimodal distribution, overlapping with that of the LLO free bilayer. Although, we did not observe any pore-like features either...
in AFM or confocal microscopic images, the decrease in diffusivities could be due to lipid–toxin interactions which led to the ordering of alkyl chains as observed from our diffusing-wave spectroscopy (DWS) data shown in Fig. S4, ESI†. Our results are consistent with recent observation by Sharma et al.55 where the addition of melittin, an antimicrobial peptide, did not affect the membrane dynamics of a DMPC/cholesterol bilayer membrane.

3.3 Probing lipid dynamics around pore complexes using STED-FCS

Although it is well known that cholesterol is a necessary ingredient for binding of a CDC such as LLO,56 little is known about the molecular role played by different phospholipids towards the propensity for CDCs to bind and initiate pore-formation.57,58 Overall, our confocal FCS data provide evidence of large scale (~μm) heterogeneous lipid dynamics induced by LLO interaction with DOPC:Chl and POPC:Chl membranes. Variations in the probability and width of the diffusivity distributions (Fig. 3d and e) after LLO binding onto POPC and DOPC membranes suggest the presence of possible sub-micron scale lipid dynamics around pore assemblies which are not captured by the optical diffraction limited FCS data. Therefore, probing lipid dynamics on the scale of single or coalesced pores could provide information about the rigidity or flexibility of the pores as well as their ability to re-organize the lipids around these self-assembled structures. For this purpose, we performed FCS in super-resolution STED mode53 by varying the STED excitation power to reduce the observation volume to well below the diffraction limit (Fig. S5, ESI†). Figure 4a–c describes the dependence of the transit time \( t_D \) as a function of the various observation spots \( \left(d^2\right) \) created by STED laser power, as extracted from the respective STED power dependent autocorrelation data before and after LLO incubation. In order to present a unified picture of the lipid dynamics in the absence and presence of LLO, and to highlight the origin of crossover to the non-Brownian regime,59 the \( \tau_D \) versus \( d^2 \) variation can provide the underlying diffusion mechanism in the studied system. To unravel this, eqn (2) can be re-written as

\[
\tau_D = \frac{d^2}{8D\ln 2} + t_0
\]

where \( d \) is the diameter of the confocal or STED focal spot and \( t_0 \) is an intercept.60–65 For free diffusion, \( t_0 = 0 \) while it can take positive or negative values according to various possible mechanisms of hindered diffusion involving the presence of nanodomains or a meshwork structure.54,60,64–69 The details of the fitting procedure used to obtain the values of \( \tau_D \) and \( d^2 \) are given in Fig. S6 (ESI†) and Fig. 4d–f respectively.

The \( \tau_D \) values, before LLO incubation, as shown in Fig. 4 decrease in the order of DMPC:Chl > POPC:Chl > DOPC:Chl and the magnitude of \( \tau_D \) is always less when compared with the LLO incubated samples irrespective of any STED power used. The linear decrease in \( \tau_D \) with the decrease in the focal spot area with a zero intercept \( (t_0 = 0) \) for pristine bilayers signifies Brownian diffusion52,70–72 at all observed length scales \( d \) ranging from 80 to 200 nm (cf. open circle in Fig. 4a–c). In contrast, for LLO incubated DOPC:Chl and POPC:Chl bilayers, a distinct dynamical crossover is observed at a length scale, \( \zeta \), of ~125 and ~108 nm respectively (cf. vertical line in Fig. 4a and b). Further, the onset of this dynamical crossover is also associated with a decrease in the value of \( x \) for LLO incubated DOPC:Chl and POPC:Chl bilayers, indicative of anomalous diffusion in this regime. Such an anomalous diffusive regime is not observed for DMPC:Chl bilayers down to the smallest \( d^2 \) values accessible using our STED microscope. Further, we fitted the data in the respective dynamical regimes using eqn (3) to extract the values of intercept, \( t_0 \), as indicated in the respective panels of Fig. 4a–c. Interestingly, it is also clear that the region of the data below \( \zeta \) does not follow a Brownian diffusion law53,60,64,67 with clear positive intercepts of \( t_0 \sim 1.9 \) and ~1 for DOPC:Chl and POPC:Chl, after LLO incubation, respectively, suggestive of
The presence of nanodomains. No such crossover is observed for LLO incubated DMPC:Chl bilayers and the linear fit yielded a $t_0$ of $\sim 0.1$ (Fig. 4c) indicative of a possible weak deviation from Brownian diffusion but it was not significant enough to warrant further evaluation. In addition, a distinct change of slope was not discernible at the smaller $d$ values. However, it is difficult to conclude whether a dynamical crossover is clearly absent in LLO incubated DMPC:Chl bilayers or it lies below the spatial resolution of our STED microscope. Although the essential nature of membrane lipid dynamics is captured by the data presented in Fig. 4, the universal properties of the lipid dynamics can be obtained from the diffusivity data. To ascertain the origin of crossover from the Brownian to the non-Brownian regime as seen in Fig. 4a and b, we continued to evaluate diffusion coefficient values using eqn (2).

Fig. 5 shows the extracted diffusion coefficient values as a function of focal spot area for the lipids before LLO incubation (see, red, olive and magenta (open circles), respectively, for cholesterol containing DOPC, POPC and DMPC lipids). Consistent with the Brownian diffusion law (of $x \sim 1$, refer Fig. 4d-f) followed in the corresponding data in Fig. 4, we find a length scale independent diffusivity, $D$, for LLO free lipids down to the smallest values of $d^2$ that could be obtained with our STED microscope. In contrast, in the presence of LLO in the membranes the diffusivity no longer remains constant, as assumed in eqn (2), below $\xi$ (cf. filled circles in Fig. 5a and b), but a diffusivity which depends on the observation time scale emerges, i.e., $D_{\text{app}}$ is actually $D(t_D)$ when $x < 1$. We recently observed such a dynamical crossover in DOPC bilayers with an increase in cholesterol concentration and ascribed this to the emergence of nanoscale cholesterol enriched lipid domains. The fact that very similar behavior is observed with LLO incubation, which, as mentioned already, has strong affinity for cholesterol, strongly suggests possible nanoscale cholesterol enriched lipid domain formation around the LLO pores. In addition, we have also extracted spatially averaged diffusivity from the slopes of the so-called diffusion law data in Fig. 4, using eqn (3) for the non-Brownian regimes as well, and referred to this as an effective diffusivity, $D_{\text{eff}}$. The extracted $D_{\text{eff}}$ values in the $d^2 < \xi$ regime are also shown for DOPC:Chl and POPC:Chl after LLO incubation in Fig. 5a and b respectively. It is interesting to note that the extent of change observed for DOPC:Chl bilayers upon LLO incubation is maximum when compared to that of the corresponding POPC:Chl and DMPC:Chl bilayers. The decrease in the magnitude of apparent diffusivities, $D_{\text{app}}$, with LLO incubation as a function of different lipids is particularly revealing. The value of $D_0/D$ decreases from $3.5 > 2.3 > 2$ with decreasing fluidity of lipids DOPC > POPC > DMPC, respectively, as shown in the free Brownian diffusion regime I ($d > \xi$) in Fig. 5d. For $d < \xi$ (see non-Brownian diffusion regime II, shaded region), the greatest perturbation of the lipid dynamics is observed in the case of DOPC:Chl bilayers as compared to POPC:Chl and DMPC:Chl bilayers. When the phospholipid's unsaturation decreases, i.e.,
in the case of POPC:Chl bilayers, the extent of cholesterol sequestration also decreases. The observed decrease in length scale heterogeneities for POPC:Chl (see vertical line in Fig. 5b) than DOPC:Chl bilayers could be due to the differences in pore formation where pore coalescence is significantly perturbed for the former bilayer. On the other hand, for fully saturated lipid bilayers (DMPC:Chl), we did not observe any pore-like features either in AFM or in confocal microscopic images.

4 Discussion

We now try to correlate the information we obtained about the morphology of LLO pores on the lipid membranes from AFM and confocal/STED images with the results of the confocal- and STED-FCS data. It seems clear that LLO forms regular, more heterogeneous and smaller compact pores in POPC:Chl SLBs as compared to DOPC:Chl consistent with earlier observations.29 If we couple this with the well established fact that LLO, in particular, and CDCs, in general, bind to cholesterol in membranes, it seems logical to assume that the formation of oligomerized pre-pore assembly and inserted pores should result in cholesterol enrichment around these LLO structures. Our earlier work74 connects this cholesterol enrichment to the observed length scale dependent emergence of anomalous dynamics in the DOPC membrane upon interaction with LLO. The enrichment was found to be most pronounced at 25% cholesterol which is the composition used in this study.74 One can then view the role of LLO as a creator of nanodomain regions where cholesterol concentration is enhanced around the pore assemblies, although the native lipid–cholesterol combination in two-component mixtures is not expected to show such domains or phase separation at the cholesterol concentrations used in our study.75–77 Even this nanodomain formation is undetected in our conventional confocal FCS measurements except for the occurrence of lipid regions with smaller diffusivity. However, STED-FCS not only reveals such domain formation but also provides some quantitative estimation of the length scale, $\xi$, of such domains below which the lipid diffusivities are significantly perturbed. It is also possible to estimate the domain sizes from the FCS diffusion law (Fig. 4) using the value of the extracted intercept, $t_0$, as has been alluded to earlier.53,67 However, we have refrained from carrying out this analysis here. Further, we find that this dynamical crossover length scale, for the same LLO and cholesterol concentration in membranes, depends on the lipid mobility. This suggests that the ability to sequester cholesterol around the membrane by LLO protomers to facilitate their oligomerization and subsequent pore formation depends crucially on the fluidity of the underlying membrane. Large lipid fluidity, as in the case of
DOPC, while being beneficial for cholesterol migration, and ease of pore formation also imply highly mobile and less compact pores which can easily coalesce often resulting in larger pores with lower stability as observed from our AFM images.\textsuperscript{29,30} Apart from these aspects, it is also likely to lead to polydispersity in pore sizes and morphological heterogeneity and pore–pore migration as evidenced in our AFM and confocal images in Fig. 1 and 2 as well as in other studies.\textsuperscript{45,78} On the other hand, POPC with intermediate fluidity allows sufficient mobility of cholesterol to form optimal, stable, compact pores but the reduced mobility of the pores (embedded in a lower mobility lipid membrane) significantly decreases the opportunity for pore coalescence. What this also implies is that the length scale, $\zeta$, of crossover to anomalous diffusion, is correlated with the region around LLO pores where cholesterol and lipid density heterogeneity exist. The smaller value of $\zeta$ for POPC membranes compared to DOPC signifies this subtle interplay between lipid mobility, pore morphology and stability.

Earlier it has also been proposed that the LLO could behave as a potent lipid domain aggregator\textsuperscript{79} but the mechanism of membrane response to LLO incubation was not clear therein due to the small length scale involved for both pore formation (10–50 nm) or lipid nanodomains (also of similar size) and the dynamic nature of the potential interaction and response. With our STED-FCS measurements we are able to connect our length scale dependent data and our proposed mechanism of cholesterol sequestration, which depends solely on the nature and physical properties of the bilayer during LLO interaction. Hence, our STED excitation power dependent FCS results clearly suggest that in the presence of LLO, the formation of nanoscale domains is driven by cholesterol sequestration, when the fluidity of the bilayer is high. The lateral diffusivity and the length scale dependent dynamic heterogeneity at a sub-optimal cholesterol content are in line with the recent theoretical predictions by Hub et al.\textsuperscript{80} suggesting the variation of local cholesterol enrichment modulating the probability of pore formation of LLO. It is interesting to note that an optimal membrane lipid fluidity seems to be most suitable for stable, membrane inserted, LLO pore formation. Higher lipid mobility seems to lead to a larger length scale of cholesterol sequestration and a consequent formation of larger, possibly, partially inserted and unstable pores which are able to further fuse to create pore complexes due to both higher lipid mobility and lipid induced protein–protein interactions.\textsuperscript{81,82} On the other hand, stiffer lipids or lipids with lower mobility such as DMPC are clearly not favorable to play an important role in regulating signal transduction, inter- and intra-cellular transport, and lipid sorting.\textsuperscript{83,84}

5 Conclusions

Using super-resolution STED-FCS we have elucidated the LLO induced lipid reorganization pathways on supported bilayer platforms. Our results reveal the intricate coupling between protein oligomerization and emergence of nanoscale regions ($\sim$100 nm) around pore complexes. Using STED-FCS we have shown that the induced lipid dynamical heterogeneity is most prominent in bilayers with greater fluidity than the gel-like lipids upon incubation with LLO. Our microscopic images revealed pore–pore coalescence which is more prominent for lipids with greater fluidity and is completely arrested when the fluidity of the bilayer is decreased. Thus there seems to be a connection which emerges between membrane fluidity, onset of cholesterol and LLO mediated lipid dynamical heterogeneity and the stability and nature of the resultant pores formed. Our study highlights the subtle interplay of lipid membrane mediated protein assembly and lipid fluidity in determining proteo-lipidic complexes formed in biomembranes and the significant insight that STED microscopy provides in unraveling the critical aspects of nanoscale membrane biophysics.

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