

Concentration dependence of lipopolymer self-diffusion in supported bilayer membranes

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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 $D_s = D_0/(1 + \alpha c)$, where $D_0 \approx 3.36 \mu\text{m}^2 \text{s}^{-1}$ and $\alpha \approx 0.56$ (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 $a_e \approx 2.41$ nm. On the other hand, the Bussell–Koch–Hammer theory, which includes hydrodynamic interactions, yields $a_e \approx 2.92$ 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, $a_l \approx 0.46$ nm. The diffusion coefficient at infinite dilution D_0 was interpreted using the Evans–Sackmann theory, furnishing an inter-leaflet frictional drag coefficient $b_s \approx 1.33 \times 10^8 \text{ N s m}^{-3}$. 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 Δt 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.

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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 bicelles 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 glycocalyx 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 (H₂SO₄) and 30 per cent hydrogen peroxide (H₂O₂). 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 $I = QS_0(1 - e^{-A})$, 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, $I = QS_0(1 - e^{-kc}) \approx QS_0kc$ 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 1*a*. These were obtained using a Nikon 2000-U inverted microscope with a $10 \times /0.25$ 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 μm 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 1*b*, 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 $63 \times /1.4$ 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 $68 \times 68 \mu\text{m}$ with resolution 256×256 pixels. FRAP spots (circles with radius $r_0 \approx 5.13 \mu\text{m}$) 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_∞ . 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, thereby 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_s , 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

$$\frac{\partial I}{\partial t} = -kI, \quad (3.1)$$

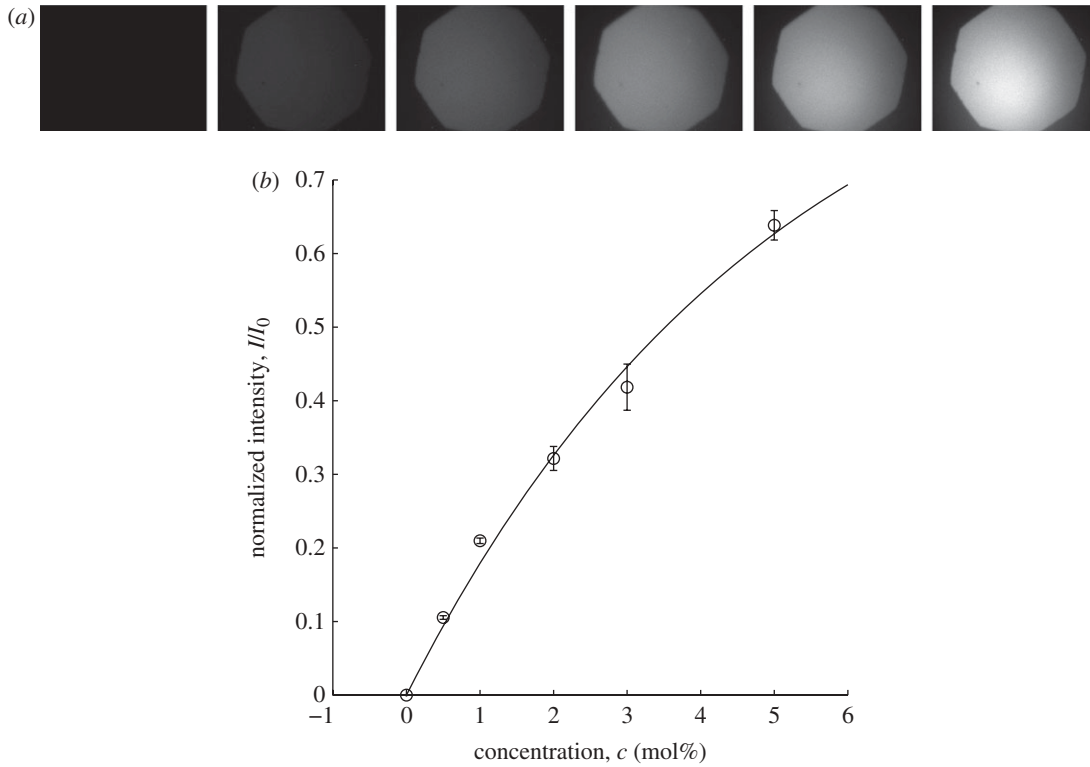


Figure 1. (a) Epifluorescence images ($592 \times 442 \mu\text{m}$) of DOPC bilayers with DSPE-PEG2k-CF at concentrations $c = 0, 0.5, 1, 2, 3$ and 5 mol%. (b) Normalized intensity I/I_0 in the central circular regions with radius $8.5 \mu\text{m}$ versus DSPE-PEG2k-CF concentration c with fit $I/I_0 \approx 1 - e^{-0.19c}$ (solid line). Error bars are the standard deviation from three replicates.

where k is the photobleaching rate constant. This yields an exponential decay in intensity, with a time constant $\tau_k = k^{-1}$. 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 $c \approx 1$ 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 $\tau_k \approx 6.3$ 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_∞ . 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_0 .

For a bilayer with mobile fraction f_m , the reaction–diffusion equation governing the concentration of *mobile fluorochromes* is

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

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

$$n^m(r, 0) = n_0 f_m, \quad 0 < r \leq r_0 \quad (3.3)$$

and

$$n^m(r, 0) = n_\infty f_m, \quad r_0 < r \leq r_2, \quad (3.4)$$

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

$$n^i(r, t) = n^i(r, 0) e^{-kt} \quad (3.5)$$

with initial conditions

$$n^i(r, 0) = n_0(1 - f_m), \quad 0 < r \leq r_0 \quad (3.6)$$

and

$$n^i(r, 0) = n_\infty(1 - f_m), \quad r_0 < r \leq r_2. \quad (3.7)$$

Note that $n^i(r, t) = n_\infty(1 - f_m)$ 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_∞ , radial position with r_0 and time with $\tau_D = r_0^2 / (4D_s)$. The dimensionless reaction–diffusion equation for the mobile fluorochromes then presents a single dimensionless parameter

$$\epsilon = k\tau_D = \frac{kr_0^2}{(4D_s)}. \quad (3.8)$$

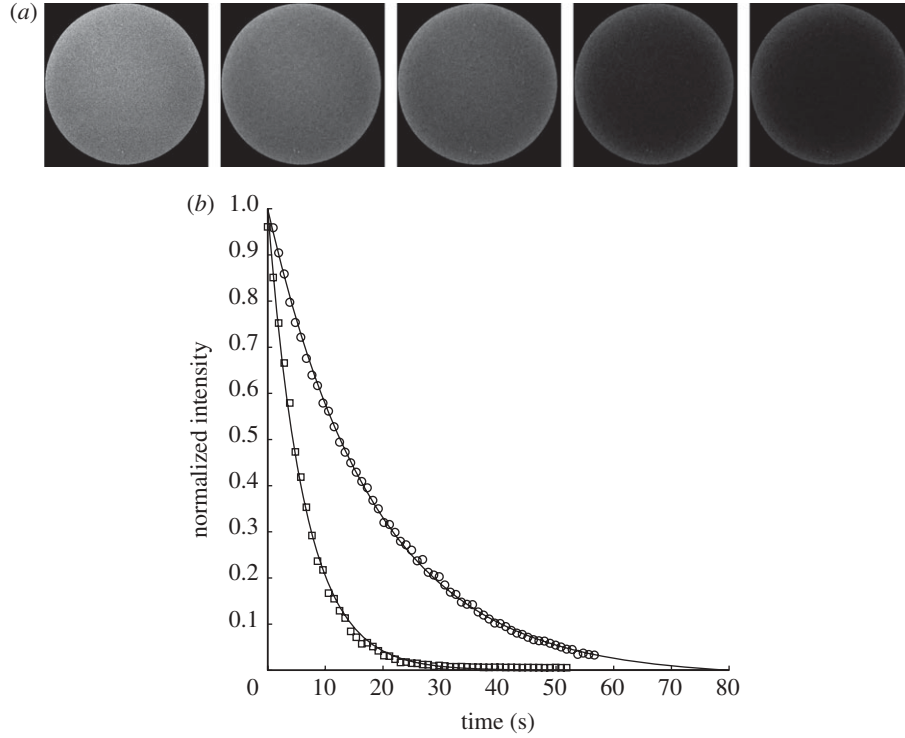


Figure 2. First-order kinetics of photobleaching. (a) Representative confocal laser scanning images of a circular domain (radius $102\text{ }\mu\text{m}$) with $c \approx 1\text{ mol}\%$ fluorescent lipopolymer DSPE-PEG2k-CF in a DOPC bilayer. (b) Circles and squares are average intensities in a small concentric circle (radius $4\text{ }\mu\text{m}$) within the images illuminated using low- and high-power laser intensities, respectively. Solid lines are exponential fits (first-order kinetics) giving decay constants $\tau_k = k^{-1} \approx 6.3$ and 18.5 s .

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_∞ , imaging size r_1/r_0 and boundary size $r_2/r_0 \geq r_1/r_0$.

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

$$I = \frac{\int_0^{r_0} 2\pi r n(r) dr}{\int_0^{r_0} 2\pi r dr}, \quad (3.9)$$

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

$$I = I^\infty - (I_\infty - I_0) \times \left\{ 1 - e^{-2\tau_D/t} \left[J_0\left(\frac{2\tau_D}{t}\right) + J_1\left(\frac{2\tau_D}{t}\right) \right] \right\}, \quad (3.10)$$

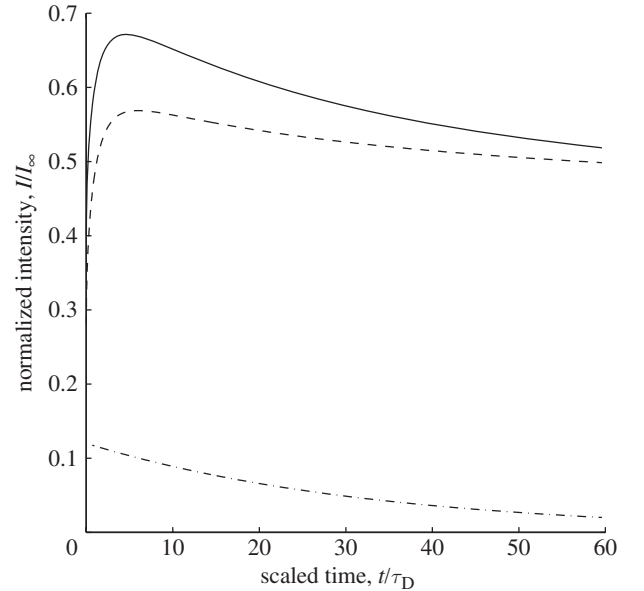


Figure 3. FRAP-spot intensity time series with $\epsilon = k\tau_D = 0.03$, $f_m = 0.7$, $n_0/n_\infty = 0.4$, $r_1/r_0 = 2$ and $r_2/r_0 = 100$. The solid line is the average intensity of fluorochromes in the FRAP spot, and the dashed and dash-dotted lines are, respectively, the contributions from the mobile and immobile fractions.

where I^∞ 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].

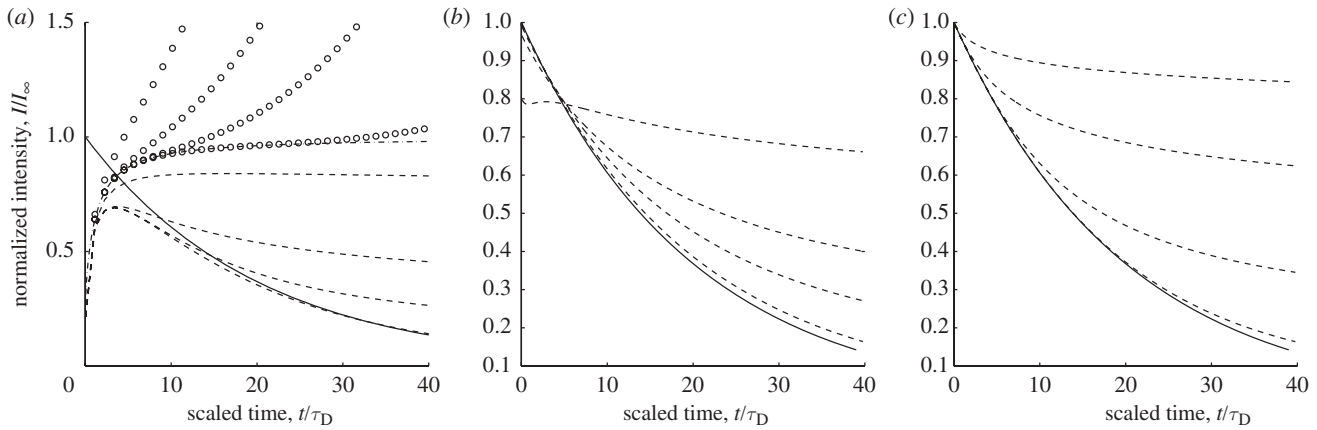


Figure 4. Correcting acquisition photobleaching with partial imaging ($r_1 < r_2$): $\epsilon = 0.05$, $f_m = 1$, $n_0/n_\infty = 0.2$ and $r_2/r_0 = 100$. Solid lines are the bleaching curve $I/I_\infty = e^{-kt}$. (a) Correction according to equation (4.2): Soumpasis's analytical solution (dash-dotted line); numerical FRAP time series for $r_1/r_0 = 1, 3, 5$ and 10 (dashed lines, top to bottom); corrected FRAP time series for $r_1/r_0 = 1, 3, 5$ and 10 (circles, top to bottom). (b) Phair *et al.*'s method of collecting reference data: reference time series with $r_1/r_0 = 2, 5, 10$ and 50 (dashed lines, top to bottom). (c) Mueller *et al.*'s method of collecting reference data: reference time series with $r_1/r_0 = 1, 2, 4$ and 8 (dashed lines, top to bottom).

4. RESULTS AND DISCUSSION

4.1. Correcting FRAP acquisition photobleaching

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

$$I_c = \frac{I_f}{I_r}, \quad (4.1)$$

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

$$I_c = I_f e^{kt} \quad (4.2)$$

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. $I_r = e^{-kt}$.

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 FRAP 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 micro-photolysis (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 $\epsilon = k\tau_D = 0.05$ 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 $I/I_\infty = e^{-kt}$. 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 $I/I_\infty = e^{-kt}$. 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 $r_1/r_0 \gtrsim 10$; otherwise there is ostensible 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 $r_1/r_0 \gg 1$. 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 $r_1/r_0 \gg 1$, 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 $r_2/r_0 = r_1/r_0 \gtrsim 10$. Moreover, both Phair *et al.*'s and Mueller *et al.*'s methods can be adopted to collect reference

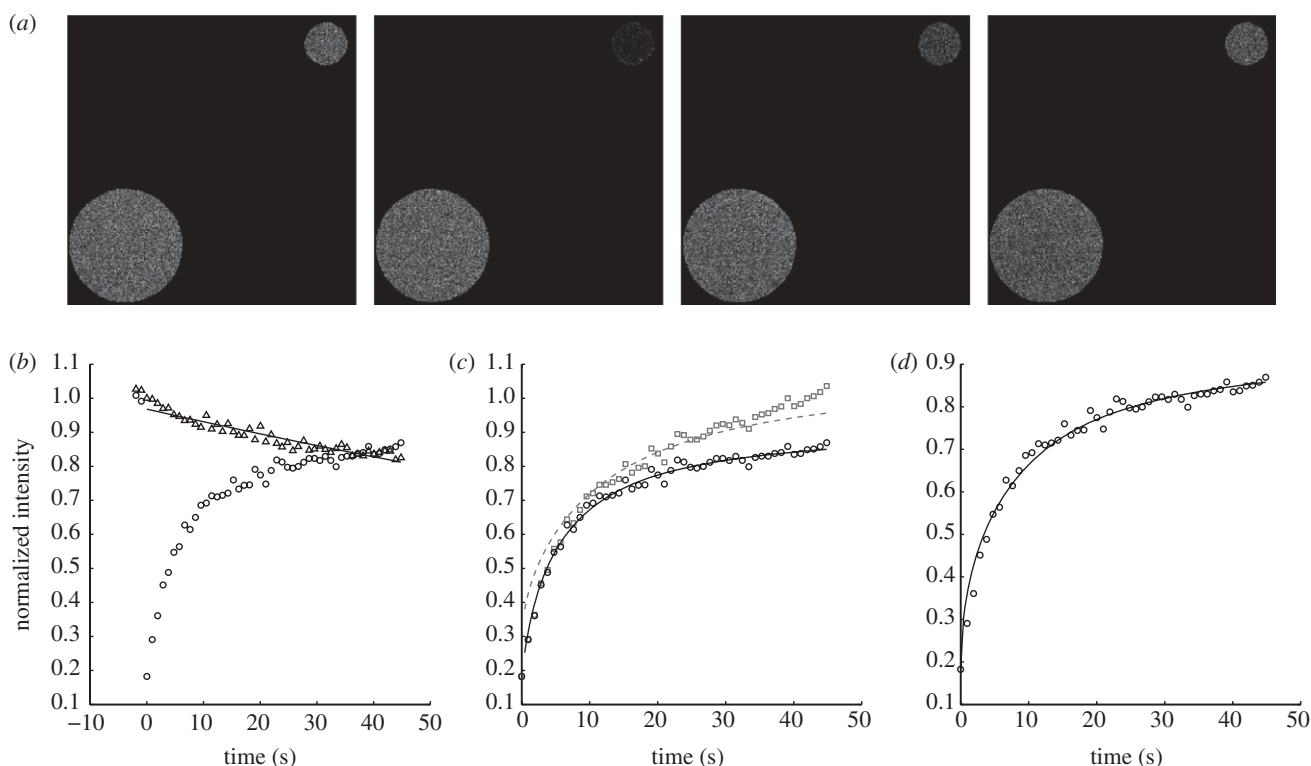


Figure 5. (a) Laser scanning confocal microscopy of FRAP (radius $r_0 \approx 5.13 \mu\text{m}$, upper right) and CFM (radius $r_1 \approx 2.7r_0$, lower left) spots. (b) FRAP time series (circles), reference intensity time series (triangles), and an exponential fit (solid line). (c) Least-squares fit of Soumpasis's solution to the raw (solid line, circles) and corrected (dashed line, squares) FRAP time series. (d) Least-squares fit of the reaction–diffusion FRAP model (solid line) to the raw FRAP time series (circles) furnishing $D_s \approx 1.05 \mu\text{m}^2 \text{s}^{-1}$, $k \approx 0.0013 \text{s}^{-1}$ and $f_m \approx 0.98$ with $\chi^2 \approx 0.02$.

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. $\tau_k \ll \tau_D$ (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 τ_k . 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 $r_0 \approx 5.13 \mu\text{m}$, and CFM spot (lower left) has radius $r_1 \approx 2.7r_0$. The imaged regions are separated by a distance $L \sim 25 \mu\text{m}$, so the characteristic time for lipopolymers with diffusivity $D_s \sim 1 \mu\text{m}^2 \text{s}^{-1}$ to traverse the gap is $\sim L^2/D_s \approx 150 \text{s}$, which is much longer than the characteristic FRAP recovery time $\tau_D \sim r_0^2/$

$D_s \approx 7$ s. Figure 5*b* 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 $I/I_\infty \approx 0.97e^{-0.0039t}$. 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 5*c* shows corrected FRAP time series (squares) obtained by applying equation (4.2) with $k \approx 0.0039$ 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 4*a*. Fitting Soumpasis's equation (3.10) (solid line) to the uncorrected FRAP data (circles) yields $D_s \approx 1.42 \mu\text{m}^2 \text{s}^{-1}$ and $n^\infty/n_\infty \approx 0.92$ 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 $D_s \approx 0.71 \mu\text{m}^2 \text{s}^{-1}$ and $n^\infty/n_\infty \approx 1.1$ 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 5*d* shows the least-squares fit (solid line) of our reaction–diffusion FRAP model to the uncorrected FRAP data (circles). This furnishes $D_s \approx 1.05 \mu\text{m}^2 \text{s}^{-1}$, $k \approx 0.0013$ 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 I^∞/I_∞ 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 τ_D/τ_k (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 α 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 in figure 6 furnishes $D_0 \approx 3.36 \mu\text{m}^2 \text{s}^{-1}$ 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 $D_0 \approx 3.81 \mu\text{m}^2 \text{s}^{-1}$ 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 $D_0 \approx 3.36 \mu\text{m}^2 \text{s}^{-1}$ 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, $D_0 \approx 3.36 \mu\text{m}^2 \text{s}^{-1}$, 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.

Table 1. Summary of FRAP data for bilayers containing $c \approx 1$ 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 .

bilayer synthesis	measurement	D_s measured	D_s average	D_s weighted average	f_m measured	f_m average	f_m weighted average
1	1	2.68	2.27 ± 0.51		96.79	94.44 ± 2.94	
	2	2.09			95.83		
	3	2.17			97.44		
	4	2.02			89.28		
	5	1.39			96.15		
	6	2.63			92.35		
	7	2.90			93.22		
2	1	2.27	2.09 ± 0.24		94.86	93.25 ± 3.34	
	2	2.42			98.63		
	3	1.93			89.97		
	4	2.22			90.05		
	5	1.83			94.24		
	6	1.88			91.73		
3	1	2.15	2.19 ± 0.09		96.63	98.08 ± 1.52	
	2	2.31			97.11		
	3	2.20			98.60		
	4	2.09			99.99		
4	1	2.10	1.91 ± 0.26		98.13	95.90 ± 2.01	
	2	1.60			95.33		
	3	2.03			94.24		
				2.16 ± 0.04			
					96 ± 0.51		

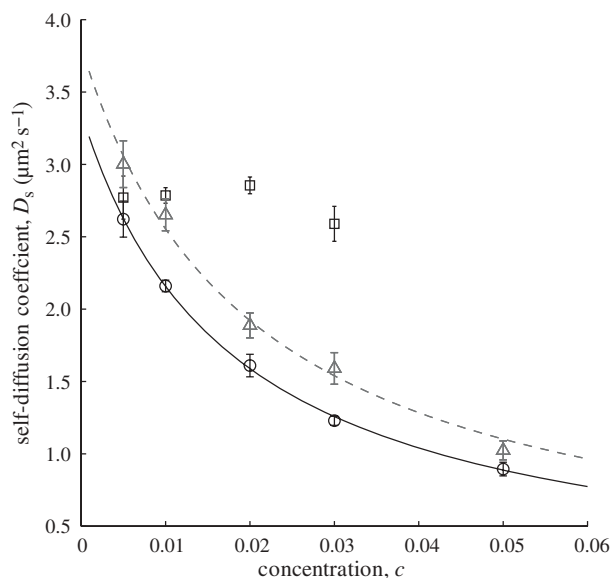


Figure 6. Self-diffusion coefficients D_s of lipopolymer DSPE-PEG2k-CF in DOPC. Circles are from least-squares fitting of the reaction-diffusion FRAP model to uncorrected FRAP time series, with least-squares fitting of interpolation formula 4.3 (solid line) furnishing $D_0 \approx 3.36 \mu\text{m}^2 \text{s}^{-1}$ and $\alpha \approx 0.56$. Triangles are from fitting Soumpasis's solution to uncorrected FRAP time series, with interpolation formula 4.3 (dashed line) furnishing $D_0 \approx 3.81 \mu\text{m}^2 \text{s}^{-1}$ and $\alpha \approx 0.49$. Squares are self-diffusion coefficients of DOPE-NBD in DOPC.

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 ($c \lesssim 5$ mol%) with mobile fractions $f_m \geq 95\%$. [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 ≥ 5 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 $c \gtrsim 1.5$ mol%. According to Kaufmann *et al.* [57], the mole fraction for the PEG mushroom-to-brush transition [60] is

$$c_t = \frac{A_1}{R_F^2}, \quad (4.4)$$

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

$$R_F = a_m n_p^{3/5}, \quad (4.5)$$

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

Table 2. Summary of literature-reported self-diffusion coefficients in lipopolymer-doped bilayers formed by vesicle fusion.

system	I D_s, f_m	II-1 D_s	II-2 D_s	III-1 D_s, f_m	III-2 D_s, f_m
$c \approx 0$ mol%	—	4.0 ± 0.1	—	$1.2 \pm 0.4, 95 \pm 2$	—
$c \approx 0.5$ mol%	$2.62 \pm 0.12,$ 97.89 ± 0.75	3.8 ± 0.2	4.0 ± 0.25	—	—
$c \approx 1$ mol%	$2.16 \pm 0.04,$ 96.20 ± 0.51	—	4.0 ± 0.15	$1.5 \pm 0.1,$ 97 ± 0.4	—
$c \approx 1.5$ mol%	—	3.9 ± 0.2	3.0 ± 0.15	$2.2 \pm 0.4, 97 \pm 2$	—
$c \approx 2$ mol%	$1.61 \pm 0.08,$ 96.92 ± 0.94	—	—	—	$1.1 \pm 0.4,$ 99 ± 1
$c \approx 3$ mol%	$1.23 \pm 0.03,$ 94.62 ± 0.65	—	2.4 ± 0.12	$1.9 \pm 0.2,$ 98 ± 0.3	—
$c \approx 5$ mol%	$0.90 \pm 0.03,$ 97.58 ± 0.41	3.9 ± 0.3	1.4 ± 0.10	no recovery	—
bulk lipids	DOPC	egg-PC	egg-PC	POPC	POPC
polymer	DSPE-PEG2k	DOPE-PEG2k	DSPE-PEG2k	DOPE-PEG2k	DSPE-PEG2k
fluorephore	PEG2k-CF	0.5 mol% TR- PE	Alexa Fluor-594- PEG2k	1 mol% NBD-PC	PEG2k-CF
substrate	cover-slips	glass slides	—	cover-slips	—
substrate cleaning	Piranha etching	baked	—	UVO cleaning	—
FRAP fitting	numerical	[27]	—	[42]	—
bleaching	fitting	neglected	—	corrected ^a	—
reference	this work	[18]	[18]	[57]	[57]

^aIntensity in a region of the FRAP image was used as reference data.

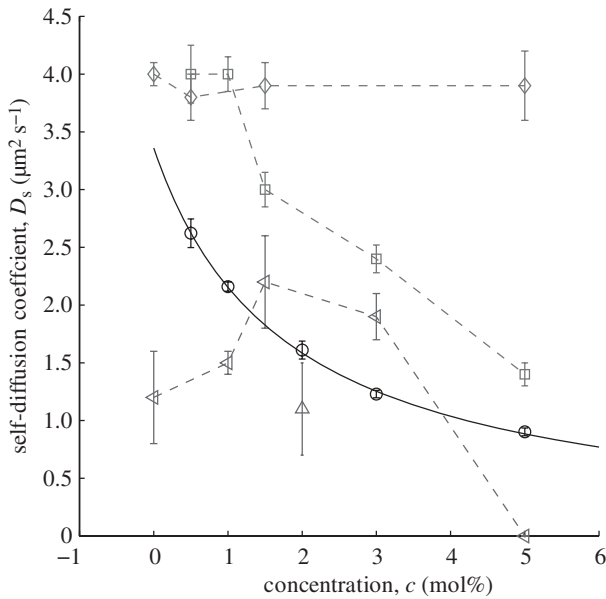


Figure 7. Summary of measured self-diffusion coefficients with lines to guide the eye. Circles: DSPE-PEG2k-CF from this study at various DSPE-PEG2k-CF concentrations. Squares: DSPE-PEG2k-Alexa Fluor-594 from [18] at various DSPE-PEG2k-Alexa Fluor-594 concentrations. Up-triangle: DSPE-PEG2k-CF from [57] 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.

$A_1 \approx 0.60\text{--}0.70$ nm² [61,62], so the mushroom-to-brush transition for PEG2k ($n_p = 45$) would occur at concentrations in the range $c_t \approx 3.5\text{--}6$ mol%. For example, with

$a_m = 0.39$ nm, $A_1 = 0.65$ nm² and $R_F \approx 3.83$ nm, we find $c_t \approx 4.4$ mol%. On the other hand, if we interpret the mushroom-to-brush transition as occurring when $c_t = A_1/(\pi R_F^2)$ (full coverage based on the area occupied by circles with radius R_F), then $c_t \approx 1.4$ 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) (i) 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 studies 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

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

where ϕ 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 $\phi_e = c\pi a_e^2/A_1$, where c is the lipopolymer mole fraction, and $A_1 \approx 0.65 \text{ nm}^2$ is the lipid cross-

sectional area corresponding to a lipid radius $a_1 \approx 0.46 \text{ nm}$. Equation (4.6) then becomes

$$\frac{D_s}{D_0} = 1 - \frac{2\pi a_e^2 c}{A_1}, \quad (4.7)$$

which, when compared with our empirical fitting formula in equation (4.3) as $c \rightarrow 0$, furnishes $a_e = a_1\sqrt{\alpha/2}$. Thus, with $\alpha \approx 0.56$ (figure 6), the effective radius $a_e \approx 2.41 \text{ nm}$.

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

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

where $\gamma \approx 0.577216$ is Euler's constant, and $\lambda = h\mu_f/(a\mu_1)$ with a the hard-cylinder radius, h the lipid–bilayer thickness, μ_1 the lipid–bilayer viscosity and μ_f 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_s/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], $\mu_1 = 1.2 \times 10^{-1} \text{ kg m}^{-1} \text{ s}^{-1}$ [69] and $\mu_f = 8.9 \times 10^{-4} \text{ kg m}^{-1} \text{ s}^{-1}$ (water; [6]). Comparing this value with the effective radius $a_s \approx 2.41 \text{ nm}$ 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

$$\lambda_t = k_B T / D_0, \quad (4.9)$$

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

$$\lambda_t = \lambda_p + \lambda_l, \quad (4.10)$$

where λ_p is the drag coefficient of the polymer chain, and λ_l the drag coefficient of the anchoring lipid. Furthermore, approximating the polymer as a sphere with radius a_p gives

$$\lambda_p = 6\pi\mu_f a_p, \quad (4.11)$$

where μ_f is the viscosity of the supporting fluid (water).

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

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

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

$$\epsilon \equiv a \sqrt{\frac{b_s}{\eta}} \quad (4.13)$$

is a dimensionless disc (lipid head) radius (not to be confused with the dimensionless bleaching constant) with b_s the inter-leaflet friction coefficient. Setting $a \approx a_l \approx 0.46 \text{nm}$ and $\eta \approx 0.08 \text{nN s m}^{-1}$ (half the value for a free lipid bilayer $\eta \approx 0.16 \text{nN s m}^{-1}$ [71]), equation (4.13) furnishes the values of ϵ and b_s 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 ϵ and b_s 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-stearoyl-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

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).

a_p (nm)	λ_p (N s m ⁻¹)	λ_l (N s m ⁻¹)	ϵ	b_s (N s m ⁻³)
0	0	1.21×10^{-9}	0.68	1.48×10^8
2.41	4.04×10^{-11}	1.17×10^{-9}	0.65	1.35×10^8
2.92	4.90×10^{-11}	1.16×10^{-9}	0.64	1.33×10^8
3.83	6.43×10^{-11}	1.15×10^{-9}	0.63	1.29×10^8

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.

R.J.H. gratefully acknowledges support from the Natural Sciences and Engineering Research Council of Canada and the Canada Research Chairs program; H.-Y.Z. thanks McGill University and the Faculty of Engineering, McGill University, for generous financial support through a Richard H. Tomlinson Fellowship and a McGill Engineering Doctoral Award (Hatch Graduate Fellowships in Engineering), respectively.

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 $r_1 = r_2 \gg r_0$. 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

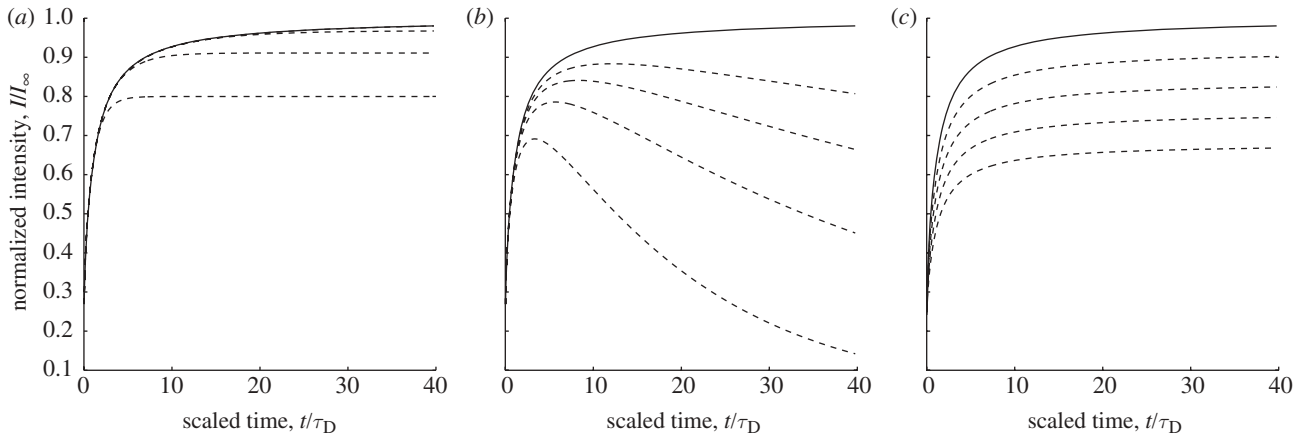


Figure 8. FRAP time series as predicted by the reaction–diffusion FRAP model (dashed lines) with partial imaging ($r_1 < r_2$). In all examples, the initial bleaching intensity $n_0/n_\infty = 0.2$. Solid lines are Soumpasis's analytical solution for FRAP without photobleaching ($\epsilon = 0$) and without an immobile fraction ($f_m = 1$). (a) Varying the reservoir radius $r_2/r_0 = 2, 3, 5$ and 10 (bottom to top) with $r_1/r_0 = 1$, $\epsilon = 0$ and $f_m = 1$. (b) Varying the bleaching constant $\epsilon = 0.005, 0.01, 0.02$ and 0.05 (top to bottom) with $f_m = 1$, $r_1/r_0 = 10$ and $r_2/r_0 = 100$. (c) Varying the mobile fraction $f_m = 0.6, 0.7, 0.8$ and 0.9 (bottom to top) with $\epsilon = 0$, $r_1/r_0 = 10$ and $r_2/r_0 = 100$.

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 $\epsilon \rightarrow 0$ 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

$$n^m(r, 0) = n_\infty f_m, \quad 0 \leq r \leq r_2 \quad (\text{B1})$$

and for the immobile fluorochromes

$$n^i(r, 0) = n_\infty(1 - f_m), \quad 0 \leq r \leq r_2. \quad (\text{B2})$$

A representative CFM time series is presented in figure 9. The intensity of immobile fluorochromes (dash-

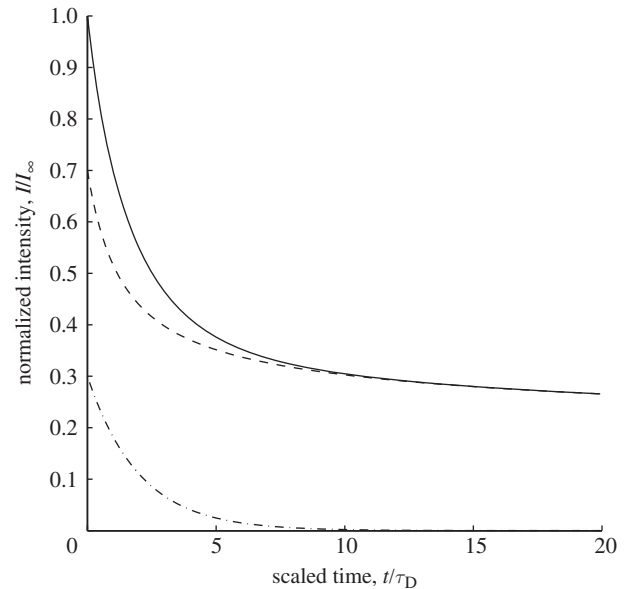


Figure 9. Continuous fluorescence micro-photolysis (CFM) time series as predicted by the reaction–diffusion model: $\epsilon = 0.5$, $f_m = 0.7$, $r_1/r_0 = 1$ and $r_2/r_1 = 100$. The solid line is the average intensity of fluorochromes in the CFM spot, and the dashed and dash-dotted lines are, respectively, the contributions from the mobile and immobile fractions.

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 $e^{-\epsilon t/\tau_D}$ (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

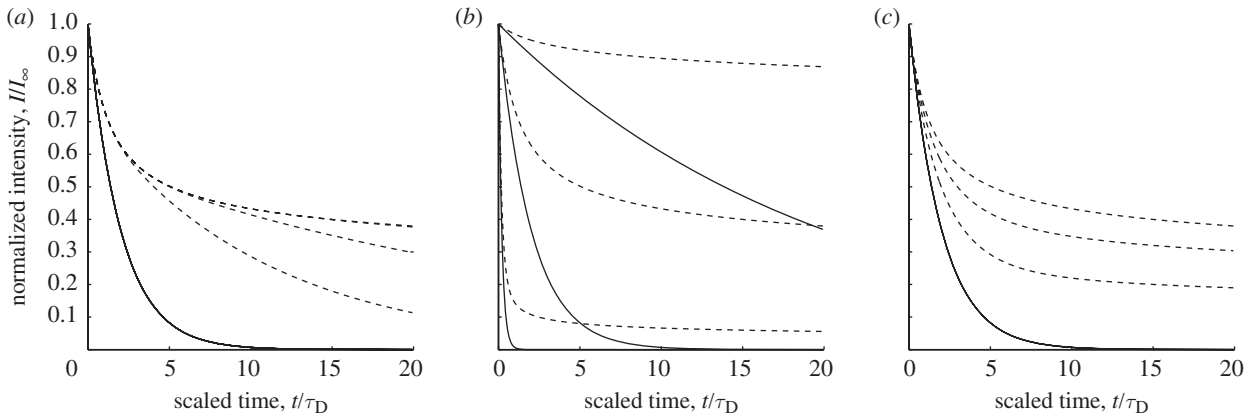


Figure 10. CFM time series as predicted by the reaction–diffusion model (dashed lines). Bleaching curves (solid lines) have corresponding values of ϵ . (a) Varying the reservoir radius $r_2/r_1 = 1, 2, 3, 5$ and 10 (bottom to top) with $\epsilon = 0.5$ and $f_m = 1$. Note that the time series with $r_2/r_1 = 1$ 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 $\epsilon = 0.05, 0.5$ 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 $\epsilon = 0.5$ and $r_2/r_1 = 100$.

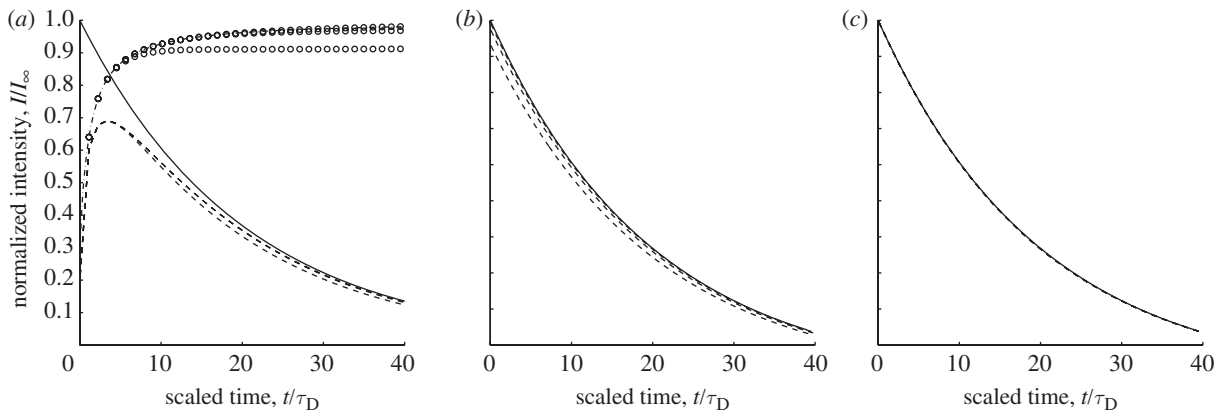


Figure 11. Correcting acquisition bleaching when the entire reservoir is imaged ($r_1 = r_2$): $\epsilon = 0.05$, $f_m = 1$ and $n_0/n_\infty = 0.2$. Solid lines are the bleaching curve $I/I_\infty = e^{-\epsilon t/\tau_D}$. (a) Correction according to equation (4.2): Soumpasis's analytical solution (dash-dotted line); numerical FRAP time series for $r_2/r_0 = r_1/r_0 = 3, 5$ and 10 (dashed lines, top to bottom); corrected FRAP time series for $r_2/r_0 = r_1/r_0 = 3, 5$ and 10 (circles, bottom to top). (b) Phair *et al.*'s method of collecting reference data: reference time series with $r_2/r_0 = r_1/r_0 = 3, 5$ and 10 (dashed lines, bottom to top). (c) Mueller *et al.*'s method of collecting reference data: reference time series with $r_2/r_0 = r_1/r_0 = 1, 5$ and 10 .

independent of the reservoir size. Figure 10b shows solutions for several values of $\epsilon = 0.05, 0.5$ and 5 (dashed lines, top to bottom) with $f_m = 1$ and $r_2/r_1 = 100$. As ϵ 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 $\epsilon = 0.5$ and $r_2/r_1 = 100$. In the absence of diffusion ($f_m = 0$), the CFM time series overlaps 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 $r_2/r_0 = r_1/r_0 = 3, 5$ and 10 (circles, bottom to top). With the given bleaching constant $\epsilon = 0.05$, 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 $r_2/r_0 = r_1/r_0 = 3, 5$ 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 $r_2/r_0 = r_1/r_0 = 1, 5$ and 10 (dashed lines). All the reference curves overlap the bleaching curve (solid line) calculated using the known bleaching constant $\epsilon = 0.05$.

REFERENCES

- 1 Janmey, P. A. & Stossel, T. P. 1989 Gelsolin–polyphosphoinositide interaction—full expression of gelsolin-

- inhibiting function by polyphosphoinositides in vesicular form and inactivation by dilution, aggregation, or masking of the inositol head group. *J. Biol. Chem.* **264**, 4825–4831.
- 2 McCloskey, M. A. & Poo, M. 1986 Contact-induced redistribution of specific membrane-components—local accumulation and development of adhesion. *J. Cell Biol.* **102**, 2185–2196. (doi:10.1083/jcb.102.6.2185)
 - 3 Chan, P. Y., Lawrence, M. B., Dustin, M. L., Ferguson, L. M., Golan, D. E. & Springer, T. A. 1991 Influence of receptor lateral mobility on adhesion strengthening between membranes containing I α -3 and cd2. *J. Cell Biol.* **115**, 245–255. (doi:10.1083/jcb.115.1.245)
 - 4 Saxton, M. J. 1999 Lateral diffusion of lipids and proteins. In *Membrane permeability*, vol. 48, *Current topics in membranes*. San Diego, CA: Academic Press.
 - 5 Saffman, P. G. & Delbruck, M. 1975 Brownian motion in biological membranes. *Proc. Natl Acad. Sci. USA* **72**, 3111–3113. (doi:10.1073/pnas.72.8.3111)
 - 6 Peters, R. & Cherry, R. J. 1982 Lateral and rotational diffusion of bacteriorhodopsin in lipid bilayers—experimental test of the Saffman–Delbruck equations. *Proc. Natl Acad. Sci. USA* **79**, 4317–4321. (doi:10.1073/pnas.79.14.4317)
 - 7 Scalettar, B. A., Abney, J. R. & Owicki, J. C. 1988 Theoretical comparison of the self-diffusion and mutual diffusion of interacting membrane-proteins. *Proc. Natl Acad. Sci. USA* **85**, 6726–6730. (doi:10.1073/pnas.85.18.6726)
 - 8 Abney, J. R., Scalettar, B. A. & Owicki, J. C. 1989 Self-diffusion of interacting membrane-proteins. *Biophys. J.* **55**, 817–833. (doi:10.1016/S0006-3495(89)82882-6)
 - 9 Batchelor, G. K. 1976 Brownian diffusion of particles with hydrodynamic interaction. *J. Fluid Mech.* **74**, 1–29. (doi:10.1017/S0022112076001663)
 - 10 Abney, J. R., Scalettar, B. A. & Owicki, J. C. 1989 Mutual diffusion of interacting membrane-proteins. *Biophys. J.* **56**, 315–326. (doi:10.1016/S0006-3495(89)82678-5)
 - 11 Pink, D. A. 1985 Protein lateral movement in lipid bilayers—stimulation studies of its dependence upon protein-concentration. *Biochim. Biophys. Acta* **818**, 200–204.
 - 12 Saxton, M. J. 1987 Lateral diffusion in an archipelago—the effect of mobile obstacles. *Biophys. J.* **52**, 989–997. (doi:10.1016/S0006-3495(87)83291-5)
 - 13 Bussell, S. J., Koch, D. L. & Hammer, D. A. 1992 The resistivity and mobility functions for a model system of 2 equal-sized proteins in a lipid bilayer. *J. Fluid Mech.* **243**, 679–697. (doi:10.1017/S002211209200288X)
 - 14 Bussell, S. J., Hammer, D. A. & Koch, D. L. 1994 The effect of hydrodynamic interactions on the tracer and gradient diffusion of integral membrane-proteins in lipid bilayers. *J. Fluid Mech.* **258**, 167–190. (doi:10.1017/S0022112094003289)
 - 15 Bussell, S. J., Koch, D. L. & Hammer, D. A. 1995 Effect of hydrodynamic interactions on the diffusion of integral membrane-proteins—tracer diffusion in organelle and reconstituted membranes. *Biophys. J.* **68**, 1828–1835. (doi:10.1016/S0006-3495(95)80359-0)
 - 16 Deverall, M. A., Gindl, E., Sinner, E. K., Besir, H., Ruehe, J., Saxton, M. J. & Naumann, C. A. 2005 Membrane lateral mobility obstructed by polymer-tethered lipids studied at the single molecule level. *Biophys. J.* **88**, 1875–1886. (doi:10.1529/biophysj.104.050559)
 - 17 Soong, R. & Macdonald, P. M. 2005 Lateral diffusion of peg-lipid in magnetically aligned bicelles measured using stimulated echo pulsed field gradient 1 h NMR. *Biophys. J.* **88**, 255–268. (doi:10.1529/biophysj.104.043620)
 - 18 Albertorio, F., Diaz, A. J., Yang, T., Chapa, V. A., Kataoka, S., Castellana, E. T. & Cremer, P. S. 2005 Fluid and air-stable lipopolymer membranes for biosensor applications. *Langmuir* **21**, 7476–7482. (doi:10.1021/la050871s)
 - 19 Low, M. G. & Saltiel, A. R. 1988 Structural and functional roles of glycosyl-phosphatidylinositol in membranes. *Science* **239**, 268–275. (doi:10.1126/science.3276003)
 - 20 Allen, T. M., Hansen, C., Martin, F., Redemann, C. & Yaouyoung, A. 1991 Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives *in vivo*. *Biochim. Biophys. Acta* **1066**, 29–36.
 - 21 Vermette, P. & Meagher, L. 2003 Interactions of phospholipid- and poly(ethylene glycol)-modified surfaces with biological systems: relation to physico-chemical properties and mechanisms. *Colloids Surf. B* **28**, 153–198. (doi:10.1016/S0927-7765(02)00160-1)
 - 22 Albertorio, F., Daniel, S. & Cremer, P. S. 2006 Supported lipopolymer membranes as nanoscale filters: simultaneous protein recognition and size-selection assays. *J. Am. Chem. Soc.* **128**, 7168–7169. (doi:10.1021/ja062010r)
 - 23 Tanakan, M. & Sackmann, E. 2005 Polymer-supported membranes as models of the cell surface. *Nature* **437**, 656–663.
 - 24 Albersdorfer, A., Feder, T. & Sackmann, E. 1997 Adhesion-induced domain formation by interplay of long-range repulsion and short-range attraction force: a model membrane study. *Biophys. J.* **73**, 245–257. (doi:10.1016/S0006-3495(97)78065-2)
 - 25 Jeppesen, C., Wong, J. Y., Kuhl, T. L., Israelachvili, J. N., Mullah, N., Zalipsky, S. & Marques, C. M. 2001 Impact of polymer tether length on multiple ligand–receptor bond formation. *Science* **293**, 465–468. (doi:10.1126/science.293.5529.465)
 - 26 Chen, Y., Lagerholm, B., Yang, B. & Jacobson, K. 2006 Methods to measure the lateral diffusion of membrane lipids and proteins. *Methods* **39**, 147–153. (doi:10.1016/j.ymeth.2006.05.008)
 - 27 Axelrod, D., Koppel, D. E., Schlessinger, J., Elson, E. & Webb, W. W. 1976 Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. *Biophys. J.* **16**, 1055–1069. (doi:10.1016/S0006-3495(76)85755-4)
 - 28 Chary, S. R. & Jain, R. K. 1989 Direct measurement of interstitial convection and diffusion of albumin in normal and neoplastic tissues by fluorescence photobleaching. *Proc. Natl Acad. Sci. USA* **86**, 5385–5389. (doi:10.1073/pnas.86.14.5385)
 - 29 Boxer, S. G. 2000 Molecular transport and organization in supported lipid membranes. *Curr. Opin. Chem. Biol.* **4**, 704–709. (doi:10.1016/S1367-5931(00)00139-3)
 - 30 Phair, R. D. & Misteli, T. 2000 High mobility of proteins in the mammalian cell nucleus. *Nature* **404**, 604–609.
 - 31 Reits, E. A. J. & Neefjes, J. J. 2001 From fixed to frap: measuring protein mobility and activity in living cells. *Nat. Cell Biol.* **3**, E145–E147. (doi:10.1038/35078615)
 - 32 Lippincott-Schwartz, J., Altan-Bonnet, N. & Patterson, G. H. 2003 Photobleaching and photoactivation: following protein dynamics in living cells. *Nat. Rev. Mol. Cell Biol.* **5**, S7–S14.
 - 33 Owen, M. D., Williamson, D., Rentero, C. & Gaus, K. 2009 Quantitative microscopy: protein dynamics and membrane organisation. *Traffic* **10**, 962–971. (doi:10.1111/j.1600-0854.2009.00908.x)

- 34 Shav-Tal, Y., Darzacq, X., Shenoy, S. M., Fusco, D., Janicki, S. M., Spector, D. L. & Singer, R. H. 2004 Dynamics of single mrnps in nuclei of living cells. *Science* **304**, 1797–1800. (doi:10.1126/science.1099754)
- 35 Giese, B. *et al.* 2003 Long term association of the cytokine receptor gp130 and the janus kinase jak1 revealed by frap analysis. *J. Biol. Chem.* **278**, 39 205–39 213. (doi:10.1074/jbc.M303347200)
- 36 Dorsch, S., Klotz, K. N., Engelhardt, S., Lohse, M. J. & Bunemann, M. 2009 Analysis of receptor oligomerization by frap microscopy. *Nature Methods* **6**, 225–230. (doi:10.1038/nmeth.1304)
- 37 Phair, R. D. & Misteli, T. 2001 Kinetic modelling approaches to *in vivo* imaging. *Nat. Rev. Mol. Cell Biol.* **2**, 898–907. (doi:10.1038/35103000)
- 38 Sprague, B. L. & McNally, J. G. 2005 Frap analysis of binding: proper and fitting. *Trends Cell Biol.* **15**, 84–91. (doi:10.1016/j.tcb.2004.12.001)
- 39 Mueller, F., Wach, P. & McNally, J. G. 2008 Evidence for a common mode of transcription factor interaction with chromatin as revealed by improved quantitative fluorescence recovery after photobleaching. *Biophys. J.* **94**, 3323–3339. (doi:10.1529/biophysj.107.123182)
- 40 Weiss, M. 2004 Challenges and artifacts in quantitative photobleaching experiments. *Traffic* **5**, 662–671. (doi:10.1111/j.1600-0854.2004.00215.x)
- 41 Dushek, O. & Coombs, D. 2008 Improving parameter estimation for cell surface frap data. *J. Biochem. Biophys. Meth.* **70**, 1224–1231. (doi:10.1016/j.jbbm.2007.07.002)
- 42 Soumpasis, D. M. 1983 Theoretical analysis of fluorescence photobleaching recovery experiments. *Biophys. J.* **41**, 95–97. (doi:10.1016/S0006-3495(83)84410-5)
- 43 Seiffert, S. & Oppermann, W. 2005 Systematic evaluation of frap experiments performed in a confocal laser scanning microscope. *J. Microscopy* **220**, 20–30. (doi:10.1111/j.1365-2818.2005.01512.x)
- 44 Ellenberg, J., Siggia, E. D., Moreira, J. E., Smith, C. L., Presley, J. F., Worman, H. J. & Lippincott-Schwartz, J. 1997 Nuclear membrane dynamics and reassembly in living cells: targeting of an inner nuclear membrane protein in interphase and mitosis. *J. Cell Biol.* **138**, 1193–1206. (doi:10.1083/jcb.138.6.1193)
- 45 Jonsson, P., Jonsson, M. P., Tegenfeldt, J. O. & Hook, F. 2008 A method improving the accuracy of fluorescence recovery after photobleaching analysis. *Biophys. J.* **95**, 5334–5348. (doi:10.1529/biophysj.108.134874)
- 46 Phair, R. D., Gorski, S. A. & Misteli, T. 2004 Measurement of dynamic protein binding to chromatin *in vivo*, using photobleaching microscopy. In *Chromatin and chromatin remodeling enzymes*, Pt A, vol. 375, *Methods in enzymology*, pp. 393–414. San Diego, CA: Academic Press.
- 47 Endress, E., Weigelt, S., Reents, G. & Bayerl, T. M. 2005 Derivation of a closed form analytical expression for fluorescence recovery after photo bleaching in the case of continuous bleaching during read out. *Eur. Phys. J. E* **16**, 81–87. (doi:10.1140/epje/e2005-00010-5)
- 48 Ren, C. L., Nap, R. J. & Szleifer, I. 2008 The role of hydrogen bonding in tethered polymer layers. *J. Phys. Chem. B* **112**, 16 238–16 248. (doi:10.1021/jp8080904)
- 49 Groves, J. T. & Boxer, S. G. 1995 Electric field-induced concentration gradients in planar supported bilayers. *Biophys. J.* **69**, 1972–1975. (doi:10.1016/S0006-3495(95)80067-6)
- 50 van Oostveldt, P. & Bauwens, S. 1990 Quantitative fluorescence in confocal microscopy: the effect of the detection pinhole aperture on the re-absorption and inner filter phenomena. *J. Microscopy* **158**, 121–132.
- 51 Herman, B. 1998 *Fluorescence microscopy*. Oxford, UK: Bios Scientific Publishers.
- 52 Kang, M., Day, C. A., Drake, K., Kenworthy, A. K. & DiBenedetto, E. 2009 A generalization of theory for two-dimensional fluorescence recovery after photobleaching applicable to confocal laser scanning microscopes. *Biophys. J.* **97**, 1501–1511. (doi:10.1016/j.bpj.2009.06.017)
- 53 Widengren, J. & Rigler, R. 1996 Mechanisms of photobleaching investigated by fluorescence correlation spectroscopy. *Bioimaging* **4**, 149–157.
- 54 Rabut, G. & Ellenberg, J. 2004 *Live cell imaging, a laboratory manual: photobleaching techniques to study mobility and molecular dynamics of proteins in live cells: FRAP, iFRAP and FLIP*. Ann Arbor, MI: Cold Spring Harbor Laboratory Press.
- 55 Peters, R., Brunger, A. & Schulten, K. 1981 Continuous fluorescence microphotolysis—a sensitive method for study of diffusion-processes in single cells. *Proc. Natl Acad. Sci. USA* **78**, 962–966. (doi:10.1073/pnas.78.2.962)
- 56 Kam, L. & Boxer, S. G. 2001 Cell adhesion to protein-micropatterned-supported lipid bilayer membranes. *J. Biomed. Mater. Res.* **55**, 487–495. (doi:10.1002/1097-4636(20010615)55:4<487::AID-JBM1041>3.0.CO;2-7)
- 57 Kaufmann, S., Papastavrou, G., Kumar, K., Textor, M. & Reimhult, E. 2009 A detailed investigation of the formation kinetics and layer structure of poly(ethylene glycol) tether supported lipid bilayers. *Soft Matter* **5**, 2804–2814. (doi:10.1039/b901874c)
- 58 Irrechukwu, O. N. & Levenston, M. E. 2009 Improved estimation of solute diffusivity through numerical analysis of frap experiments. *Cell. Mol. Bioeng.* **2**, 104–117. (doi:10.1007/s12195-009-0042-1)
- 59 Bevington, P. R. & Robinson, D. K. 2002 *Data reduction and error analysis for the physical sciences*, 3rd edn. London, UK: McGraw-Hill.
- 60 de Gennes, P. G. 1980 Conformations of polymers attached to an interface. *Macromolecules* **13**, 1069–1075. (doi:10.1021/ma60077a009)
- 61 Hristova, K., Kenworthy, A. & McIntosh, T. 1995 Effect of bilayer composition on the phase behavior of liposomal suspensions containing poly(ethylene glycol)-lipids. *Macromolecules* **28**, 7693–7699. (doi:10.1021/ma00127a015)
- 62 Nagle, J. F. & Tristram-Nagle, S. 2000 Lipid bilayer structure. *Curr. Opin. Struct. Biol.* **10**, 474–480. (doi:10.1016/S0959-440X(00)00117-2)
- 63 Przybylo, M., Sykora, J., Humpolickova, J., Benda, A., Zan, A. & Hof, M. 2006 Lipid diffusion in giant unilamellar vesicles is more than 2 times faster than in supported phospholipid bilayers under identical conditions. *Langmuir* **22**, 9096–9099. (doi:10.1021/la061934p)
- 64 Scomparin, C., Lecuyer, S., Ferreira, M., Charitat, T. & Tinland, B. 2009 Diffusion in supported lipid bilayers: influence of substrate and preparation technique on the internal dynamics. *Eur. Phys. J. E* **28**, 211–220. (doi:10.1140/epje/i2008-10407-3)
- 65 Seu, K. J., Cambrea, L. R., Everly, R. M. & Hovis, J. S. 2006 Influence of lipid chemistry on membrane fluidity: tail and headgroup interactions. *Biophys. J.* **91**, 3727–3735. (doi:10.1529/biophysj.106.084590)
- 66 Seu, K. J., Pandey, A. P., Haque, F., Proctor, E. A., Ribbe, A. E. & Hovis, J. S. 2007 Effect of surface treatment on diffusion and domain formation in supported lipid

- bilayers. *Biophys. J.* **92**, 2445–2450. (doi:10.1529/biophysj.106.099721)
- 67 Lis, L. J., Mcalister, M., Fuller, N., Rand, R. P. & Parsegian, V. A. 1982 Interactions between neutral phospholipid–bilayer membranes. *Biophys. J.* **37**, 657–665.
- 68 Marsh, D. 1990 *Handbook of lipid bilayers*. Boca Raton, FL: CRC Press.
- 69 Ishii, C. Y. & Boxer, S. G. 2006 Controlling two-dimensional tethered vesicle motion using an electric field: interplay of electrophoresis and electro-osmosis. *Langmuir* **22**, 2384–2391.
- 70 Evans, E. & Sackmann, E. 1988 Translational and rotational drag coefficients for a disk moving in a liquid membrane-associated with a rigid substrate. *J. Fluid Mech.* **194**, 553–561. (doi:10.1017/S0022112088003106)
- 71 Kuhner, M., Tampe, R. & Sackmann, E. 1994 Lipid monolayer and bilayer supported on polymer-films—composite polymer–lipid films on solid substrates. *Biophys. J.* **67**, 217–226. (doi:10.1016/S0006-3495(94)80472-2)
- 72 Tanaka, M., Hermann, J., Haase, I., Fischer, M. & Boxer, S. G. 2007 Frictional drag and electrical manipulation of recombinant proteins in polymer-supported membranes. *Langmuir* **23**, 5638–5644. (doi:10.1021/la0628219)