Concentration dependence of lipopolymer self-diffusion in supported bilayer membranes

Huai-Ying Zhang and Reghan J. Hill*

Department of Chemical Engineering, McGill University, Montreal, Quebec, Canada H3A 2B2

Self-diffusion coefficients of poly(ethylene glycol)2k-derivatized lipids (DSPE-PEG2k-CF) in glass-supported DOPC phospholipid bilayers are ascertained from quantitative fluorescence recovery after photobleaching (FRAP). We developed a first-order reaction—diffusion model to ascertain the bleaching constant, mobile fraction and lipopolymer self-diffusion coefficient $D_s$ at concentrations in the range $c \approx 0.5$–5 mol%. In contrast to control experiments with 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt) (DOPE-NBD) in 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), the lipopolymer self-diffusion coefficient decreases monotonically with increasing concentration, without a distinguishing mushroom-to-brush transition. Our data yield a correlation $D_s = D_0/(1 + ac)$, where $D_0 \approx 3.36 \mu m^2 \text{s}^{-1}$ and $a \approx 0.56$ (with $c$ expressed as a mole percent). Interpreting the dilute limit with the Scalettar–Abney–Owicki statistical mechanical theory for transmembrane proteins yields an effective disc radius $a_0 \approx 2.41$ nm. On the other hand, the Bussell–Koch–Hammer theory, which includes hydrodynamic interactions, yields $a_0 \approx 2.92$ nm. As expected, both measures are smaller than the Flory radius of the 2 kDa poly(ethylene glycol) (PEG) chains, $R_F \approx 3.83$ nm, and significantly larger than the nominal radius of the phospholipid heads, $a_0 \approx 0.46$ nm. The diffusion coefficient at infinite dilution $D_0$ was interpreted using the Evans–Sackmann theory, furnishing an inter-leaflet frictional drag coefficient $b_\perp \approx 1.33 \times 10^8 \text{ N s m}^{-3}$. Our results suggest that lipopolymer interactions are dominated by the excluded volume of the PEG-chain segments, with frictional drag dominated by the two-dimensional bilayer hydrodynamics.

Keywords: lipopolymers; phospholipid bilayer membranes; self-diffusion coefficient; fluorescence recovery after photobleaching

1. INTRODUCTION

The well-defined and easily controlled composition of reconstituted phospholipid bilayers makes them excellent models for studying cellular membranes. Lateral diffusion is of particular interest because it plays an important role in cellular processes such as cell signalling [1] and adhesion [2,3]. Consequently, there has been a concerted effort in the literature to understand the connection between lateral diffusion and membrane physics. A detailed discussion of the functional role of lateral diffusion is found in a review by Saxton [4].

Saffman & Delbruck [5] first modelled lateral diffusion of a single molecule in membranes using continuum hydrodynamics. The Saffman–Delbruck equation successfully describes transmembrane protein diffusion at vanishingly small concentrations [6]. At finite concentrations, however, the diffusion coefficient is ostensibly concentration dependent [6]. This concentration dependence is due to non-specific protein–protein interactions, including direct (thermodynamic) and hydrodynamic interactions [7,8].

In interacting systems, self-diffusion and gradient diffusion must be distinguished. Self-diffusion—also termed tracer diffusion—quantifies the mean-squared displacement of a single molecule. In two dimensions, the self-diffusion coefficient $D_s$ is defined by $\langle r^2 \rangle = 4D_s \Delta t$, where $\langle r^2 \rangle$ is the mean-squared displacement in a time interval $\Delta t$ under Brownian forces. Gradient diffusion—also termed mutual diffusion—is the macroscopic flux of particles due to Brownian forces and a concentration gradient. The gradient diffusion coefficient $D_g$ appears in Fick’s law [9].

Theoretical modelling of self- and gradient diffusion is extensive. Direct interactions, including hard-core repulsion and soft interactions, were analysed by Scalettar et al. [7] and Abney et al. [8,10] using the statistical mechanical theory of fluids. The influence of excluded volume on self-diffusion from the continuum approach is in close agreement with the lattice models of Pink [11] and Saxton [12]. In addition to thermodynamic interactions, Bussell et al. [13–15] studied hydrodynamic interactions, achieving better agreement with the measured concentration dependence of protein self-diffusion coefficients.
Recently, Deverall et al. [16] experimentally observed self-diffusion of lipids and proteins obstructed in lipopolymer-containing bilayers. The obstacles were hydrophobic lipopolymers (lipid-mimicking dioctadecylamine moieties) in the bottom leaflet of model membranes, with bulk lipids (without polymer attached) in the upper leaflet. Theoretical interpretation of the results suggested that lipopolymers act as immobile obstacles to lipid and protein diffusion. Soong and coworkers, however, have shown (using NMR spectroscopy) that the mobility of poly(ethylene glycol) (PEG)-lipids in magnetically aligned birefringence decreases with increasing lipopolymer concentration [17]. Most recently, Albertorio et al. [18] studied mobile PEG-lipids in planar supported lipid bilayers (SLBs). Fluorescence recovery after photobleaching (FRAP) experiments revealed a lipopolymer self-diffusion coefficient that decreases with increasing concentration, but only at concentrations above the mushroom-to-brush transition.

In contrast to transmembrane protein diffusion, theoretical understanding of lipopolymer diffusion is poor, and few systematic experimental studies have been undertaken. Such studies may help to understand the dynamics of phospholipid-anchored membrane proteins that bear large head groups, which are known to obstruct membrane fluidity [19]. Such knowledge would also guide the engineering of technological devices based on lipopolymer-grafted bilayers. For example, phospholipids bearing PEG chains can be used to prolong vesicle circulation time in drug delivery [20], enhance bio-compatibility on the surfaces of implantable materials [21], provide air and fluid stability of bilayers used in microfluidic diagnostics [18,22], eliminate bilayer–substrate interactions that immobilize proteins in solid supported bilayers [23], and mimic glycolalix in cell adhesion [24] and ligand–receptor binding [25].

FRAP is the most widely adopted technique for measuring self-diffusion coefficients, mainly because it can be conducted using readily available confocal microscopes. Other techniques, such as fluorescence correlation spectroscopy (FCS) and single particle tracking (SPT), require more specialized instrumentation [26]. FRAP is also used for studying transport in cellular membranes [27–29], protein dynamics [30–33], mRNA mobility [34], signal transduction [35,36] and in vivo cellular binding [37–39].

Qualitative assessment of FRAP is often simple and straightforward, but quantitative interpretation requires special care. For example, failing to meet the requirements for accurate FRAP has hindered understanding dynamics in biological systems, and has even lead to erroneous results [see 39–41, for examples]. Accordingly, much effort has been devoted to improve FRAP accuracy. For example, FRAP fitting models have been extended from a uniformly bleached circle [27,42] to a Gaussian bleached spot [27,43], a uniformly bleached rectangle [44] and an arbitrary geometry with an arbitrary initial profile [45]. Moreover, violating the assumption of an infinite fluorochrome reservoir when working with cells has been corrected by adopting a finite sized reservoir in the fitting model [38,39,44]. Other sources of uncertainty, such as finite bleaching time, cell movement and bleaching reversibility, have been addressed to various extents [38,46].

One important issue—the intensity loss during acquisition—is not fully resolved. Acquisition bleaching must be distinguished from intentional FRAP bleaching, and should be minimized by carefully choosing experimental parameters, such as imaging time and illumination intensity.

Experimental parameters must also achieve an acceptable signal-to-noise ratio. This makes acquisition bleaching difficult to avoid and, thus, necessitates correcting raw data [46]. Several corrective methodologies have been used in the literature, but, according to Mueller et al. [39], none are completely satisfactory. Indeed, Mueller et al. [39] recently devised new approach, which has been theoretically corroborated [47], to correct acquisition bleaching. Nevertheless, the authors still find acquisition bleaching artefacts in the apparent binding rates of site-specific transcription factors to chromatin [39].

To address the foregoing gaps in the literature, we undertook a systematic study of the concentration dependence of the self-diffusion coefficient of lipopolymer DSPE-PEG2k-CF (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol) 2000-N-carboxyfluorescein]) in glass-supported DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) bilayers. To rigorously analyse FRAP, we developed a reaction–diffusion FRAP model that accounts for acquisition bleaching and a finite fluorochrome mobile fraction. This furnished a correlation that quantifies how lipopolymer interactions hinder self-diffusion. Interestingly, our experiments do not reveal a distinct mushroom-to-brush transition. Rather, we observe a smooth transition from the dilute to semi-dilute regimes, echoing the smooth variation in spreading pressure [48]. Noteworthy is that theoretical interpretation of our data at small, but finite, lipopolymer concentrations furnishes reasonable PEG-chain dimensions and other physical characteristics of the lipids. Overall, drag forces are dominated by the two-dimensional hydrodynamics of the lipid tails in their respective leaflets, with interactions in the dilute limit dominated by the excluded-volume of the PEG-chains in random coil configurations with weak hydrodynamic interactions.

The paper is set out as follows. Section 2 describes bilayer synthesis and data collection. In §3, we set out the FRAP model for handling acquisition bleaching and an immobile fraction. Section 4.1 uses simulated FRAP data to test existing methodologies for correcting acquisition bleaching. We apply the FRAP model in §4.2 to extract from the experiments the self-diffusion coefficient, mobile fraction and bleaching constant. The concentration dependence of the self-diffusion coefficient is examined in §4.3. We compare our results with the available literature (§4.4) and theoretically interpret these data to furnish PEG-chain dimensions (§4.5) and inter-leaflet friction coefficient (§4.6). Section 5 provides a concluding summary.

2. Experimental

2.1. Bilayer synthesis

SLBs were prepared by vesicle fusion following literature procedures [49] with minor modifications. First, a
mixture of lipids containing 2 mg 1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, Avanti Polar Lipids, Alabaster, AL, USA) and a desired concentration of lipopolymer 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol)2000-N’-carboxyfluorescein] (DSPE-PEG2k-CF, Avanti Polar Lipids, Alabaster, AL, USA) in chloroform was dried under a stream of nitrogen gas, followed by desiccation under vacuum for 2 h before reconstituting in buffer (10 mM phosphate, 100 mM NaCl, pH 7.4) to 2 mg ml\(^{-1}\). The lipid mixture was extruded 20 times through a 100 nm polycarbonate membrane, and then another 20 times through a 50 nm polycarbonate membrane (Avanti Polar Lipids, Alabaster, AL, USA) to form small unilamellar vesicles (SUVs). The SUVs were deposited on pre-cleaned glass cover-slips (VWR, Ville Mont-Royal, QC, Canada) to form lipid bilayers by vesicle fusion. The cover-slips were first boiled in 7X solution (MP Biomedical, Solon, OH, USA) for 30 min, rinsed excessively with reverse osmosis (RO) water, dried under a stream of nitrogen gas, and further cleaned by piranha etching for 20 min in a solution of 3:1 (v/v) concentrated sulphuric acid (H\(_2\)SO\(_4\)) and 30 per cent hydrogen peroxide (H\(_2\)O\(_2\)). The cover-slips were then rinsed excessively with RO water, dried under a stream of nitrogen gas and used immediately. Control experiments were performed with 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2,1,3-benzoxadiazol-4-yl) (ammonium salt) (DOPE-NBD) (Avanti Polar Lipids, Alabaster, AL, USA) in DOPC. All chemicals, except where otherwise stated, were used as purchased (Sigma Aldrich, Oakville, Ontario, Canada).

### 2.2. Fluorescence intensity and fluorochrome concentration

FRAP interpretation is based on an assumption that fluorescence intensity is proportional to fluorochrome concentration. This is valid only when the fluorochrome concentration is sufficiently low. Otherwise, photons are subject to quenching by multiple absorption and emission [50]. Generally, the fluorescence intensity can be expressed as \(I = QS_0 (1 - e^{-kt})\), where \(S_0\) is the illumination intensity, \(Q\) is the fluorochrome quantum yield and \(A\) is the absorbance [51]. The amount of light absorbed by the fluorophores is proportional to their concentration, so \(A = kc\), where \(k\) is a constant and \(c\) is the concentration. Accordingly, \(I = QS_0 (1 - e^{-kt}) \approx QS_0 kc\) when \(kc \ll 1\).

Epifluorescence images of DOPC bilayers doped with \(c \approx 0, 0.5, 1, 2, 3\) and 5 mol\% DSPE-PEG2k-CF are shown in figure 1a. These were obtained using a Nikon 2000-U inverted microscope with a 10 × /0.25 air-immersion objective. The specimen was illuminated with a mercury lamp filtered through two neutral filters (ND8 and ND4). Fluorescence was imaged with a green filter (C82465, Chroma Technology, Brattleboro, VT, USA) and a digital CCD camera (CoolSNAP ES, Photometrics, Tucson, AZ, USA). Intensity in the central circular region with radius 8.5 \(\mu\)m of each image was collected, and intensity in the image for \(c = 0\) mol\% was subtracted as a background level for all images of bilayers containing DSPE-PEG2k-CF. In figure 1b, an exponential fit gives \(I/I_0 \approx 1 - e^{-0.19c}\) (solid line), suggesting that a linear approximation is acceptable when \(c \lesssim 5\) mol\%. Similar measurements for DOPE-NBD in DOPC bilayers with the same filters give \(I/I_0 \approx 1 - e^{-0.11c}\). Based on these data, subsequent self-diffusion coefficient measurements were limited to concentrations \(c \lesssim 5\) mol\%.

### 2.3. FRAP data collection

Bilayers were imaged with a Zeiss LSM510 confocal laser scanning microscope using a 63 × /1.4 oil-immersion objective and a 488 nm argon ion laser (25 mW) with intensities in the range 0.1—0.5%. The image size was 68 × 68 \(\mu\)m with resolution 256 × 256 pixels. FRAP spots (circles with radius \(r_0 \approx 5.13 \mu\)m) were produced using laser intensities in the range 10—100%. Two images were captured immediately before FRAP bleaching to ascertain the initial average intensity \(I_0\). To minimize the FRAP bleaching time, a single bleach of approximately 45 ms duration was adopted. The scan time to acquire the first post-bleaching image is approximately 500 ms if the entire image is scanned. However, this provides an initial condition that is closer to a Gaussian profile, which is problematic when fitting FRAP models for uniformly bleached circles [40,52]. Thus, for the diffusion coefficients reported in §4.3, only the FRAP spot and a circular reference spot were imaged (e.g. figure 5). This achieves a threefold reduction of the imaging time to approximately 150 ms, there by achieving a nearly uniform initial bleach.

### 2.4. FRAP data analysis

Raw images (16 bits) were transferred to tag image file format (TIFF), which guarantees lossless compression, using the IMAGEJ software (W. Rasband, National Institutes of Health, Bethesda, MD, USA), with quantitative analysis undertaken using Matlab (The MathWorks, Natick, MA, USA). Numerical solutions of a cylindrically symmetric reaction–diffusion model were fitted to the FRAP time series to obtain the self-diffusion coefficient \(D_m\), mobile fraction \(f_m\) and a photobleaching rate constant \(k\). Details of the reaction–diffusion model are provided in §4.3. Fitting the model to experimental data was undertaken using the Matlab function ‘lsqcurvefit’. Further details of the fitting procedure are provided in §4.2.

### 3. REACTION–DIFFUSION FRAP MODEL

We model fluorescence intensity by defining \(n\) as the concentration (number per unit area) of active fluorochromes in the bilayer. Assuming the fluorescence intensity \(I\) is proportional to \(n\), the intensity of a uniform, uniformly illuminated bilayer satisfies

\[
\frac{\partial I}{\partial t} = -kI, \tag{3.1}
\]
where \( k \) is the photobleaching rate constant. This yields an exponential decay in intensity, with a time constant \( \tau_b = k^{-1} \). Although the photobleaching mechanism is much more complicated [53], the first-order model faithfully captures the photobleaching kinetics in our experiments. For example, time series for \( c \approx 1 \text{ mol}\% \) fluorescent lipopolymer DSPE-PEG2k-CF in DOPC bilayers with uniform illumination at two laser intensities are shown in figure 2. Fitting exponential decays to these data furnishes \( \tau_b \approx 6.3 \) and 18.5 s with high- and low-power illumination, respectively.

We approximate the bilayer as a large circle with radius \( r_2 \) and uniform initial fluorochrome concentration \( n_m \). A smaller concentric circle with radius \( r_1 \) can be imaged, with FRAP bleaching within an even smaller concentric circle—termed the FRAP spot—with radius \( r_0 \). The FRAP spot is uniformly bleached to concentration \( n_m \).

For a bilayer with mobile fraction \( f_m \) the reaction–diffusion equation governing the concentration of mobile fluorochromes is

\[
\frac{\partial n^m}{\partial t} = D_s \nabla^2 n^m - k(r) n^m, \tag{3.2}
\]

where \( n^m \) is the mobile fluorochrome concentration and \( D_s \) is the self-diffusion coefficient. The bleaching rate \( k \) is a constant when \( r \leq r_1 \) and \( k(r) = 0 \) in the region \( r > r_1 \) where the bilayer is not imaged.

With cylindrical symmetry, equation (3.2) is easily solved numerically using the Matlab function ‘pdepe’.

Calculations furnishing the spatial and temporal evolution \( n^m(r, t) \) were undertaken on a domain with radial distances \( 0 < r \leq r_2 \). The initial conditions are

\[
n^m(r, 0) = n_0 f_m, \quad 0 < r \leq r_0 \tag{3.3}
\]

and

\[
n^m(r, 0) = n_0 f_m, \quad r_0 < r \leq r_2, \tag{3.4}
\]

with zero-flux boundary conditions \( \partial n^m / \partial r = 0 \) at \( r = 0 \) and \( r_2 \); and continuous radial diffusion fluxes \( -D_s \partial n^m / \partial r \) at \( r = r_0 \) and \( r_1 \). Note that situations where only the FRAP spot is imaged correspond to setting \( r_1 = r_0 \).

The concentration of immobile fluorochromes is

\[
n'(r, t) = n'(r, t)e^{-lt} \tag{3.5}
\]

with initial conditions

\[
n'(r, 0) = n_0(1 - f_m), \quad 0 < r \leq r_0 \tag{3.6}
\]

and

\[
n'(r, 0) = n_0(1 - f_m), \quad r_0 < r \leq r_2. \tag{3.7}
\]

Note that \( n'(r, t) = n_0(1 - f_m) \) when \( r_1 < r \leq r_2 \), since \( k(r) = 0 \) when \( r_1 < r \leq r_2 \).

It is expedient to scale the concentrations with \( n_0 \), radial position with \( r_0 \) and time with \( \tau_B = r_0^2 / (4D_s) \). The dimensionless reaction–diffusion equation for the mobile fluorochromes then presents a single dimensionless parameter

\[
\epsilon = k \tau_B = \frac{kr_0^2}{(4D_s)}. \tag{3.8}
\]
The other independent dimensionless parameters, which arise from the initial and boundary conditions, are the mobile fraction \( f_m \), initial bleaching intensity \( n_0/n_1 \), imaging size \( r_1/r_0 \) and boundary size \( r_2/r_0 \).

A time series of the FRAP intensity \( I \), defined as the average intensity within the FRAP spot, i.e.

\[
I = \int_0^1 \frac{2\pi r n(r) dr}{\int_0^1 2\pi r dr},
\]

is shown in figure 3. Here, the intensity of mobile fluorochromes (dashed line) initially increases due to recovery by diffusion, and later decreases due to bleaching. The intensity of immobile fluorochromes (dash-dotted line) vanishes exponentially, while the total intensity (solid line) plateaus to a value where the rate of photobleaching is balanced by diffusive restoration from the surrounding bilayer.

The accuracy of our numerical calculations is verified, in part, by Soumpasis’s analytical solution (see figure 8 in appendix A) for bilayers without an immobile fraction or acquisition photobleaching. Soumpasis’s solution of the diffusion equation in an unbounded domain gives a FRAP intensity

\[
I = I^\infty - (I_\infty - I_0) \times \left\{ 1 - e^{-2\tau_0/t} \left[ J_0 \left( \frac{2\tau_0}{t} \right) + J_1 \left( \frac{2\tau_0}{t} \right) \right] \right\},
\]

where \( I^\infty \) is the intensity after full recovery, \( I_0 \) is the intensity at \( t = 0 \), i.e. immediately following FRAP bleaching, and \( J_0 \) and \( J_1 \) are the zeroth- and first-order modified Bessel functions of the first kind [42].
4. RESULTS AND DISCUSSION

4.1. Correcting FRAP acquisition photobleaching

Acquisition photobleaching in FRAP is often corrected using reference data via \([46,54]\)

\[
I_c = \frac{I}{I_0},
\]

where \(I\) is the observed FRAP data, \(I_c\) is the corrected FRAP data (e.g. to be fitted to Soumpasis’s theory) and \(I_0\) is the so-called reference data. Recently, it was rigorously proven \([39,47]\) that acquisition photobleaching can be corrected via

\[
I_c = I_0 e^{kt}
\]

when photobleaching kinetics are first order. This suggests that the conventional method, as presented by equation (4.1), is correct if the reference intensity decays exponentially, i.e. \(I_c = I_0 e^{kt}\).

It is customary to use intensity averaged over the entire FRAP image as reference data \([46]\). We will refer to this as Phair et al.’s method. Recently, however, Mueller et al. captured a new time series following conventional FRAP, using the same FRAP collection parameters but without FRAP bleaching. They generated reference data from this new time series at the same location and in exactly the same way as for FRAP \([39]\). We will refer to this as Mueller et al.’s method. Note that the reference time series in Mueller et al.’s method is essentially continuous fluorescence microphotolysis (CFM). This is a less popular technique that uses photobleaching to measure self-diffusion coefficients \([55]\). In CFM, the bilayer is continuously illuminated at medium laser intensity, and the CFM-spot intensity indicates the rate of photobleaching and the diffusive restoration from the surrounding bilayer.

Using our reaction–diffusion FRAP model, we examined the utility of equation (4.2) for correcting acquisition bleaching. Calculations were performed with a dimensionless bleaching constant \(\epsilon = kT_D = 0.05\) and mobile fraction \(f_m = 1\), so perfectly corrected data would reproduce Soumpasis’s solution. We tested conditions under which Phair et al.’s and Mueller et al.’s methods for collecting reference data can be used to ascertain the bleaching constant. These tests compare the respective reference time series with the bleaching curve \(I/I_0 = e^{-kt}\). To model reference data according to Mueller et al.’s method, the geometry of our reaction–diffusion FRAP model is modified to simulate a CFM experiment (see appendix B for details).

Simulations with partial imaging of the reservoir, i.e. \(r_1/r_0 < r_2/r_0 = 100\), are shown in figure 4. Note that solid lines, shown for convenient reference, are the bleaching curve \(I/I_0 = e^{-kt}\). Figure 4a shows FRAP time series for various image sizes \(r_1/r_0 = 1, 3, 5\) and 10 (dashed lines, top to bottom), and the accompanying corrected time series (circles, top to bottom) according to equation (4.2). The corrections agree with Soumpasis’s solution (dash-dotted line) when the image radius \(r_1/r_0 > 10\); otherwise there is observable over-correction. Figure 4b shows reference time series collected according to Phair et al.’s method with \(r_1/r_0 = 2, 5, 10\) and 50 (dashed lines, top to bottom). As expected, the reference time series approach the bleaching curve (solid line) when \(r_1/r_0 > 1\). Figure 4c shows reference data collected using Mueller et al.’s method with \(r_1/r_0 = 1, 2, 4\) and 8 (dashed lines, top to bottom). Again, the reference time series approach the bleaching curve (solid line) when \(r_1/r_0 > 1\), but the method achieves closer correspondence to the bleaching curve with smaller values of \(r_1/r_0\) than with Phair et al.’s method.

Another situation of interest is when the entire reservoir is imaged, i.e. \(r_1 = r_2\). This is often the case when imaging cells \([39,46]\) or finely patterned bilayers \([56]\). Again, we find that acquisition bleaching can be accurately corrected using equation (4.2) when \(r_2/r_0 = r_1/r_0 > 10\). Moreover, both Phair et al.’s and Mueller et al.’s methods can be adopted to collect reference
data even when \( r_2/r_0 = r_1/r_0 < 10 \) (see figure 11 in appendix C).

To accurately obtain the bleaching constant, reference time series must decay exponentially and, thus, overlap the bleaching curve. This requires diffusion to be absent during the collection of reference data. When the entire reservoir is imaged, Mueller et al.’s method can accurately reproduce the bleaching constant (reference curve overlaps exactly with the bleaching curve) for all image sizes, because bleaching occurs uniformly over the entire reservoir. In this case, only bleaching dynamics can be resolved in a CFM process (see figure 10a in appendix B). Furthermore, the reference region does not need to be coincident with the FRAP imaging. In fact, intensity collected over the whole or any sub-region of the CFM spot can be used as reference data. Phair et al.’s method still requires a finite image size to approximate the bleaching constant due to the effect of the FRAP-bleaching in the image centre.

With partial imaging, diffusion is negligible in a CFM process only when \( \epsilon \gg 1 \), i.e. \( \tau_0 \ll \tau_D \) (see figure 10b in appendix B). For a CFM time series to be satisfactorily adopted as reference time series in Mueller et al.’s method, the illumination and FRAP imaging intensities must be identical, thereby fixing \( \tau_0 \). Thus, we can then only increase the image size \( r_1 \) to achieve \( \epsilon \gg 1 \). By using only the central region of a CFM spot as the reference region, the image size \( r_1 \) required to achieve \( \epsilon \gg 1 \) is reduced. As shown in figure 2, the central location in a CFM spot bleaches faster than in the periphery. On the other hand, Phair et al.’s method requires a larger image size, not only because of the FRAP-bleached spot in the centre, but also because of diffusion at the image boundary.

Mueller et al. [39] still found their acquisition bleaching correction affects apparent protein binding rates. This might be owing to violation of assumptions in the theoretical model. For example, fluorochromes in the cell (whole cell imaged) are not uniformly distributed, so there remains diffusion surrounding the reference region. We also note that when FRAP image series are used as reference data, a sub-region of the FRAP image is sometimes used as the reference region [57]. However, the position and size of such a region has to be carefully chosen so that diffusion from the bleached fluorochromes in the FRAP spot and the unbleached ones at the image boundary are both negligible.

### 4.2. Self-diffusion coefficient from FRAP time series

To minimize the scan time, we imaged the FRAP spot and a CFM reference spot. Representative snapshots of a \( c \approx 3 \text{ mol}\% \) DSPE-PEG2k-CF doped DOPC bilayer are shown in figure 5a. The FRAP spot (upper right) has radius \( r_0 \approx 5.13 \text{ µm} \), and CFM spot (lower left) has radius \( r_1 \approx 2.7r_0 \). The imaged regions are separated by a distance \( L \approx 25 \text{ µm} \), so the characteristic time for lipopolymers with diffusivity \( D_0 \approx 1 \text{ µm}^2 \text{s}^{-1} \) to traverse the gap is \( \approx L^2/D_0 \approx 150 \text{ s} \), which is much longer than the characteristic FRAP recovery time \( \tau_D \approx r_0^2/2D_0 \).
$D_s \approx 7$ s. Figure 5b shows the corresponding FRAP time series (circles) and reference time series (triangles). Here, the reference data are the average intensities within the central circular region (with radius $r_0$) of the CFM spot. The solid line is an exponential fit to the reference data giving $I/I_0 \approx 0.97e^{-0.0039t}$. Note that the reference time series does not decay exponentially, which suggests that $r_1 \approx 2.7r_0$ is not large enough for diffusion to be negligible within the central circular region of the CFM spot. Figure 5c shows corrected FRAP time series (squares) obtained by applying equation (4.2) with $k = 0.0039$ s$^{-1}$. Here, the corrected intensity—normalized with the initially uniform intensity—is greater than one, indicating that acquisition bleaching is over-corrected, as expected from figure 4a. Fitting Soumpasis’s equation (3.10) (solid line) to the uncorrected FRAP data (circles) yields $D_s \approx 1.42$ μm$^2$ s$^{-1}$ and $n_\infty/n_0 \approx 0.92$ with the squared 2-norm of the residuals $\chi^2 \approx 0.01$. The least-squares fit (dashed line) of Soumpasis’s solution to the corrected time series (squares) furnishes $D_s \approx 0.71$ μm$^2$ s$^{-1}$ and $n_\infty/n_0 \approx 1.1$ with $\chi^2 \approx 0.08$. Here, fitting Soumpasis’s solution to the corrected time series is unsatisfactory due to over-correcting of acquisition bleaching. Finally, figure 5d shows the least-squares fit (solid line) of our reaction–diffusion FRAP model to the uncorrected FRAP data (circles). This furnishes $D_s \approx 1.05$ μm$^2$ s$^{-1}$, $k \approx 0.0013$ s$^{-1}$, and $f_m \approx 0.98$ with $\chi^2 \approx 0.02$.

Note that fitting our FRAP model to experimental data requires reasonable initial guesses of the fitting parameters $D_s$, $k$ and $f_m$ to converge upon the correct values. Using simulated FRAP data, we found that $D_s$, from fitting Soumpasis’s equation (3.10) to the FRAP time series, $k$ from fitting an exponential decay to the reference time series, and setting $f_m = 1$ furnished satisfactory starting values. Comparing the least-squares fits of $D_s$ from the reaction–diffusion FRAP model with the values obtained from Soumpasis’s solution reveals that acquisition bleaching manifests as a faster recovery (see figure 8), thereby overestimating $D_s$. Note that Soumpasis’s solution does not yield a bleaching constant, and $F'/F_m$ may be reasonably taken to be the mobile fraction $f_m$ only when the bleaching intensity $n_0/n_\infty = I_0/I_\infty = 0$.

In principle, fitting the reaction–diffusion CFM model to experimental CFM time series furnishes $D_s$, $k$ and $f_m$. In practice, however, we found the fitting to be sensitive to initial estimates of the least-squares fitting parameters. One reason is that the accuracy of CFM depends on the ratio $r_d/r_c$ (see [55]). Our CFM images were obtained at the same time as FRAP imaging, so the bleaching is not strictly continuous due to line-scanning limitations of the confocal microscope. Similarly to Irrechukwu & Levenston [58], the bleaching constant must be treated as an apparent value, since it depends on instrument-specific settings, such as laser-illumination intensity.

### 4.3. Self-diffusion coefficient concentration dependence

We synthesized bilayers containing various concentrations of DSPE-PEG2k-CF in DOPC. For each bilayer synthesis, we performed FRAP replicates in separate regions, each time furnishing $D_s$, $k$ and $f_m$ as detailed in §4.2. Least-squares fits were rejected if the squared 2-norm of the residuals $\chi^2 > 0.02$. Next, the average and standard deviation of $D_s$ and $f_m$ from the replicates were computed. Finally, data from several separately synthesized bilayers—with the same lipopolymer concentration—were combined to obtain weighted average of $D_s$ and $f_m$ using the standard deviations as weights (see [59, p. 57]). Representative data are summarized in table 1 for bilayers with $c \approx 1$ mol% DSPE-PEG2k-CF in DOPC. We found that PEG-lipids are mobile at concentrations up to $c \approx 5$ mol%, with the mobile fraction $f_m \gtrsim 0.95$. As a control, we applied a similar methodology to obtain self-diffusion coefficients and mobile fractions of DOPE-NBD in DOPC.

Self-diffusion coefficients $D_s$ are plotted as a function of fluorochrome concentration $c$ in figure 6 for DSPE-PEG2k-CF (circles) and DOPE-NBD (squares) in DOPC. In striking contrast to the DOPE-NBD control, the self-diffusion coefficient of the lipopolymer decreases monotonically with increasing concentration. Interestingly, the DOPE-NBD control has a very weak, non-monotonic concentration dependence. We fit the lipopolymer diffusion coefficient data to an empirical interpolation formula

$$D_s = \frac{D_0}{1 + \alpha c}, \quad (4.3)$$

where $D_0$ is the diffusion coefficient at infinite dilution, and $\alpha$ is a constant that characterizes the degree to which diffusion is hindered at finite lipopolymer concentrations. Least-squares fitting (solid line) of equation (4.3) to the data furnishes $D_0 \approx 3.36$ μm$^2$ s$^{-1}$ and $\alpha \approx 0.56$ when $c$ is expressed as a mole percent. Also shown in figure 6 are lipopolymer self-diffusion coefficients (triangles) obtained by fitting Soumpasis’s solution to the uncorrected FRAP time series. As expected, $D_s$ from Soumpasis’s solution is larger than that obtained from the reaction–diffusion FRAP model. Least-squares fitting (dashed line) of equation (4.3) to the data furnishes $D_0 \approx 3.81$ μm$^2$ s$^{-1}$ and $\alpha \approx 0.49$. For reasons discussed in §4.2, we consider the results derived from the reaction–diffusion FRAP model to be more accurate, so the values $D_0 \approx 3.36$ μm$^2$ s$^{-1}$ and $\alpha \approx 0.56$ will be adopted for the following theoretical interpretation.

Note that the diffusion coefficient of lipopolymer DSPE-PEG2k-CF at infinite dilution, $D_0 \approx 3.36$ μm$^2$ s$^{-1}$, is somewhat higher than the value inferred for the DOPE–NBD control. While it is difficult to provide a conclusive interpretation of this discrepancy without invoking membrane elasticity and curvature, for example, the lower mobility of DOPE-NBD might reflect a stronger hydrodynamic coupling of the DOPE-NBD doped bilayer with the glass support. This is not unreasonable given that the bilayer hosting DOPE-NBD can be much closer to the glass than the lipids in DSPE-PEG2k-CF doped bilayers.
4.4. Comparison with literature

Literature-reported self-diffusion coefficients for lipopolymer-doped bilayers formed by vesicle fusion are summarized in Table 2 with the accompanying lipid composition, substrate cleaning procedure, and other parameters known to affect bilayer fluidity. For clarity, the data are plotted in Figure 7. Note that we have indicated a vanishing self-diffusion coefficient in Figure 7 from the study of Kaufmann et al. [57], whereas they actually reported that there was no recovery after photobleaching.

Recall, the DSPE-PEG2k-CF lipids in our work are mobile at all concentrations studied \( c \lesssim 5 \text{ mol\%} \) with mobile fractions \( f_m / C_{21} \approx 95\% \). [18] also observed mobile DSPE-PEG2k lipids in supported egg-PC bilayers at concentrations up to 5 mol\%. However, [57] found that 1 mol\% NBD-PC bilayers are ostensibly inhomogeneous, with no discernable recovery after bleaching, when the mole fraction of DOPE-PEG2k \( c \approx 5 \text{ mol\%} \). Thus, mounting evidence suggests that qualitative differences in bilayer structure often manifest only when well into the brush regime, perhaps further than inferred by Albertorio et al. [18].

Now turning to the concentration dependence of lipopolymer self-diffusion coefficients, Albertorio et al. [18] reported self-diffusion coefficients of Alexa Fluor-594-labelled NH2-PEG2k-DSPE that decrease with increasing concentration at concentrations \( c \gtrsim 1.5 \text{ mol\%} \). According to Kaufmann et al. [57], the mole fraction for the PEG mushroom-to-brush transition [60] is

\[
c_t = \frac{A_1}{R_F^2}, \tag{4.4}
\]

where \( A_1 \) is the cross-sectional area of a lipid, and the PEG-chain Flory radius

\[
R_F = a_m n_p^{3/5}, \tag{4.5}
\]

where \( a_m \) is the monomer size and \( n_p \) the degree of polymerization. Literature suggests that \( a_m \approx 0.35 - 0.43 \text{ nm} \) and

### Table 1. Summary of FRAP data for bilayers containing \( c \approx 1 \text{ mol\%} \) DSPE-PEG2k-CF in DOPC. Here, data from four separately synthesized bilayers, each with several replicate FRAP experiments with in each bilayer, are combined to furnish the weighted average self-diffusion coefficient \( D_s \) and mobile fraction \( f_m \).

<table>
<thead>
<tr>
<th>bilayer synthesis</th>
<th>measurement</th>
<th>( D_s ) measured</th>
<th>( D_s ) average</th>
<th>( f_m ) measured</th>
<th>( f_m ) average</th>
<th>( f_m ) weighted average</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>2.68</td>
<td>2.27 ± 0.51</td>
<td>96.79</td>
<td>94.44 ± 2.94</td>
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</tr>
<tr>
<td></td>
<td>2</td>
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<tr>
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<td></td>
<td>93.22</td>
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<tr>
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<td>94.86</td>
<td>93.25 ± 3.34</td>
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<td>95.90 ± 2.01</td>
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<td></td>
<td>94.24</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>2.16 ± 0.04</td>
<td></td>
<td></td>
<td>96 ± 0.51</td>
<td></td>
</tr>
</tbody>
</table>

\[ f_m / C_{21} \approx 95\% \]
Table 2. Summary of literature-reported self-diffusion coefficients in lipopolymer-doped bilayers formed by vesicle fusion.

<table>
<thead>
<tr>
<th>System</th>
<th>I</th>
<th>II-1</th>
<th>II-2</th>
<th>III-1</th>
<th>III-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>D_{sc}</td>
<td>D_{f}</td>
<td>D_{sc}</td>
<td>D_{f}</td>
<td>D_{sc}</td>
<td>D_{f}</td>
</tr>
<tr>
<td>c ≈ 0 mol%</td>
<td>—</td>
<td>4.0 ± 0.1</td>
<td>—</td>
<td>1.2 ± 0.4, 95 ± 2</td>
<td>—</td>
</tr>
<tr>
<td>c ≈ 0.5 mol%</td>
<td>2.62 ± 0.12, 97.89 ± 0.75</td>
<td>3.8 ± 0.2</td>
<td>4.0 ± 0.25</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>c ≈ 1 mol%</td>
<td>2.16 ± 0.04, 96.20 ± 0.51</td>
<td>—</td>
<td>4.0 ± 0.15</td>
<td>1.5 ± 0.1, 97 ± 0.4</td>
<td>—</td>
</tr>
<tr>
<td>c ≈ 1.5 mol%</td>
<td>—</td>
<td>3.9 ± 0.2</td>
<td>3.0 ± 0.15</td>
<td>2.2 ± 0.4, 97 ± 2</td>
<td>—</td>
</tr>
<tr>
<td>c ≈ 2 mol%</td>
<td>1.61 ± 0.08, 96.92 ± 0.94</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1.1 ± 0.4, 99 ± 1</td>
</tr>
<tr>
<td>c ≈ 3 mol%</td>
<td>1.23 ± 0.03, 94.62 ± 0.65</td>
<td>—</td>
<td>2.4 ± 0.12</td>
<td>1.9 ± 0.2, 98 ± 0.3</td>
<td>—</td>
</tr>
<tr>
<td>c ≈ 5 mol%</td>
<td>0.90 ± 0.03, 97.58 ± 0.41</td>
<td>3.9 ± 0.3</td>
<td>1.4 ± 0.10</td>
<td>—</td>
<td>no recovery</td>
</tr>
<tr>
<td>Bulk lipids</td>
<td>DOPC</td>
<td>egg-PC</td>
<td>egg-PC</td>
<td>POPC</td>
<td>POPC</td>
</tr>
<tr>
<td>Fluorophore</td>
<td>DSPE-PEG2k</td>
<td>DSPE-PEG2k</td>
<td>Alexa Fluor-594-PEG2k</td>
<td>DSPE-PEG2k</td>
<td>PEG2k-CF</td>
</tr>
<tr>
<td>Substrate</td>
<td>cover-slips</td>
<td>glass slides</td>
<td>cover-slips</td>
<td>baked</td>
<td>UVO cleaning</td>
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<tr>
<td>FRAP fitting</td>
<td>numerical</td>
<td>[27]</td>
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<td>corrected*</td>
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<tr>
<td>Bleaching</td>
<td>fitting</td>
<td>neglected</td>
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<tr>
<td>Reference</td>
<td>this work</td>
<td>[18]</td>
<td>[18]</td>
<td>[57]</td>
<td>[57]</td>
</tr>
</tbody>
</table>

*Intensity in a region of the FRAP image was used as reference data.

Figure 7. Summary of measured self-diffusion coefficients with lines to guide the eye. Circles: DSPE-PEG2k-CF from this study at various DSPE-PEG2k-CF concentrations. Squares: DSPE-PEG2k-Alexa Fluor-594 from [18] at various DSPE-PEG2k-Alexa Fluor-594 concentrations. Up-triangles: DSPE-PEG2k-CF from [57] at one DSPE-PEG2k-CF concentration. Diamonds: 0.5 mol% TR-PE from [18] at various DOPE-PEG2k concentrations. Left-triangles: 1 mol% NBD-PC from [57] at various DOPE-PEG2k concentrations. Note that [57] reported an absence of recovery after photobleaching at 5 mol% DOPE-PEG2k, which we depict here as a vanishing diffusion coefficient. Data are listed in table 2.

A_i ≈ 0.60–0.70 nm^2 [61,62], so the mushroom-to-brush transition for PEG2k (n_p = 45) would occur at concentrations in the range c_i ≈ 3.5–6 mol%. For example, with a_m = 0.39 nm, A_i = 0.65 nm^2 and R_p ≈ 3.83 nm, we find c_i ≈ 4.4 mol%. On the other hand, if we interpret the mushroom-to-brush transition as occurring when c_i = A_i/(πR_f^2) (full coverage based on the area occupied by circles with radius R_f), then c_i ≈ 1.4 mol%. Indeed, this estimate is consistent with the abrupt transition in the self-diffusion coefficient observed by Albertorio et al. [18].

Interestingly, our data for DSPE-PEG2k-CF in DOPC decrease continuously with increasing lipopolymer concentration, similarly to the smooth manner in which the spreading pressure varies according to self-consistent mean-field theory [48]. This suggests that the mushroom-to-brush transition in our experiments is considerably more gradual than inferred by Albertorio et al. [18]. Moreover, the PEG2k-CF chains in our experiments may not adopt an ostensibly brush-like configuration until concentrations are reached that are closer to or higher than the 4.4 mol% suggested by equation (4.4). If this is indeed the case, then it would be reasonable to consider our experiments as having been performed with the PEG2k-CF chains predominantly in mushroom-like configurations.

We emphasize that quantitative comparison of lateral diffusion coefficients in SLBs is difficult because diffusion is easily affected by bilayer composition, support properties, and other parameters [63,64]. In recent years, several studies have tried to systematically address the influences of such parameters on diffusion. For example, Seu et al. [65] found that the diffusion of NBD-DOPC in egg phosphatidylcholine (egg-PC) decreases significantly upon the addition of egg phosphatidylethanolamine (egg-PE), (ii) increases significantly with addition of lyso-phosphatidylcholine (LPC), and (iii) decreases slightly with addition of lyso-phosphatidylethanolamine (LPE). They concluded
that both lipid head group and tail structure affect diffusion. In another study, Seu et al. [66] found that piranha etched glass-slide supported bilayers are three-fold more fluidic than on baked glass. Finally, Scomparin et al. [64] demonstrated that dynamics in SLBs depend on the type of substrate (glass and mica) and the method of bilayer preparation (Langmuir–Blodgett deposition and vesicle fusion).

The main differences between the three studies listed in Table 2 are (i) lipopolymer tail structure; (ii) bulk lipids; (iii) substrate cleaning procedures; and (iv) FRAP fitting methodology. In the study of Albertorio et al., DPPE-PEG2k and DSPE-PEG2k have similar influences on the diffusion of Texas Red-DHPE in egg-PC membranes [18]. This shows that differences in lipopolymer tail structure might not have a significant influence on lipopolymer mobilities. Bulk lipids used in the three studies listed in Table 2 are DOPC, egg-PC, and POPC. The main component of egg-PC is POPC, so the bulk lipids in system II and III in Table 2 are similar. The striking difference in the dynamics of those two systems indicates that the bulk/supporting lipid plays a minor role. Also, self-diffusion coefficients fitted using Soumpasis’s solution agree qualitatively with our reaction–diffusion FRAP model (figure 6), indicating that the qualitative differences in the three systems are more likely due to the substrate cleaning procedures. However, in contrast to the observations of Seu et al. [66], lipopolymer self-diffusion coefficients in our study (system I) on piranha etched glasses are actually smaller than the values from system II using baked glasses. At present, it seems that every parameter plays a role in the dynamics of the three systems, and that the principal contribution remains elusive.

4.5. Theoretical interpretation of hindered self-diffusion

Theory is available to interpret the hindered diffusivity of membrane proteins [16], and their dynamics are reasonably well understood. The dynamics of lipopolymers in SLBs is more challenging, because each lipopolymer comprises a small lipid anchor embedded in a viscous, two-dimensional fluid, with a large grafted polymer chain immersed in a low-viscosity, three-dimensional half-space.

For discs with excluded-volume interactions in lipid bilayers, the [7] theory predicts a self-diffusion coefficient at low concentrations

$$\frac{D_1}{D_0} = 1 - 2\phi, \quad (4.6)$$

where \(\phi\) is the disc area fraction. To compare theory and experiments for proteins, the experimentally measured protein concentration must be converted to an area fraction. This conversion rests on accurately specifying a disc radius \([8,10,15]\). For lipopolymers, however, the area fraction is not easy to specify, because lipopolymers have a large polymer coil grafted to a hydrodynamically small lipid anchor. Nevertheless, if we treat the lipopolymer as a disc with an effective radius \(a_e\), then the effective area fraction is \(\phi_e = c\pi a_e^2 / A_l\), where \(c\) is the lipopolymer mole fraction, and \(A_l \approx 0.65 \text{ nm}^2\) is the lipid cross-sectional area corresponding to a lipid radius \(a_l \approx 0.46 \text{ nm}\). Equation (4.6) then becomes

$$\frac{D_1}{D_0} = 1 - \frac{2\pi c^2}{A_l}, \quad (4.7)$$

which, when compared with our empirical fitting formula in equation (4.3) as \(c \to 0\), furnishes \(a_e = a_l \sqrt{\gamma}/2\). Thus, with \(\gamma \approx 0.56\) (figure 6), the effective radius \(a_e \approx 2.41 \text{ nm}\).

By including hydrodynamic interactions, Bussell et al. [14] derived

$$\frac{D_1}{D_0} = 1 - 2\phi \left(1 - \frac{1 + \ln 29/23}{\ln \lambda - \gamma} \right) - 0.07, \quad (4.8)$$

where \(\gamma = 0.577216\) is Euler’s constant, and \(\lambda = h\mu_l/(a_0\mu_t)\) with \(a\) the hard-cylinder radius, \(h\) the lipid–bilayer thickness, \(\mu_l\) the lipid–bilayer viscosity and \(\mu_t\) the viscosity of the supporting fluid. To furnish an effective cylinder radius for lipopolymers according to Bussell et al.’s theory, we specify an effective radius \(a_e\) to calculate \(D_1/D_0\) according to equation (4.8). Next, comparing the dilute limit of the theory to the experimental data, we obtain an improved estimate of \(a_e\). Repeating this procedure until successive values of \(a_e\) change by less than one percent furnishes \(a_e \approx 2.92 \text{ nm}\) when we set \(h = 3.2 \text{ nm}\) [67,68], \(\mu_l = 1.2 \times 10^{-11} \text{ kg m}^{-1} \text{s}^{-1}\) [69] and \(\mu_t = 8.9 \times 10^{-5} \text{ kg m}^{-1} \text{s}^{-1}\) (water; [6]). Comparing this value with the effective radius \(a_e \approx 2.41 \text{ nm}\) from the Scalettar–Abney–Owicki theory (equation (4.7)) suggests that hydrodynamic interactions play a small role.

It is reassuring that the effective radii for PEG2k-CF are much larger than the lipid head radius. This confirms that thermodynamic interactions between the tethered polymer chains are significant. Moreover, the effective radii are both smaller than the PEG-chain Flory radius, which is reasonable when acknowledging that the effective hard-core radius here is representing a large, soft polymer coil that is attached to a very small and compact anchor. The reasonable size of the PEG2k-CF chains also supports our assumption that the surface concentration of lipopolymers equals the stoichiometric amount used in the bilayer synthesis.

Note that DSPE-PEG2k-CF bears a charge \(- e\) at the lipid–PEG junction, and a charge \(- e\) at the PEG2k-CF junction. Thus, DOPE-NBD in DOPC serves as a valuable control because it also bears a negative charge without a grafted PEG chain. Control experiments do not exhibit significant changes in the self-diffusion coefficient with concentration, indicating that electrostatic interactions between the charged lipid heads do not significantly influence lipopolymer dynamics. Rather, the polymer–polymer interaction seems to play an important role. However, the hindered self-diffusion coefficient is not sufficient to distinguish direct lipopolymer interactions (thermodynamic interactions) from lipopolymer-induced perturbations to flow (hydrodynamic interactions). This competition, with other influences, such as substrate–bilayer interactions, will hopefully become clearer with complementary knowledge of the gradient diffusion coefficient.
4.6. Frictional drag in polymer-grafted bilayers

From the diffusion coefficient at infinite dilution, the Einstein relation furnishes a lipopolymer drag coefficient

$$\lambda_i = k_B T / D_0,$$  \hspace{1cm} (4.9)

where $k_B T$ is the thermal energy. With $D_0 \approx 3.36 \text{ mm}^2 \text{s}^{-1}$, we have $\lambda_0 \approx 1.21 \times 10^{-9} \text{ N m}^{-1}$ at $T \approx 295 \text{ K}$. Let us now consider the total drag coefficient on a lipopolymer as the sum

$$\lambda_i = \lambda_p + \lambda_l,$$  \hspace{1cm} (4.10)

where $\lambda_p$ is the drag coefficient of the polymer chain, and $\lambda_l$ the drag coefficient of the anchoring lipid. Furthermore, approximating the polymer as a sphere with radius $a_p$ gives

$$\lambda_p = 6 \pi \mu_l a_p,$$  \hspace{1cm} (4.11)

where $\mu_l$ is the viscosity of the supporting fluid (water).

As an extension of the [5] theory, the [70] theory gives

$$\lambda_l = 4 \pi \eta \left[ \frac{\epsilon^2}{3} + \frac{\epsilon K_1(\epsilon)}{K_0(\epsilon)} \right],$$  \hspace{1cm} (4.12)

where $\eta$ is the membrane viscosity; $K_0$ and $K_1$ are zeroth- and first-order modified Bessel functions of the second kind; and

$$\epsilon = a_p \sqrt{\frac{b_l}{\eta}}$$  \hspace{1cm} (4.13)

is a dimensionless disc (lipid head) radius (not to be confused with the dimensionless bleaching constant) with $b_l$ the inter-leaflet friction coefficient. Setting $a \approx a_p \approx 0.46 \text{ nm}$ and $\eta \approx 0.08 \text{ nN s}^{-1}$ (half the value for a free lipid bilayer $\eta \approx 0.16 \text{ nN s}^{-1}$ [71]), equation (4.13) furnishes the values of $\epsilon$ and $b_l$ listed in table 3 for several estimates of $a_p$.

Evidently, all reasonable values of $a_p$ yield $\lambda_p \ll \lambda_l$, which, in turn, furnishes values of $\epsilon$ and $b_l$ that are practically independent of the polymer size. This demonstrates that the total frictional drag on lipopolymers is overwhelmingly dominated by hydrodynamic friction within the leaflet that hosts the lipid anchor. A similar conclusion can be drawn from histidine-tagged EGFP anchored to mono-layers [72]. Note also that the dimensionless lipopolymer anchor radius $\epsilon \approx 0.65$ corresponds closely with $\epsilon \approx 0.73$ for NBD-PE in 1-stearyl-2-oleoyl-sn-glycero-3-phosphocholine (SOPC; [72]).

5. CONCLUSIONS

We developed a reaction–diffusion FRAP model that integrates a finite immobile fraction and first-order photobleaching kinetics. We used this model to evaluate commonly adopted photobleaching correction methodologies for quantitative FRAP in the literature, and applied it to furnish self-diffusion coefficients (and mobile fraction) for a fluorescent lipopolymer DSPE-PEG2k-CF in DOPC supported bilayers over a wide range of DSPE-PEG2k-CF concentrations. To quantify the influence of the grafted PEG chains on thermodynamic and hydrodynamic interactions, we performed similar experiments furnishing the self-diffusion coefficient of DOPE-NBD in DOPC over a similar range of DOPE-NBD concentrations.

We conclude that the limiting diffusion coefficient $D_0$ reflects—almost entirely—hydrodynamic friction within the two-dimensional leaflet in which the lipopolymer is anchored. This supports the hypothesis that there is a negligible difference between the dynamics of lipids in the top and bottom leaflets of these SLBs, assuming lipopolymers are equally distributed between both leaflets.

Perhaps surprisingly, the lipopolymer self-diffusion coefficient is significantly hindered by thermodynamic interactions between the grafted PEG chains. Moreover, theoretical interpretation of the data suggests that hydrodynamic interactions between the PEG chains are weak compared to thermodynamic (excluded-volume) interactions. Thus, based on the [7] theory of diffusion, we expect an enhancement of the gradient diffusion coefficient of DSPE-PEG2k-CF in DOPC with increasing concentration. This may have important practical implications for interpreting the dynamics of biologically relevant lipopolymers, and technological applications involving lipopolymer membranes.

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The self-diffusion coefficient of the anchored lipopolymer provides a measure of hydrodynamic friction (and steric interactions) between the monolayers and the surrounding medium. This measure is important for understanding the dynamics of lipopolymers in confined environments, such as lipid bilayers and micelles. The self-diffusion coefficient can also be used to infer the viscoelastic properties of the surrounding medium, which is relevant for applications in drug delivery and biotechnology.

### Table 3. Frictional drag characteristics for DSPE-PEG2k-CF in DOPC leaflets according to theoretical interpretations of measured $D_0$ (see text for details) using equations (4.12) and (4.13).

<table>
<thead>
<tr>
<th>$a_p$ (nm)</th>
<th>$\lambda_p$ (N s m$^{-1}$)</th>
<th>$\lambda_l$ (N s m$^{-1}$)</th>
<th>$\epsilon$</th>
<th>$b_l$ (N s m$^{-3}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1.21 x 10$^{-9}$</td>
<td>0.68</td>
<td>1.48 x 10$^8$</td>
</tr>
<tr>
<td>2.41</td>
<td>4.04 x 10$^{-11}$</td>
<td>1.17 x 10$^{-9}$</td>
<td>0.65</td>
<td>1.35 x 10$^8$</td>
</tr>
<tr>
<td>2.92</td>
<td>4.90 x 10$^{-11}$</td>
<td>1.16 x 10$^{-9}$</td>
<td>0.64</td>
<td>1.33 x 10$^8$</td>
</tr>
<tr>
<td>3.83</td>
<td>6.43 x 10$^{-11}$</td>
<td>1.15 x 10$^{-9}$</td>
<td>0.63</td>
<td>1.29 x 10$^8$</td>
</tr>
</tbody>
</table>

### APPENDIX A. GENERAL SOLUTION OF THE REACTION–DIFFUSION FRAP MODEL

General solutions of the reaction–diffusion FRAP model are examined in figure 8 for partial imaging ($r_1 < r_2$). Note, however, that the results are practically the same as when imaging the entire domain, i.e. when $r_1 = r_2 > r_0$. Figure 8a shows the FRAP spot time series for $r_1/r_0 = 1$ and several values of $r_2/r_0 = 2$, 3, 5 and 10 (bottom to top), without acquisition.
photobleaching ($\epsilon = 0$) and without immobile fluorochromes ($f_m = 1$). Increasing $r_2/r_0$ decreases the overall recovery rate. Model predictions (dashed lines) closely agree with Soumpasis’s theory (solid lines) when $r_2/r_0 \geq 10$. Figure 8b shows model predictions for several dimensionless photobleaching rate constants $\epsilon = 0.005, 0.01, 0.02$ and 0.05 (top to bottom), also without immobile fluorochromes ($f_m = 1$). As expected, the model reproduces Soumpasis’s theory (solid line) as $\epsilon \to 0$ with $r_2/r_0 \gg 1$. However, there are significant qualitative differences when $t/\tau_D \gtrsim 1$ and $\epsilon > 0$. Note that even weak photobleaching affects the recovery rate and plateau. Finally, figure 8c shows model predictions for several mobile fractions $f_m = 0.6, 0.7, 0.8$ and 0.9 (bottom to top), without acquisition photobleaching ($\epsilon = 0$). Again, there are significant deviations from Soumpasis’s theory when $f_m < 1$, with the intensity after recovery reflecting the mobile fraction.

APPENDIX B. APPLICATION OF THE REACTION–DIFFUSION FRAP MODEL TO CONTINUOUS FLUORESCENCE MICRO-PHOTOLYSIS

Continuous fluorescence micro-photolysis (CFM) is another methodology for measuring self-diffusion coefficients using photobleaching [55]. The CFM spot is uniformly and continuously illuminated at medium laser intensity. Modifying the initial condition of the reaction–diffusion FRAP model captures CFM-spot intensity dynamics. The CFM model has $r_0 = r_1$ with initial conditions for the mobile fluorochromes

$$n^m(r, 0) = n_0 f_m, \quad 0 \leq r \leq r_2$$

and for the immobile fluorochromes

$$n'(r, 0) = n_0 (1 - f_m), \quad 0 \leq r \leq r_2.$$ (B2)

A representative CFM time series is presented in figure 9. The intensity of immobile fluorochromes (dash-dotted line) decays exponentially with the mobile fluorochrome intensity (dashed line) decaying more slowly due to recovery by diffusion. Note that the total intensity (solid line) approaches a plateau where the rate of photobleaching is balanced by diffusive restoration.

The influence of several model parameters on CFM time series are examined in figure 10. For convenient reference, all panels show bleaching curves $e^{-\epsilon t/r_0}$ (solid lines). Figure 10a shows solutions for several values of $r_2/r_1 = 1, 2, 3, 5$ and 10 (dashed lines, bottom to top) with $\epsilon = 0.5$ and $f_m = 1$. As expected, when imaging the entire domain ($r_2/r_1 = 1$), the CFM time series overlaps the bleaching curve. Moreover, when $r_2/r_1 \gtrsim 5$, the time series are practically...
Figure 10. CFM time series as predicted by the reaction–diffusion model (dashed lines). Bleaching curves (solid lines) have corresponding values of $\varepsilon$. (a) Varying the reservoir radius $r_2/r_1 = 1, 2, 3, 5$ and 10 (bottom to top) with $\varepsilon = 0.5$ and $f_m = 1$. Note that the time series with $r_2/r_1 = 1$ overlaps the bleaching curve (solid line), and time series with $r_2/r_1 = 5$ and 10 overlap each other. (b) Varying the bleaching constant $\varepsilon = 0.05, 0.5$ and 5 (dashed lines, top to bottom) with $f_m = 1$ and $r_2/r_1 = 100$. (c) Varying the mobile fraction $f_m = 0.5, 0.8$ and 1 (bottom to top) with $\varepsilon = 0.5$ and $r_2/r_1 = 100$.

Figure 11. Correcting acquisition bleaching when the entire reservoir is imaged ($r_1 = r_2$): $\varepsilon = 0.05$, 0.5 and 5 (dashed lines, top to bottom) with $f_m = 1$ and $r_2/r_1 = 100$. As $\varepsilon$ increases, bleaching dominates and the CFM time series decay to lower plateaus. Figure 10c shows solutions for several values of $f_m = 0.5, 0.8$ and 1 (dashed lines, bottom to top) with $\varepsilon = 0.5$ and $r_2/r_1 = 100$. In the absence of diffusion ($f_m = 0$), the CFM time series overlap the bleaching curve. Moreover, increasing $f_m$ furnishes CFM times series that decay toward increasingly higher plateaus.

**APPENDIX C. CORRECTING ACQUISITION BLEACHING WHEN IMAGING THE ENTIRE RESERVOIR**

The results of correcting acquisition bleaching for the case when whole reservoir is imaged, i.e. $r_1 = r_2$, are presented in figure 11. Figure 11a shows corrected FRAP curves for $r_2/r_1 = r_1/r_0 = 3, 5$ and 10 (circles, bottom to top). With the given bleaching constant $\varepsilon = 0.05$, the corrected FRAP curve agrees with Soumpasis’s solution (dash-dotted line) when $r_2/r_0 = r_1/r_0 \gtrsim 10$. Figure 11b shows the reference curves collected using Phair et al.’s method for $r_2/r_1 = r_1/r_0 = 3, 5$ and 10 (dashed lines, bottom to top). The reference curves agree with the actual bleaching curve (solid line) when $r_2/r_0 \gtrsim 10$. Figure 11c shows reference data collected using Mueller et al.’s method for $r_2/r_1 = r_1/r_0 = 1, 5$ and 10 (dashed lines). All the reference curves overlap the bleaching curve (solid line) calculated using the known bleaching constant $\varepsilon = 0.05$.

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