

the difference in D between lipids and prothrombin is growing with an increasing content of dioleoyl-phosphoserine (DOPS) in the bilayer [116] indicating a specific interaction between prothrombin and DOPS [164]. Forstner *et al.* investigated the binding of cholera toxin subunit B to dimyristoyl-phosphocholine (DMPC) SLBs containing ganglioside GM₁. The decrease in D was most pronounced close to the main phase transition of the lipids, when the crosslinking of GM₁ by cholera toxin has the greatest impact on the lipid order [165]. A recent FCS study of protein diffusion in GUVs reported a linear decrease of the protein and lipid diffusion coefficients with an increasing protein concentration. The authors concluded that at protein densities $\sim 25,000 \mu\text{m}^{-2}$ typical for biological membranes, the diffusion coefficients would be about an order of magnitude lower than the values measured in GUVs at protein density $3,000 \mu\text{m}^{-2}$ [158].

Binding of antimicrobial or cytolytic peptides to lipid membranes also affects the lipid diffusion coefficient in a manner dependent on the mechanism of interaction of the given peptide with membranes. The presence of the peptide molecules is not the only cause for the change in D since most antimicrobial and cytolytic peptides are known to create pores or other perturbations in the membranes [166–168]. Sheynis *et al.* reported a decrease in D caused by melittin and magainin II, but no effect of an artificial peptide KAL (KKA(LA)₇KK), which corresponds to deeper insertion and smaller surface effects of KAL [169]. We have observed a decrease in lipid D to approximately 60% of its original value after treatment of a SLB with 1 μM melittin. The conclusion that pores are responsible for the large decrease is supported by a significant loss of lipids from the bilayer [170]. A large decrease in D was also observed in SLBs treated with 1 μM cryptdin-4 [171]. Removal of cryptdin-4 from the sample by washing it with an excess of a clean buffer resulted in a partial recovery towards the original values of D . Washing away melittin, however, did not change the D suggesting a difference in the membrane perturbations induced by the two peptides [170,171].

5.2. Supported versus Free-standing Planar Lipid Membranes

GUVs representing free-standing lipid membranes and being in size similar to cells are certainly the most realistic artificial model of plasma membrane of living cells. However, their preparation protocols are rather demanding and the most widespread ones are limited to low ionic strengths [54,55,172], although protocols allowing GUV preparation under physiological conditions have been also described [173]. SLBs are, on the other hand, a considerably less realistic model system, but very easy to prepare and stable and, thanks to their very well-defined geometry, accessible to characterisation by a wide range of experimental techniques [58,174–176]. They are formed on hydrophilic surfaces such as mica, glass, fused silica [51,175] or self-assembled alkanethiol monolayers [177,178] via adsorption and fusion of lipid vesicles [175,179,180] or via Langmuir–Blodgett and Langmuir–Schaefer techniques [59,181,182]. Although the lipid bilayer is separated from the solid surface by a thin aqueous layer (in the order of nm), thanks to which the bilayer retains its fluidity [183–185], the proximity of the support has a significant influence on the properties of the lipid membrane.

The first direct quantitative comparison of lipid diffusion in free-standing and supported lipid membranes was published by Przybylo *et al.* [107]. The relatively broad distribution of previously published values of lipid diffusion coefficients in GUVs (ranging from 3–6.5 $\mu\text{m}^2\text{s}^{-1}$ for the same lipid

composition [87,153,186]) and SLBs (for example 2.6 or 4.2 $\mu\text{m}^2\text{s}^{-1}$ [22,187]) did not allow quantitative conclusions on the effect of the solid support on lateral lipid mobility. Apart from possible errors caused by inaccurate calibration in single-point FCS, the different experimental conditions such as ionic strength and sugar concentration are probably responsible for the incomparability of the individual results. While GUVs are usually investigated under very low ionic strengths (required in the typical preparation protocols) [55,60,188], physiologically more relevant conditions (100 or 150 mM NaCl) are common in SLB studies [22,97,136,170,187]. A decrease in lipid diffusion coefficient with an increasing concentration of NaCl has been observed both by FCS and molecular dynamics simulations [47,189]. Furthermore, GUVs are often stabilized by sugars such as glucose or sucrose [29,107,190,191]. FCS experiments and molecular dynamics simulations have shown slower lipid diffusion in the presence of various monosaccharides and disaccharides attributed to hydrogen bonding between a sugar molecule and phosphate groups of several lipid molecules [190,192,193]. Sucrose produces the strongest effect reducing the diffusion coefficient of lipids up to 3 times (at 1.5 M concentration) [190,193]. Przybylo *et al.* performed Z-scan FCS on GUVs and SLBs under identical conditions (150 mOsm glucose solution) and found that the lipid diffusion coefficient in DOPC GUVs $D_{GUV} = (7.8 \pm 0.8) \mu\text{m}^2\text{s}^{-1}$ is more than 2 times higher than in SLBs of identical lipid composition on mica with $D_{SLB} = (3.1 \pm 0.3) \mu\text{m}^2\text{s}^{-1}$. The finding is supported by the results of later studies [77].

Another limitation of SLBs is the fact that the very small distance between the proximal leaflet and the solid surface may prevent correct reconstitution of transmembrane proteins into the bilayer. To increase the space available on both sides of the membrane while maintaining the convenient geometry of SLBs, membranes on soft polymer layers (polymer-cushioned bilayers) [180,194–196] or linear polymer spacers covalently coupled to lipid head groups (polymer-tethered bilayers) [197–200] have been developed. Polymer-tethered bilayers were used for example in a recent scanning-FCS study of G protein-coupled receptor diffusion [201]. However, the tethered lipids may act as obstacles and hinder the diffusion in the planar membrane [146,198]. An alternative free-standing planar lipid membrane can be prepared by spreading a bilayer over an aperture (40–150 μm in diameter) in a polytetrafluoroethylene septum. The diffusion coefficient of lipids in such a model membrane determined by FCS ($8.1 \pm 0.4) \mu\text{m}^2\text{s}^{-1}$ corresponds to the values measured in GUVs [202].

5.3. Inter-leaflet Coupling and Membrane Asymmetry

The membranes of living cells are known to be asymmetric; the two leaflets of the membrane differ in their lipid and protein composition and they also face different aqueous phases [203,204]. It is, therefore, very interesting to know how strong is the interaction between the membrane leaflets and how are the dynamic properties of one leaflet related to those of the other. Such questions are of a high relevance for biology; namely the question of how do the structural and dynamic parameters of the cytosolic leaflet of the plasma membrane reflect the changes in the outer leaflet induced by lipid phase separation or peripheral binding of other molecules to the membrane.

In terms of artificial lipid membranes, the question of inter-leaflet coupling was addressed by Przybylo *et al.*, who concluded that a strong inter-leaflet coupling exists in SLBs and lipids in both leaflets diffuse with the same velocity [107]. The argumentation was based on the approximately

2-fold difference in lipid diffusion among supported (SLBs) and free-standing bilayers (GUVs). In the absence of a strong inter-leaflet coupling, the lipids in the distal leaflet should diffuse like lipids in GUVs and the lipids in the proximal leaflet would have to be approximately 4 times slower. Since FCS can reliably distinguish contributions from molecules which differ at least 1.6–2 times in their diffusion coefficients [77,205], such a large difference between proximal and distal leaflet would result in two distinct values of diffusion time measured in SLBs. The measured autocorrelation curves could be, however, fitted successfully with a model containing a single diffusion time, indicating, thus, a strong inter-leaflet coupling. Zhang and Granick arrived to the same conclusions when they selectively quenched the fluorophores in the distal leaflet by iodide [68,187,206]. They acquired evidence for a strong inter-leaflet coupling in SLBs on quartz prepared both by vesicle adsorption and fusion and by Langmuir-Blodgett technique. The same effect was reproduced in SLBs on polymer cushions [206] and the strong inter-leaflet coupling was also found in experiments when the diffusion in the distal leaflet was slowed down by polymer binding [68,187].

A recent FCS study investigated the diffusion of poly-lysine in free-standing planar membranes and its influence on lipid diffusion. D for both poly-lysine and lipids decreased with the number of lysine units in the polymer and an evidence for strong inter-leaflet coupling of poly-lysine diffusion was found, indicating that two poly-lysine molecules on the two leaflets of the bilayer move together with lipids sandwiched between them forming a nanodomain [202]. Such alignment of lipid domains is likely to play an important role in transmembrane signalling.

6. Concluding Remarks

FCS methodology for investigation of the lateral mobility of molecules in planar systems has undergone a rapid development in the past decade, which has been motivated mainly by the interest in mobility of molecules in biological membranes and their artificial models. The currently available FCS approaches, which include for example Z-scan FCS, 2-focus FCS or many variants of scanning FCS and imaging FCS, have overcome all the important limitations encountered originally in FCS measurements on planar samples. They have solved the problem with exact positioning of the planar sample into the focus of the microscope and they do not need external calibration. Some of the methods, such as RICS, can access a very broad range of diffusion coefficients, practically the whole range of diffusion coefficients of molecules in lipid membranes. Other methods, such as TIR FCS or FLCS with lifetime tuning, are capable of suppressing a fluorescence background not originating from the planar sample of interest, while yet other techniques can achieve a considerable reduction of the detection volume in all directions and, thus, allow FCS measurements with a high spatial resolution and at higher concentrations of fluorescent tracer molecules. To conclude, state of the art FCS methodologies represent a versatile and efficient tool for investigation of diffusion (and other dynamic processes) in lipid membranes. They combine single molecule sensitivity with reasonably short measurement times acceptable for routine essays. Furthermore, all the FCS methods described here can be used for investigation of membranes of living cells or even multicellular organisms [207] and can, therefore, help to relate the molecular diffusion in model and native membranes. It is certain that with such potential, FCS will help to bring answers to many open questions of current membrane biology and biophysics.

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