PATTERN PHOTOBLEACHING OF FLUORESCENT LIPID VESICLES USING POLARIZED LASER LIGHT

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ABSTRACT A burst of linearly polarized laser radiation incident on a spherical lipid vesicle, liposome, or biological cell can produce a well-defined nonuniform distribution of membrane-bound fluorescent molecules, provided the absorption transition dipole moment of the fluorescent label has a nonrandom orientation relative to the membrane surface and can be photobleached by the laser radiation. The return (recovery) of fluorescent membrane-bound molecules to a uniform distribution can be monitored using the same polarized radiation source. Under appropriate conditions this recovery is characterized by a single exponential time constant τ. This time constant is related to the radius R of the vesicle and the lateral diffusion coefficient D of the fluorescent membrane-bound molecules by the equation $R^2 = 6D\tau$. In the case of vesicle membranes this result is not limited by diffraction and so should be applicable to vesicles whose radii are less than the wavelength of light. The above considerations are illustrated by the polarized light photobleaching-recovery of lipid vesicles containing a fluorescent lipid, *N*-4-nitro-benzo-2-oxa,1,3-diazole L-α-dimyristoylphosphatidylethanolamine (NBD-DMPE).

INTRODUCTION

Fluorescence recovery after photobleaching has been used in many biophysical studies of molecular motion in both model membranes and biological membranes. This work is described in a recent review by Cherry (1979). In the version of this method in use in our laboratory, a burst of laser radiation is passed through a grid consisting of transparent and opaque regions (e.g., a Ronchi ruling) and the geometrical grid pattern is photobleached onto the fluorescent sample (Smith and McConnell, 1978; Smith et al., 1979*a*, *b*). The recovery of the remaining fluorescent membrane-bound molecules to the initial distribution is then monitored using an attenuated light beam from the same laser source. A knowledge of the bleach pattern geometry and a measurement of the recovery time(s) enables the determination of the diffusion coefficient(s). A control in these experiments is to bleach the entire sample uniformly (by removing the grid) to be sure that there is no fluorescence recovery that is unrelated to the bleach pattern. In certain cases we have observed fluorescence recovery in the absence of a grid. This can be caused by a number of different phenomena, including (*i*) fluorescent material in solution diffusing into the observation area after the bleach pulse, (*ii*) spatial intensity inhomogeneities in the laser beam, and (*iii*) the anisotropic photobleaching effect discussed in the present paper. It is the purpose of the present paper to show that this anisotropic photobleaching effect can be used to measure lateral diffusion.

The qualitative basis of this method is quite simple. It is well-known that photochemical destruction of chromophores by plane-polarized light can give rise to a photoselection of molecular orientations in systems where molecules do not undergo rapid, isotropic rotational motion. It is also known that membrane-bound fluorescent lipid-like molecules have transition dipole moments that are nonrandomly distributed relative to the membrane surface (Yguerabide and Stryer, 1971; Axelrod, 1979). Thus the orientation photoselection obtained by photobleaching with polarized light also gives a lateral spatial photoselection on the surface of a spherical membrane. (This interrelated orientational and lateral spatial photoselection was not considered in the study of surface diffusion in the erythrocyte by Peters et al. [1974] and is probably not significant in their work because of the heterogeneous surface labeling.) The recovery from a nonuniform distribution of membrane-bound fluorescent molecules can be monitored with polarized exciting radiation leading to a determination of the diffusion coefficient of the fluorescent membrane-bound molecule. In the case of a spherical membrane (i.e., lipid vesicle or biological cell) of known radius, the diffusion coefficient can be determined from the measured time for fluorescence recovery.

THEORETICAL BACKGROUND

Let $\mathbf{i}'$, $\mathbf{j}'$, $\mathbf{k}'$ be a laboratory-fixed axis system of orthonormal unit vectors, such that the laser radiation propagates in the $\mathbf{j}'$-direction and is plane-polarized in the $\mathbf{k}'$-direction (Fig. 1). Let the origin O' be located at the center of a spherical lipid vesicle of radius R. A second set of orthonormal unit vectors, $\mathbf{i}$, $\mathbf{j}$, $\mathbf{k}$ has its origin O located on a fluorescent-labeled, membrane-bound molecule on the vesicle surface. The vector $\mathbf{k}$ is taken to be the outward direction normal to the vesicle surface. The location of the molecule on the vesicle surface can be described by the spherical polar angles θ, ϕ in the $\mathbf{i}'$, $\mathbf{j}'$, $\mathbf{k}'$ axis system, and the orientation of the axes $\mathbf{i}$, $\mathbf{j}$, $\mathbf{k}$ relative to $\mathbf{i}'$, $\mathbf{j}'$, $\mathbf{k}'$ is given by the Euler angles α, β, γ. (This choice of notation, axis system, and Euler angles has been used previously. For details see Gaffney and McConnell, 1974.)

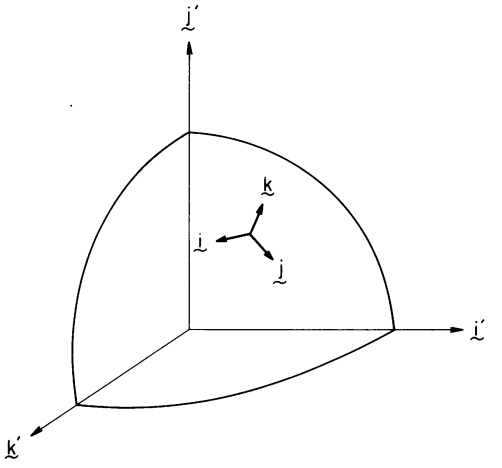

FIGURE 1 Coordinate system used in derivation of Eq. 14. Light propagates in the direction of the unit vector $\mathbf{j}'$, and the electric vector of the polarized light is in the $\mathbf{k}'$ direction.

Without loss of generality, the absorption transition dipole matrix element μ can be taken to lie in the **i**, **k** plane.

$$\mu = \mu_i \mathbf{i} + \mu_k \mathbf{k}. \tag{1}$$

The component of μ in the direct of the incident radiation electric vector $\mathbf{k}'$ is

$$\mu \cdot \mathbf{k}' = \mu_i \mathbf{i} \cdot \mathbf{k}' + \mu_k \mathbf{k} \cdot \mathbf{k}' \tag{2}$$

$$= \mu_i \sin\beta \sin\gamma + \mu_k \cos\beta. \tag{3}$$

The absorption transition probability, and by assumption, the surface concentration c of bleached molecules, is proportional to $(\mu \cdot \mathbf{k}')^2$

$$c = c_0(\mu_i^2 \sin^2\beta \sin^2\gamma + \mu_k^2 \cos^2\beta)/\mu_k^2. \tag{4}$$

Here c_0 is the surface concentration of bleached molecules where $\beta = 0$. We have assumed that the orientations of the transition moments μ are cylindrically symmetric about the radial direction **k**. That is, the membrane is either isotropic in the plane of the membrane, or two-dimensional membrane anisotropies are averaged out by in-plane isotropically distributed domain orientations. With this assumption all values of γ are equally probable; the averages of $\sin\gamma$ and $\sin^2\gamma$ are $\overline{\sin\gamma} = 0$ and $\overline{\sin^2\gamma} = 1/2$. We have used $\overline{\sin\gamma} = 0$ in going from Eq. 3 to Eq. 4. We can use $\overline{\sin^2\gamma} = 1/2$ to rewrite Eq. 4:

$$c = 1/3\, c_0[\mu^2 + 1/2(3\mu_k^2 - \mu^2)\,(3\cos^2\beta - 1)]/\mu_k^2 \tag{5}$$

Our choice of the spherical polar angles θ, ϕ and Euler angles α, β, γ is such that $\theta = \beta$; Eq. 5 can be written in terms of the normalized spherical harmonics $Y_{\ell m}(\theta, \phi)$ familiar from the quantum mechanics of the hydrogen atom (Eyring et al., 1944).

$$c = A_{00}Y_{00}(\theta, \phi) + A_{20}Y_{20}(\theta, \phi), \tag{6}$$

where

$$A_{00} = 2/3\ \sqrt{\pi}\mu^2 c_0/\mu_k^2 \tag{7}$$

$$A_{20} = 2/3\ \sqrt{\pi/5}\ (3\mu_k^2 - \mu^2)c_0/\mu_k^2. \tag{8}$$

Note that if the fluorescent molecules have an isotropic orientational distribution relative to the membrane surface, $\overline{(3\mu_k^2 - \mu^2)} = 0$ and the angular dependence of the concentration of bleached molecules disappears. The same is true if the dipoles are oriented at the "magic angle" relative to the membrane surface such that $3\mu_k^2 - \mu^2 = 0$.

Eq. 5 thus gives the concentration profile immediately after the initial bleaching burst of radiation. The concentration distribution of the photobleached molecules on the surface of the vesicle sphere $c(\theta, \phi)$ will then change with time according to the diffusion equation, as will the nonbleached fluorescent molecules whose concentration is $\rho\,(\theta, \phi) = c' - c\,(\theta, \phi)$, where c' is the initial concentration of membrane-bound fluorescent molecules before photobleaching. $c\,(\theta, \phi)$, as well as $\rho\,(\theta, \phi)$, follows the two-dimensional (isotropic) surface diffusion equation,

$$\partial c/\partial t = D\nabla^2 c, \tag{9}$$

where

$$\nabla^2 = 1/R^2\left[\frac{1}{\sin\theta}\frac{\partial}{\partial\theta}\left(\sin\theta\frac{\partial}{\partial\theta}\right) + \frac{1}{\sin^2\theta}\frac{\partial^2}{\partial\phi^2}\right]. \tag{10}$$

From Eyring et al. (1944) it follows that

$$\nabla^2 Y_{\ell m}(\theta, \phi) = \frac{-\ell(\ell+1)}{R^2} Y_{\ell m}(\theta, \phi). \tag{11}$$

Thus, a general solution to the problem of diffusion on the surface of a sphere is

$$c(\theta, \phi, t) = \sum_{\ell m} A_{\ell m} Y_{\ell m}(\theta, \phi) \exp\{-t/\tau_\ell\} \tag{12}$$

and

$$\tau_\ell = R^2/D\ell(\ell + 1). \tag{13}$$

When we compare Eqs. 12 and 13 with the initial condition following photobleaching Eqs. 6–8, it follows that the photobleach recovery curve is described by a single exponential $\tau_2 \equiv \tau$, where

$$\tau = R^2/6D\,. \tag{14}$$

Thus, a measurement of fluorescent recovery can be related to the lateral diffusion coefficient D and the vesicle radius R by Eq. 14.

If the laser radiation incident on the spherical sample is only partially polarized, or completely depolarized, spherical harmonics $Y_{2m}(\theta, \phi)$ with $m \neq 0$ must also be included in Eqs. 6 and 12. This does not change the recovery time $\tau_2 \equiv \tau$, since this depends only on ℓ (=2). On the other hand, the recovery amplitude is affected. For example, if the linearly polarized laser radiation were converted to circularly polarized (or depolarized) radiation, the axis of symmetry of the bleach pattern is changed from $\mathbf{k}'$ to $\mathbf{j}'$ and $Y_{20}(\theta, \phi)$ in Eq. 2 and 12 must be replaced by the appropriate linear combination of terms $Y_{2m}(\theta, \phi)$. Alternatively, we may introduce new polar angles θ', ϕ', where θ' is the angle between $\mathbf{k}$ and $\mathbf{j}'$. In this case the change of linearly polarized to circularly polarized radiation requires that $Y_{20}(\theta, \phi)$ in Eq. 6 be replaced by $-1/2\ Y_{20}(\theta', \phi')$. Thus, the bleach amplitude (and hence recovery amplitude) is expected to be reduced by a factor of 2, for a given number of bleach photons incident on the sample.

EXPERIMENTAL

Fig. 2 gives epifluorescence photomicrographs of a liposome containing 20 mol % cholesterol, 80 mol % DMPC,[1] and 0.02 mol % NBD-DMPE. The laser radiation is vertically polarized, as indicated. The photomicrographs show two perpendicular liposome orientations, an unsymmetrical liposome having been deliberately chosen to show this liposome rotation. The nonuniform fluorescence of the liposome directly demonstrates that the absorption transition dipole moment (resulting ultimately in NBD-

Abbreviations used in this paper: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; NBD-DMPE, a fluorescent-labeled dimyristoylphosphatidylcholine, *N*-4-nitro-benzo-2-oxa-1,3-diazole L-α-dimyristoylphosphatidylethanolamine.

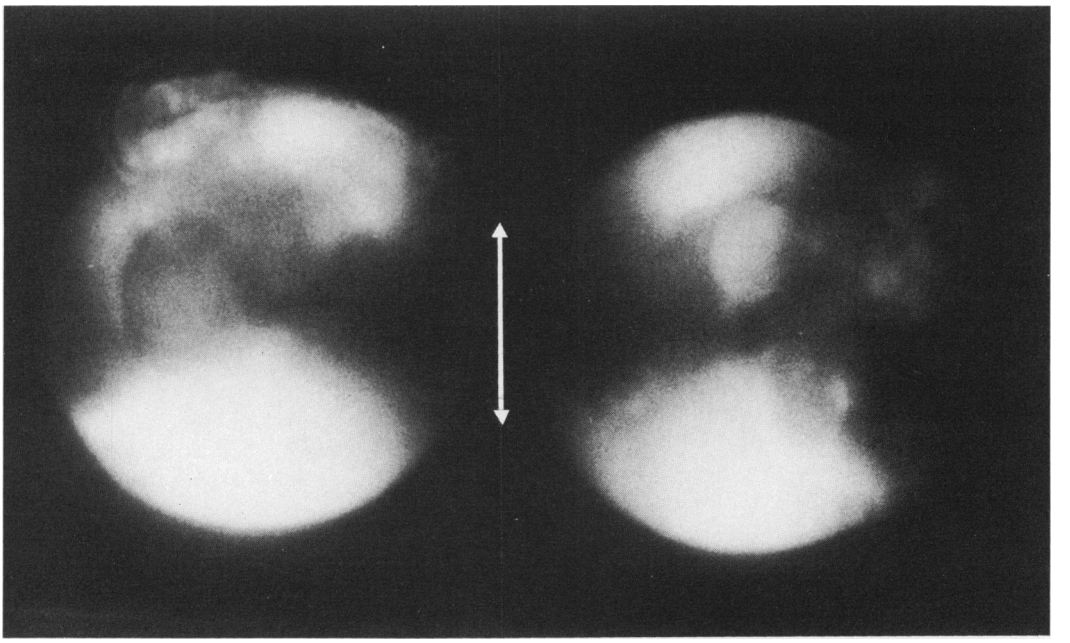

FIGURE 2 Epifluorescence photomicrographs of a liposome containing 20 mol % cholesterol, 80 mol % DMPC, and 0.02 mol % NBD-DMPE. Polarization of the exciting radiation (488 nm) is as shown (±5°). Two perpendicular liposome orientations are shown to illustrate that the nonuniform fluorescence observed is a function of the polarization of the light rather than any geometrical property of the liposome. Similar results are obtained with polarized incoherent excitation from an ultraviolet lamp and also when the plane of polarization of the incident laser light is rotated. Liposomes of this type typically consist of a number of concentric bilayer shells whose radii are within 10–15% of the average radius.

DMPE fluorescence) has a nonrandom orientation relative to the polarization of the incident light and thus a nonrandom orientation relative to the membrane surface. Photobleaching of such molecules should give rise to a nonuniform distribution of bleached molecules, and remaining fluorescent molecules, as discussed above. The simple distribution in Eq. 6 of course is only valid if the degree of bleaching at every point on the sample surface is proportional to $(\mu_{k'}^2)$, where $\mu_{k'}$ is the component of μ in the direction of electric vector of the polarized light.

To test the validity of Eq. 14, photobleach recovery curves were recorded (in the absence of any grid) for a number of liposomes of different sizes prepared as given in the legend to Fig. 3. Fig. 3 gives recovery time constants as a function of R^2. As expected, τ is proportional to R^2. The value of D obtained from the slope of the line in Fig. 3 is identical to that obtained in previous work (Rubenstein et al., 1979; Smith et al., 1980). The values of R were measured using a calibrated eyepiece reticle. The objective lens was a 40× planachromat, numerical aperture 0.65. Experiments were carried out in which the sample was deliberately strongly defocused, and equivalent results were obtained.

One important potential advantage of this method for the measurement of lateral motion is its applicability to small vesicles that are often obtained as a result of membrane reconstitution experiments. In Fig. 4 is shown the fluorescence recovery curve obtained from 15–20 vesicles with diameters of the order of 1 μm. The value of D which may be calculated from this curve using the value $R = 0.5$ μm is 5×10^{-10} cm^2/s. This result agrees with the previously measured values for this lipid composition and temperature (Rubenstein et al., 1979; Smith et al., 1980). Vesicles were prepared by the cholate dilution method (Smith et al., 1979*c*). Since the recovery time in Eq. 14 is proportional to R^2, and the Brownian rotational correlation time of a sphere is proportional to R^3, it follows that the present

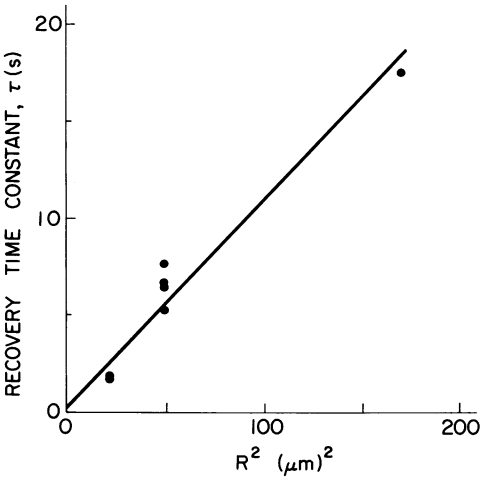

FIGURE 3

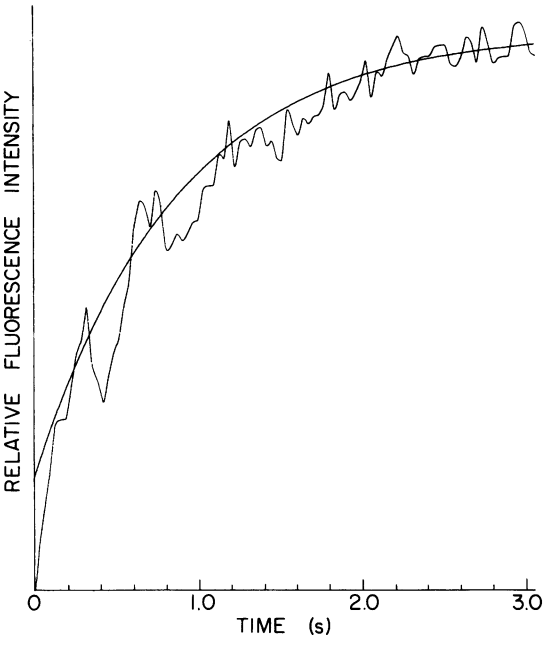

FIGURE 4

FIGURE 3 Fluorescence recovery time τ vs. R^2 for liposomes of different radii. Liposomes are 35 mol % cholesterol, 65 mol % DMPC, 0.02 mol % NBD-DMPE. Liposomes were prepared as described in Smith et al. (1980). Lipids (1 μmol) were roto-evaporated to a thin film from chloroform, 1 ml of phosphate-buffered saline added, the flask kept at 40°C for 15 min and then vortexed vigorously for 15 s to form the liposomal suspension. Samples were diluted twofold with distilled water before measurements to make the external buffer hypotonic and eliminate surface wrinkling of the liposomes.

FIGURE 4 Fluorescence recovery of NBD-DMPE in 15–20 vesicles of ~ 1 μm diam. Vesicles were prepared by the cholate dilution method (Smith et al., 1979*c*). Vesicles are 10% cholesterol, 90% DMPC, 0.2% NBD-DMPE, in phosphate-buffered saline. Temperature is 18°C. A value of $D = 5 \times 10^{-10}$ cm^2/s is obtained from this curve if the value of R is taken as 0.5 μm, and agrees with previously measured values at this lipid composition and temperature (Smith et al., 1980; Rubenstein et al., 1979).

method is not applicable to small vesicles undergoing Brownian motion. For a diffusion coefficient of $D = 5 \times 10^{-10}$ cm^2/s, and a solvent viscosity of 1 cp, these two times are comparable when $R = 3.3$ μm. In the present experiments, however, the vesicles are not in suspension but are resting on the surface of the microscopic slide. The interaction of these vesicles with the glass surface is sufficient to eliminate Brownian motion as assessed by visual observation.

The data in Figs. 3 and 4 were obtained using the general procedure described elsewhere (Smith et al., 1979*b*). Briefly, the liposome is centered in the microscope field of view, and the pattern produced by illumination with polarized laser light is focused. A fluorescent intensity signal level measured with a cooled photomultiplier tube (RCA Solid State, Somerville, N.J.; model #C31034-02) is set to full scale on an oscilloscope screen. The sample is exposed to a burst of intense (~500 W/cm^2) 4,880 Å polarized laser radiation for ~ 100 ms, giving an approximate twofold reduction in fluorescence intensity. The liposome fluorescence emission is then immediately recorded as a function of time using a highly attenuated (~10^3) probe beam. Data were recorded by a PDP-8/E computer (Digital Equipment Corp., Marlboro, Mass.), and the best least squares fit to a single exponential calculated. The calculated time constant τ is then used to calculate D according to Eq. 14. The calculated recovery curve is displayed

superimposed on the observed recovery curve on an oscilloscope screen or, as in Fig. 4, on a chart recorder record. In the plot in Fig. 4 the recovery amplitude is expanded to full scale, and therefore the baseline fluorescence intensity is the fluorescence intensity of the sample at zero time (onset of probe beam).

DISCUSSION

In the present work we have shown how linearly polarized light can be used to photobleach molecules on the surface of a lipid vesicle, leaving a nonuniform spatial distribution of nonbleached molecules on the vesicle surface. The decay of the nonuniform fluorescence to a uniform distribution of fluorescent molecules has a simple exponential time-dependence, where the recovery time constant τ is related to the radius R of the vesicle and the lateral diffusion coefficient of the membrane-bound fluorescent molecules by Eq. 14. These statements require two important qualifications. For linearly polarized light photobleaching to yield a nonuniform distribution of molecules on a spherical membrane surface, the photosensitive molecules must have a nonrandom molecular orientation relative to the surface of the membrane. More specifically, the (vector) absorption transition dipole matrix elements must be nonrandomly oriented relative to the membrane surface. In order that the fluorescence recovery after a single burst of bleaching radiation be accounted for in terms of a single exponential time constant, the surface bleach pattern must be described by a single angular-dependent spherical harmonic (or a linear combination of spherical harmonics with the same ℓ-values). This is the case if the bleaching is proportional to the square of the component of the electronic absorption transition dipole moment in the direction of light polarization for each point on the surface of the sphere. These conditions have evidently been adequately approximated for the experiments described in the present paper. The largest sources of error in these experiments apparently arise from nonspherical vesicles and/or spatial noise in the laser illumination.

Perhaps one of the most interesting potential applications of this "pattern" photobleaching using polarized laser light concerns the study of membrane structures having dimensions less than the wavelength of light. Not only is there the possibility of studying lateral diffusion in small immobilized lipid vesicles, but there is also the possibility of studying anisotropic domain structures in planar membranes. That is, it may be possible to detect molecular ordering in both model membranes and cellular membranes that is long-range on the scale of molecular distances, but short-range on the scale of the wavelength of light.

Throughout the present paper we have emphasized the use of polarized light rather than depolarized light for several practical reasons. The nonuniform distribution of fluorescence from a spherical vesicle is much more easily visualized in a microscope equipped for epifluorescence using linearly polarized radiation. Also, recovery amplitudes are larger, as discussed above. Finally, linearly polarized light offers the possibility of studying anisotropies in planar membranes.

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