Determination of Diffusion Coefficients of Fluorescently Labelled Molecules in Lipid Membranes Using Confocal Scanning Laser Microscopy

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A series of liposomes has been studied by the Fluorescence Recovery after Photobleaching technique using confocal scanning laser microscopy. Both mobile and immobile specimens of lipid membranes have been examined. The specimens consisted of liposomal suspensions incorporating mixtures of the phospholipids dioleoyl phosphatidylcholine (DOPC) and distearoyl phosphatidylcholine (DSPC) with the aminolipid dihexadecanol glycerophospho-ethanolamine (DHPE). The measurements reported provide information about the effective diffusion coefficients, which vary from $0.2 \cdot 10^{-10}$ cm^{2}s^{-1} to $3.2 \cdot 10^{-10}$ cm^{2}s^{-1}. They were highly dependent on the ratio DSPC:DOPC. A simple theoretical model of 1-D diffusion was applied for the calculation of the effective diffusion coefficients. The measurements show that it is possible to use straightforward procedures with an unmodified commercial confocal microscope to determine diffusion coefficients of biological specimens.

Key words: Phospholipid; Lateral Diffusion; CSLM.

1. Introduction

Much of the current knowledge about the physical properties of lipid bilayers has been gained from the study of the Mueller-Rudin bilayer [1], the phospholipid liposome [2 – 8], and the sonicated phospholipid vesicle [9]. Molecular motion of phospholipids in bilayers is likely to be somewhat restricted by the amphiphilic nature of the molecule. Two kinds of translational motion of lipids in bilayers have been demonstrated: (i) the lateral diffusion of the molecule, and (ii) the movement of the molecule from one bilayer leaflet to the other, a process that has been called flip-flop. Lateral diffusion rates of phosphopholipids in bilayers depend on the physical state of the bilayer and are much more rapid for lipids in the liquid crystalline state than for lipids in the gel state. Several different techniques have been applied to the study of lateral diffusion in phospholipid bilayers [10 – 13].

Fluorescence Recovery after Photobleaching (FRAP) is a technique that has been used to investigate molecular mobilities within cells and membrane systems in situ. The diffusion of fluorescein isothiocyanate (FITC) labelled macromolecules measured by FRAP determines a lateral diffusion coefficient [14, 15]. FRAP is typically applied to measurements of reconstituted membranes such as liposomes or planar bilayers. The basic idea of FRAP (and techniques derived from it) is to irreversibly bleach the fluorescent probes in a defined area with a high-intensity light flash and to monitor the subsequent fluorescence recovery. This technique yields information about the relative mobility of the fluorophore, specifically the effective diffusion coefficient (D_{eff}), and the fraction of the fluorophore that is mobile.

Typically, in FRAP, a short pulse of intense laser light irreversibly destroys (photobleaches) the fluorescence in a micron-size area. Recovery of fluorescence in the photobleached area occurs as a result of diffusional exchange between bleached and unbleached fluorescent molecules, and is monitored using low-intensity laser illumination. Photobleaching theory primarily describes solutions for finding D_{eff} from bleaching a small spot in a planar, uniform plasma membrane [16 – 19]. To determine D_{eff} as accurately as possible, theoretical models must be developed [20].

There are several FRAP studies using laser-scanning microscopy [21–23]. The confocal microscope offers many advantages over the standard fluorescence microscope, enabling photobleaching of discrete regions within the sample and generating images with a high degree of spatial resolution without interference from out-of-focus fluorescence [24–26]. In this study, the behaviour of phospholipids is investigated using a Mathcad (Mathsoft Engineering and Education Inc) worksheet to implement methods for diffusion measurements by the FRAP technique.

2. Experimental Methods

2.1. Preparation of the Specimens

Multilamellar liposomes were prepared by vortex-mixing phospholipids in an electrolyte solution. The phospholipids were dried on the inside of a test tube under a nitrogen gas atmosphere and mixed with the solution at approximately 60 °C. The solution composition was: KCl, 150 mM; $CaCl_2$, 0.1 mM; K-EDTA, 0.1 mM; Trisbuffer, 5 mM. Typically, a liposome suspension contained less than 10 mg lipid ml^{-1}. It was stored at 5 °C, where it sedimented to give a localised, dense suspension of variously aggregated liposomes.

The phospholipids used were: (i) fluorescein DHPE: dihexadecanoyl glycerophospho-ethanolamine; with fluorescein attached at the headgroup (F362 Molecular Probes [25]); (ii) DSPC: distearoyl phosphatidylcholine (Sigma); (iii) DOPC: dioleoyl phosphatidylcholine (Sigma).

The proportions of the phospholipids were varied; DHPE was generally less than 3% of the lipid, but sufficient to provide a strong fluorescence signal.

2.2. Apparatus and Measurements

Measurements were performed on a Bio-Rad MRC 1024 confocal scanning system incorporating a Nikon inverted microscope. An oil-immersion objective ($\times 60$) was used in conjunction with an iris setting of 2 mm for image collection. All samples were scanned using the 488 nm wavelength line of a Krypton/Argon ion laser. The microscope was controlled and images were recorded using Bio-Rad LaserSharp 3.0 software, running on IBM's OS/2 operating system.

In order to hold the specimens on the confocal laser microscope, glass slides were used. These had a small hole, diameter ~ 2 mm, drilled through the slide. This was sealed on the lower face by a glass cover slip to create a well. Into this well was pipetted a quantity of the specimen under examination. Once the specimen was prepared, it was mounted on the microscope.

Once a vesicle was found that was deemed suitable, two images were recorded, both at laser power of 1%: one at low zoom (zoom = 1) to show the vesicle in its surroundings, and the other at a higher zoom (zoom = 5) to record the vesicle itself in more detail. Once these images were recorded, the computer was set up to begin recording of a time series of images as promptly as possible once photobleaching had taken place. Both the laser power and zoom were then increased to effect photobleaching, typically at laser power of 100% applied at zoom = 20 for one or two scans over the targeted area of the vesicle. Immediately after photobleaching the time was noted, and a stopwatch was started for the recording of a time series of images, recorded at low power and low zoom. As expected, recovery was observed after photobleaching of mobile specimens, but not for immobile specimens.

The analysis was carried out in two stages. In the first stage a recovery time-course was extracted from the original time series image files, using Scion Image and Microsoft Excel. Time-course results were found both for a region approximating an average over the whole of the partly-bleached liposome, and for a region approximating that deliberately bleached. The time-course data were then fitted to a theoretical 1-D FRAP recovery curve, using Mathcad.

3. Results and Discussion

3.1. Mobile Liposomes

Images of the DOPC:DSPC:DHPE sample mixes were recorded for low and high zoom values over a range of laser power settings on the MRC 1024. The images recorded include vertical sections of vesicles, and also show what difference the initial fluorescence intensity makes to fluorescence recovery. The small size of the confocal aperture ensures that as much as possible of the out of focus blurring which occurs, is eliminated, and thus even at low powers a confocal microscope can gather images of a very high quality.

Figure 1 contains a series of images showing one of the mobile samples before and after photobleaching. The ratio and preparation temperature of this sample were 71.5% DSPC, 28.5% DOPC, 2% DHPE,

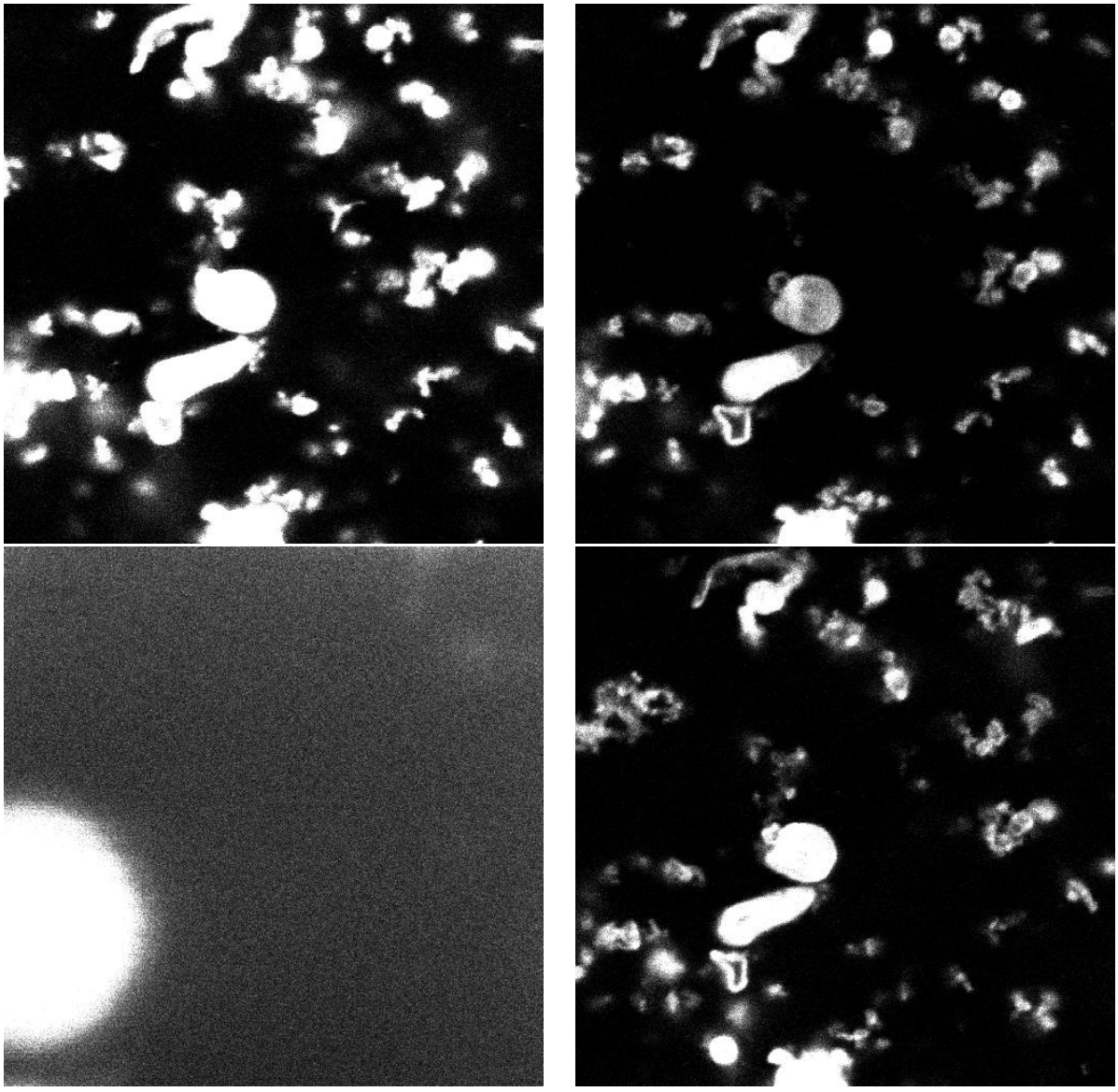

Fig. 1. A series of images showing a mobile sample (DOPC:DSPC = 71.5% : 28.5%) before and after bleaching. – *Left, top.* Pre-bleach image: taken at 1% laser power, zoom 5. *Left, bottom.* Bleach image: taken at 100% laser power, 2 sweeps, zoom 20. *Right, top.* First recovery image: taken at 1% laser, zoom 5, 26 s after bleach. Note vertical edge to bleached region, visible on near-circular vesicle close to centre of image. *Right, bottom.* Final recovery image: taken at 1% laser, zoom 5, 1548 s after bleach.

and approximately 65 °C, respectively. General operation conditions for this sample were: speed normal, objective ×60, laser 488 nm, PMT 2 analog mode, gain 1200, iris 2, direct collection at $N = 1$ (i. e. each image uses a single scan to minimise unwanted bleaching), emission filter 522DF32, box size 512×512. The intensity of fluorescence was reduced by photobleaching, and the recovery of the fluorescence intensity within the bleached region can be visualised as a time-sequence of profiles of intensity versus distance across the vesicle, as shown in Figure 2. It can be seen from the figures that, in addition to the deliberately bleached area, there was significant non-local photobleaching and corresponding recovery.

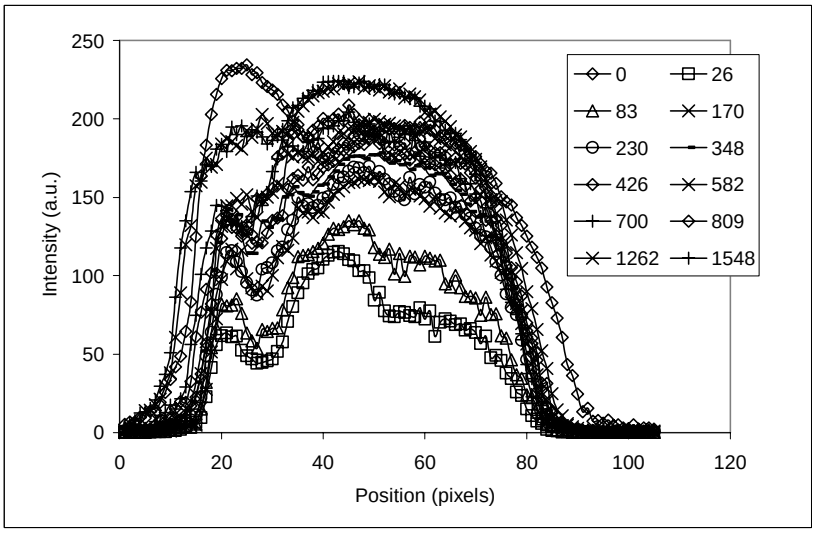

Fig. 2. Profiles across liposome: fluorescence intensity versus distance during recovery, with time as parameter (legend shows elapsed time in seconds and $t = 0$ is the pre-bleach profile).

The variation of the intensity can be interpreted in terms of time variation in fluorescent output during the transit of the scanning beam across each point in the sample. The zoom and laser power were increased to effect bleaching, and an effort was made to bleach about half of a liposome. The recovery sequences were analysed in two stages. In the first stage, a recovery time-course was extracted from the original image files, using Scion Image and Microsoft Excel. Time-course results were found for a region approximating an average over the whole of the partly bleached liposome, and for a region approximating that deliberately bleached. In the second stage, the time-course data were fitted to a theoretical 1-D FRAP recovery curve using a Mathcad worksheet that implements an analytical solution for an infinite 1-D domain in which a single region is bleached, corresponding to bleaching a stripe in a 2-D membrane. The time-course of the integrated intensity across the bleached region is then given by

$$I(t,a,D_{\mathrm{eff}},I_{\mathrm{f}}) = \tag{1}$$

$$I_{\mathrm{f}}\Big\{1+\frac{\sqrt{D_{\mathrm{eff}}t}}{a\sqrt{\pi}}\Big[1-\exp\Big(\frac{a^2}{D_{\mathrm{eff}}t}\Big)\Big]-\mathrm{erf}\Big(\frac{a}{\sqrt{D_{\mathrm{eff}}t}}\Big)\Big\},$$

where $2a$ is the width of the bleached region, and I_{f} is the final value of recovered intensity. This was fitted to the time-course data using a Mathcad least-squares fit function, either by adjusting D_{eff} and I_{eff} as parameters, or by setting I_{eff} equal to the pre-bleaching level and adjusting D_{eff} as a single parameter.

Table 1. Estimates of D_{eff} (units of 10^{-10} cm^{2}s^{-1}).

	Whole liposome	Bleached region
1-parameter fit	1.092	1.767
2-parameter fit	0.5052	0.8970

Figure 3 shows fitted results for the sample. Using a bleached-width parameter $a = 1$ μm, the estimates of D_{eff} (10^{-10} cm^{2}s^{-1}) are shown in Table 1.

For comparison, a diffusion coefficient D_{eff} can also be deduced from an approximate form of the recovery function [26] as

$$D_{\mathrm{eff}} = \frac{a^2(t_2-t_1)}{4t_1t_2}\left[\ln\frac{I(t_1)\sqrt{t_2}}{I(t_2)\sqrt{t_1}}\right]^{-1}. \tag{2}$$

Here a is the bleached-width parameter, $I(t_1)$ and $I(t_2)$ are the intensities at the initial and final recovery times, respectively. From this equation, $D_{\mathrm{eff}} = 3.2\cdot 10^{-10}$ cm^{2}s^{-1} is found, comparable with the values estimated from a full fit to the data.

For other samples, values of D_{eff} were found to vary from $0.2\cdot 10^{-10}$ cm^{2}s^{-1} to $3.2\cdot 10^{-10}$ cm^{2}s^{-1} (depending also on the method of analysis). When the ratio of DSPC:DOPC is reduced to 50% : 50%, D_{eff} decreases. A 1-parameter fit for the "bleached region" recovery of this sample gave a value of $0.248\cdot 10^{-10}$ cm^{2}s^{-1}, compared with $1.767\cdot 10^{-10}$ cm^{2}s^{-1} for the 71.5% : 28.5% sample. The 50% : 50% complex is larger than the 71.5% : 28.5% complex: the mobility of the molecules decreases as their size increases, and thus D_{eff} is expected to change according to the ratio of

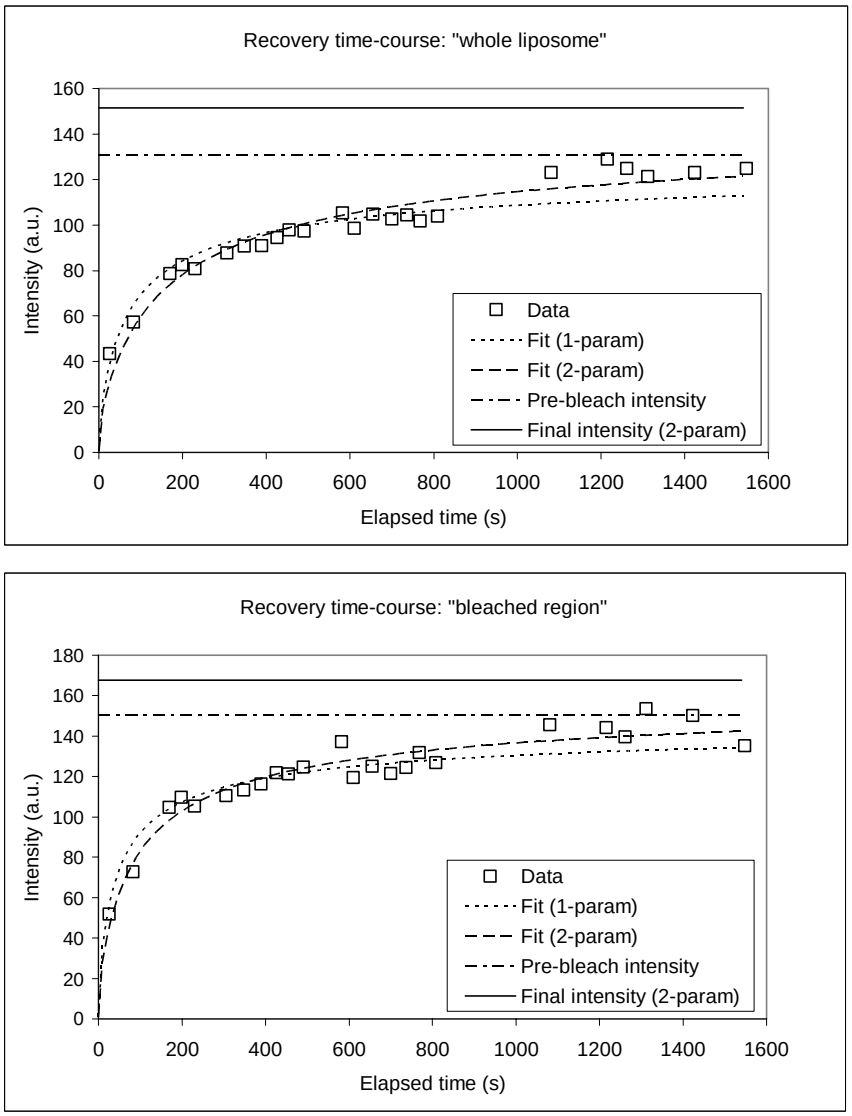

Fig. 3. Fitted results of the selected sample.

DSPC:DOPC. A number of other factors may also influence the diffusion of larger molecules. They include hydrodynamic drag, the alteration of solvent properties by the presence of DSPC:DOPC, and the bending kinetics of DSPC:DOPC, but these have not been investigated in this work.

3.2. Immobile Liposomes

The images of the DSPC:DHPE sample mixes were recorded on the confocal microscope. Due to the immobility of the fluorescently stained molecules little or no recovery is expected after photobleaching has been carried out. This should result in a graph that shows no change after photobleaching. Most of the graphs clearly show the total lack of any recovery in the fluorescence levels within the photobleached region of the vesicle, which is indicative of an immobile sample.

4. Conclusions

The behaviour of fluorescence-labelled membrane molecules has been studied. We believe that by our

experimental method the diffusion of fluorescently labelled molecules in lipid membranes can be conveniently measured. Although confocal laser scanning microscopy still faces some limitations in routine application of FRAP to biology, standard commercial instruments can be used successfully.

Diffusion coefficients of liposomes estimated by fitting a theoretical model of the FRAP recovery time-course have been compared with diffusion coefficients calculated from [26]. In future studies, this is to be exploited in biological and medical applications especially in an aqueous environment.

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