Effects of second order photobleaching on recovered diffusion parameters from fluorescence photobleaching recovery

D. W. Bjarneson and N. O. Petersen*
Department of Chemistry, The University of Western Ontario, London, Ontario, N6A 5B7, Canada

ABSTRACT In the original theoretical development of fluorescence photobleaching recovery with circular or Gaussian laser intensity profiles (Axelrod et al., 1976, *Biophys. J.*) the bleaching process is assumed to obey first order kinetics in the fluorescent probe. While this is reasonable in most cases where oxygen participates in the photolysis reaction, some processes may obey second order kinetics in the fluorophore concentration due to dimerization. Accordingly, we present here an analysis of the fluorescence recovery when the photobleaching process is taken to be second order in the probe. Analytical solutions for small bleaching levels indicate that the fluorescence recovery curve is very similar to that measured following a bleaching process first order in the probe. Numerical solutions for moderate bleaching levels show that the recovery is qualitatively similar, but quantitatively different. Because the shape of the recovery curve provides no evidence as to the order of photobleaching, we recommend continued use of the previous theoretical analysis. However, it must be borne in mind that the diffusion coefficient is increasingly underestimated as the extent of photobleaching is increased. The true diffusion coefficient is obtained in the limit of small levels of photobleaching. Estimates of the fractional recovery are not affected by this approach.

INTRODUCTION

Fluorescence Photobleaching Recovery (FPR) has been used to investigate the mobility of a large number of probes in biologically important systems. The theoretical basis behind FPR was presented by Axelrod et al. in 1976 (1). In that paper, the effects on the fluorescence recovery due to diffusion, flow, and a combination of both are discussed. In all cases, however, the kinetics of the photochemical reaction is taken to be first order in the fluorescent probe. This assumption is based on the premise that the chemistry of photobleaching involves oxidation of the fluorescent probe by singlet oxygen (2, 3). In most biological systems, the oxygen concentration is sufficient to allow the oxidation to be first order in the probe, and to occur without interference from other possible photochemical processes.

Recently, we have employed FPR to examine the mobility of naphthacene adsorbed on silica gel surfaces in vacuo (4). In this system, the photolysis reaction can lead to either oxidation of naphthacene if sufficient oxygen is present (5–9), or dimerization (10–12). In solution, we find that both of these reactions are first order in ground state naphthacene, but on the silica surface, the kinetics are more complex.

In this study we examine the effects of second order photobleaching kinetics on recovered diffusion parameters (diffusion coefficient D, and fractional recovery X_m) from an FPR experiment. The recovery portion of the simulated experiments are very similar in shape, implying that the original theory outlined by Axelrod et al. (1) can be used to fit data in most cases. Little effect is seen on the fractional recovery, but as the extent of bleaching increases, the error on the recovered diffusion coefficient increases if one uses only the original theory.

THEORETICAL SECTION

The procedure of Axelrod et al. (1) starts with the definition of the time-dependent fluorescence intensity ($f(t)$) which is measured during an FPR experiment:

$$f(t) = (g \epsilon Q/A) \int_0^\infty I(r)\, C(r,t)\, d^2r, \qquad (1)$$

where g accounts for instrumental losses, ϵ and Q are the molar extinction coefficient and fluorescence quantum yield of the probe, and A is the attenuation of the excitation source. $C(r,t)$ is the concentration of the probe, which is dependent on both radial position (r) and time (t). $I(r)$ represents the laser beam intensity profile. A laser beam of Gaussian intensity profile is defined by:

$$I(r) = I_0 \exp(-2r^2/\omega^2). \qquad (2)$$

ω represents the experimentally determined laser beam width where the intensity drops to exp (−2) of the maximum value.

Assuming the photobleaching follows a pathway first order in the fluorescent probe, the rate equation of bleaching is given by:

$$\frac{dC(r,t)}{dt} = -\lambda I(r)\, C(r,t), \qquad (3)$$

0006-3495/91/11/1128/04 $2.00

where λ represents a photochemical quantum yield. Integration of Eq. 3, where time zero is taken to be at the end of the bleaching pulse gives:

$$C(r, 0) = \overline{C} \exp(-\lambda I(r) t'), \quad (4)$$

where $\overline{C}$ is the average prebleach probe concentration and t' is the duration of the bleaching pulse. Using the form of the intensity profile given in Eq. 2 and defining K to be $(\lambda I_0 t')$, which is a measure of the extent of bleaching, Eq. 4 gives:

$$C(r, 0) = \overline{C} \exp\{-K \exp(-2r^2/\omega^2)\}. \quad (5)$$

Using Eq. 5 as the initial condition, Axelrod et al. (1) determined the solution for the time-dependent fluorescence intensity from Eq. 1 to be:

$$f(t) = \left(\frac{g \epsilon Q P \overline{C}}{A}\right) \sum_{n=0}^{\infty} \frac{(-K)^n}{n![1 + n(1 + 2t/\tau_D)]}, \quad (6)$$

where P is the power of the laser and τ_D is the characteristic diffusion time, and is related to the diffusion coefficient (D) by:

$$D = \omega^2/4\tau_D. \quad (7)$$

If we assume that the photobleaching is second order in the fluorescent probe, the rate equation (Eq. 3) must be replaced by:

$$\frac{d C(r, t)}{dt} = -\lambda' I(r) C^2(r, t). \quad (8)$$

The solution for the initial postbleach concentration then is:

$$C(r, 0) = \frac{\overline{C}}{1 + \overline{C}\lambda' t' I(r)}, \quad (9)$$

where λ' is the photochemical quantum yield for the second order process. Defining a photobleaching parameter K' to be $(\overline{C}\lambda' t' I_o)$, the initial postbleach concentration can be expressed as:

$$C(r, 0) = \frac{\overline{C}}{1 + K' \exp(-2r^2/\omega^2)}. \quad (10)$$

With these initial conditions, the solution for the time-dependent fluorescence intensity for $K' < 1$ becomes:

$$f(t) = \left[\frac{g \epsilon Q P \overline{C}}{A}\right] \sum_{n=0}^{\infty} \frac{(-K')^n}{[1 + n(1 + 2t/\tau_D)]}. \quad (11)$$

The solution follows the same procedure outlined by Axelrod et al. (1) and involves a series expansion of $[1 + K' \exp(-2r^2/\omega^2)]^{-1}$.[1] This expansion is only valid for values of $(K' \exp(-2r^2/\omega^2))$ less than unity (13), which corresponds to less than ~50% bleaching at the center of the beam. It is evident from comparisons of Eqs. 6 and 11 that the recovery curves are very similar in form. Only the $n!$ term in the denominator of Eq. 6 is missing in Eq. 11, and the bleaching parameter has a different interpretation (but is still a constant for a certain duration of bleaching and initial prebleach concentration). The effect of the $n!$ term is that the series in Eq. 6 converges more rapidly, but for small extents of bleaching this is a small effect.

For values of $K' \geq 1$, the series in Eq. 11 diverges. This greatly limits the usefulness of Eq. 11 as a solution to which data are fit because K' is a fitting variable in most cases. Analytical solutions to Eq. 1 for $K' \geq 1$ with the initial condition of Eq. 10 have escaped us so far.

We note that for small bleaching levels the recoveries are analytically similar. We further recognize that there is no physical reason to expect a singularity or unusual change in behavior at $K' = 1$. Thus, the shape of the recovery should be qualitatively similar at large K' values also. In most cases it will be advantageous to fit experimental data to the result of Eq. 6 and use an empirical formula to estimate the error in the recovered diffusion parameters. The next section is intended to illustrate this latter point.

SIMULATION SECTION

Fluorescence recovery curves ($f(t)$) can be calculated for simulated FPR experiments by using either Eq. 6 or 11. Nine values for the extent of bleaching ranging from 14 to 50% bleaching at the center of the beam, and three values of τ_D (5, 10, 25 s), are used in these simulations, with 10% random noise added to the data. In all simulations, 38.4 s of data were calculated with the count time of 100 ms/data point. All simulated data is fit using the original theory (Eq. 6) developed by Axelrod et al (1, 14).

Eq. 6 provides the recovery after a bleaching process first order in the probe. An example of a simulated recovery curve is presented in Fig. 1, curve *a*. After fitting, all recovered values of the fractional recovery are unity, as expected. Also, all recovered values of the diffusion coefficient are within 3% of the theoretical input value and are independent of the extent of bleaching. These are in effect the control data (Table 1).

Eq. 11 generates the recovery of the fluorescence intensity after a bleaching process which is second order in the probe. An example is given in Fig. 1, curve *b*. To allow for direct comparison, the same values of τ_D and

[1]The series expansion employed in the derivation of Eq. 11 is:

$$(1 + X)^{-1} = \sum_{n=0}^{\infty} (-X)^n \text{ for } |X| < 1 \quad (13),$$

where $X = K' \exp(-2r^2/\omega^2)$.

Note in the text we refer to K' being less than unity and not $K' \times \exp(-2r^2/\omega^2)$. This is valid because $r = 0$ is part of the radial component.

The analogous series expansion employed to derive Eq. 6 is:

$$\exp(-X) = \sum_{n=0}^{\infty} \frac{(-X)^n}{n!} \text{ for all } X \quad (13), \text{ where in this case}$$

$X = K \exp(-2r^2/\omega^2)$.

For small bleaching levels the relationship between the recovery curves differ only by the $(1/n!)$ term. This result is independent of the illumination profile. The discussion here will also apply, in principle, to uniform or airy disc illumination profiles.

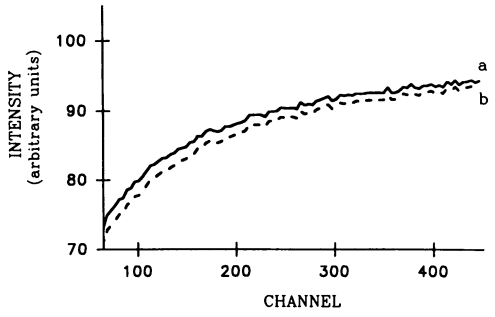

FIGURE 1 Comparison of the recovery portions from two simulated FPR experiments. (*a*) The recovery after a bleaching process first order in the probe (——). (*b*) The recovery after photobleaching second order in the probe (---). Note that the intensity is integrated over the beam and is directly comparable to the measured fluorescence intensity.

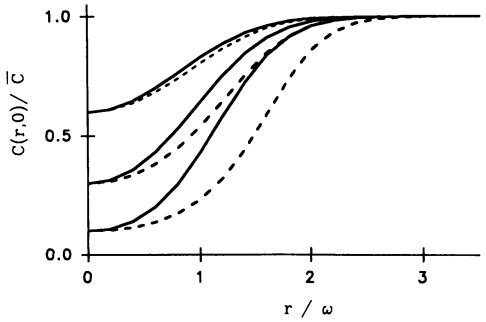

FIGURE 2 Initial postbleach concentration profiles for photobleaching processes which are first (——) and second (---) order in the probe. The profiles are generated directly from Eqs. 5 and 10, respectively. Based on an arbitrary prebleach intensity of 100, for the first order cases, the initial postbleach intensities calculated from Eq. 6 will be 78, 58, and 39. For the second order case, using Eq. 11, the initial postbleach intensities are 76, 53, and 28. These last two values are estimates based on the values from the first order case.

the same time base are used. Also, the same extent of bleaching at the center of the beam (i.e., $C(0, 0)/\overline{C}$) is used. Table 1 clearly shows that at low bleaching levels the correct estimate of the diffusion coefficient is obtained, but as the extent of bleaching increases the estimate of the diffusion coefficient decreases. In all cases the correct fractional recovery is obtained.

The origin of the difference in the recovered diffusion coefficients is easily seen by examining the initial postbleach concentration profiles for both cases (Fig. 2). For a photobleaching process, which is second order in the probe, many more probes are photolyzed at comparable r values than for the first order case. As the overall extent of bleaching increases, the differences between the two profiles also increases.

Calculation of the diffusion coefficient from recovery data depends on accurate measurement of the beam size. Fig. 2 reveals that for the same extent of bleaching at the center of the laser beam, a second order process causes the postbleach concentration at larger radial positions to be much less than in a first order process. Using the first order theory to fit recovery data, which follows a second order bleaching process, is therefore equivalent to imposing a measured beam width that is smaller than the effective beam width. Hence, the diffusion coefficient will be underestimated (cf. eq. 7). As a result, when the extent of bleaching increases, the difference between the initial postbleach concentration profiles also increases, i.e., the error on D increases.

For all data examined, we have restricted ourselves to valid choices of K', and comparable values of K. Due to the restriction imposed by the use of the series expansion noted above, this allows us to compare results for data with at most 50% bleach at the center of the beam. Within this region, the difference between the two diffusion coefficients is as much as 10%. However, because the extent of bleaching in actual experiments is often greater, we must, at least qualitatively, estimate the error on D at larger extents of bleaching. To achieve this goal we examine the width of the initial postbleach concentration profiles for each case (Fig. 2). For concentration profiles with 90% bleaching at the center of the beam, the difference in the measured beam width (used in the first order case and in the fitting) and the effective beam width for the second order case is ~35%. This would lead to an underestimate on D by a factor of 1.8 due to the ω^2 dependence alone (Eq. 7). At high bleaching levels, such corrections could become critical. The clue as to whether corrections are needed will arise from observations that the measured diffusion coefficient shows dependence on the extent of bleaching.

TABLE 1 Simulated diffusion parameters after first or second order photobleaching. Simulated data for various extents of bleaching and a characteristic diffusion time of 10 s ($D = 2.50 \times 10^{-10}$ cm^2 s^{-1}).

	$D/10^{-10}$ cm^2 s^{-1}		Fractional recovery*	
$C(0, 0)/\overline{C}$	First order	Second order	First order	Second order
0.86	2.49	2.51	1.15	1.13
0.82	2.57	2.50	1.00	1.00
0.78	2.51	2.47	1.05	1.05
0.74	2.55	2.48	1.02	1.02
0.68	2.52	2.42	1.05	1.04
0.64	2.57	2.45	1.02	1.02
0.56	2.54	2.38	1.04	1.04
0.51	2.54	2.33	1.02	1.02
0.50	2.54	2.18	1.02	1.03

*Fractional recoveries greater than unity are unrealistic, but arise from these values being fitted estimates.

CONCLUSIONS

By investigating the effect of the kinetics of photobleaching on diffusion parameters measured by FPR, we show that the diffusion coefficient can be drastically underestimated. The kinetics of photobleaching are rarely reported, and probably rarely measured. We show that for large extents of bleaching the diffusion coefficient can be underestimated by a factor of two if one assumes a first order process when in fact the kinetics are second order in the probe. The magnitude of the error on the diffusion coefficient depends greatly on the extent of bleaching. No apparent effect is seen on the fractional recovery.

For large extents of bleaching ($K > 10$) the initial bleach profile might be approximated by a square well model, but this will not suffice at intermediate bleaching levels. It is also possible that measurements of the bleaching kinetics will reveal neither first nor second order kinetics. It might then be necessary to solve the more general diffusion problem with combined first and second order kinetics. Alternatively, models accounting for heterogeneity in bleaching of various regions of the sample could be incorporated. We have not pursued any of these approaches. Instead we propose that the original theory outlined by Axelrod et al. (1) be used to fit all data because the effect on the diffusion coefficient is within a factor of two. If there is compelling evidence that the diffusion coefficient is dependent on the extent of bleaching, then a correction factor for the second order photobleaching may be applied. Alternatively, the true diffusion coefficient may be estimated in the limit of low extents of photobleaching ($\leq 20\%$).

This work is supported by the Natural Sciences and Engineering Research Council of Canada.

Received for publication 22 April 1991 and in final form 22 July 1991.

REFERENCES

1. Axelrod, D., D. E. Koppel, J. Schlessinger, E. Elson, and W. W. Webb. 1976. Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. *Biophys. J.* 16:1055–1069.
2. Lepock, J. R., J. E. Thompson, J. Kruuv, and D. F. Wallach. 1978. Photoinduced crosslinking of membrane proteins by fluorescein isothiocyanate. *Biochem. Biophys. Res. Commun.* 85:344–350.
3. Sheetz, M. P., and D. E. Koppel. 1979. Membrane damage caused by irradiation of fluorescent concanavilin A. *Proc. Natl. Acad. Sci. USA.* 76:3314–3317.
4. Bjarneson, D. W., and N. O. Petersen. 1990. Direct diffusion measurements of naphthacene on silica as a function of silanol density. *J. Am. Chem. Soc.* 112:988–992.
5. Stevens, B., and B. E. Algar. 1968. The photoperoxidation of unsaturated organic molecules. II. The autoperoxidation of aromatic hydrocarbons. *J. Phys. Chem.* 72:3468–3474.
6. Stevens, B. 1978. Kinetics of singlet oxygen peroxidation. *In* Singlet Oxygen Reactions with Organic Molecules and Polymers. B. Rånby and J. F. Rabek, editors. John Wiley and Sons, Chichester, UK.
7. Bloodworth, A. J., and H. J. Eggelte. 1985. Endoperoxides. *In* Singlet O_2. A. A. Frimer, editor. CRC Press, Boca Raton, FL.
8. Saito, I., and T. Matsuura. 1979. The oxidation of electron-rich aromatic compounds. *In* Singlet Oxygen. H. H. Wasserman, editor. Academic Press, London.
9. Rigaudy, J., and D. Sparfel. 1978. Transformations thermique des photooxydes meso des acenes. IV. Cas du photooxyde du naphthacene. *Tetrahedron.* 34:113–121.
10. Lapouyade, R., A. Nourmamode, and H. Bouas-Laurent. 1980. Photocycloaddition des hydrocarbures aromatiques polynucleairies en solution. V. La photodimerisation du tetrahydro-1,2,3,4 naphthacene et les deux photodimeres du naphthacene. *Tetrahedron.* 36:2311–2316.
11. Wei, K. S., and R. Livingston. 1967. Reversible photodimerization of anthracene and tetracene. *Photochem. Photobiol.* 6:229–232.
12. Fournie, G., F. Dupuy, M. Martinaud, G. Nouchi, and J. M. Turlet. 1972. Auto-association of tetracene in solution. *Chem. Phys. Lett.* 16:332–335.
13. O'Neil, P. V. 1987. Advanced Engineering Mathematics, Second Edition. J. Harrison, editor. Wadsworth, Belmont, CA.
14. Petersen, N. O., S. Felder, and E. L. Elson. 1986. Measurement of lateral diffusion by fluorescence photobleaching recovery. *In* Handbook of Experimental Immunology, Volume 1. D. M. Weir, editor. Blackwell Scientific Publications, Oxford, UK.