Photobleaching Kinetics of Optically Trapped Multilamellar Vesicles Containing Verteporfin Using Two-photon Excitation§

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ABSTRACT

Two-photon excitation photodynamic therapy (TPE-PDT) is being developed as an improved treatment for retinal diseases. TPE-PDT has advantages over one-photon PDT, including lower collateral damage to healthy tissue and more precise delivery of PDT. As with one-photon PDT, there can be local photochemical depletion of oxygen during TPE-PDT. Here, we investigate model systems and live cells to measure local photosensitizer photobleaching and through it, infer local oxygen consumption in therapeutic volumes of the order 1 μm². Multilamellar vesicles (MLV) and African green monkey kidney (CV-1) cells were used to study the TPE photobleaching dynamics of the photosensitizer, Verteporfin. It was found that in an oxygen-rich environment, photobleaching kinetics could not be modeled using a mono-exponential function, whereas in hypoxic conditions a mono-exponential decay was adequate to represent photobleaching. A biexponential was found to adequately model the oxygen-rich conditions and it is hypothesized that the fast part of the decay is oxygen-dependent, whereas the slower rate constant is largely oxygen-independent. Photobleaching recovery studies in the CV-1 cells support this hypothesis.

INTRODUCTION

Verteporfin (benzoporphyrin derivative mono acid [ring A] or BPD-MA) is a photosensitizing agent that has gained government approval in several countries for use in photodynamic therapy ( PDT) of age-related macular degeneration (AMD). Traditionally, Verteporfin is promoted into its first excited state by irradiation with continuous-wave laser light (∼680 nm) via a one-photon process. Recently, it has been shown that the photochemistry of Verteporfin is independent of the excitation pathway (1–3).

Two-photon excitation (TPE) PDT has advantages over the traditional one-photon PDT. First, the probability of TPE-PDT occurring is strongest at the focal plane of a highly focused laser beam, and therefore, it is possible to achieve excitation of the photosensitizer in volumes as small as 0.5 μm³. Potentially, this excitation volume size restriction will allow selective treatment of individual cells or blood vessels in delicate tissues such as the retina. It is important to stress that in TPE the photosensitizer is excited through a different process, but once the molecule enters the first singlet excited state, it will undergo the same photo-physical and photochemical processes that occur with one-photon excitation (1–3).

Verteporfin, like other traditional photosensitizers, is hydrophobic and therefore requires a delivery vehicle. Combining Verteporfin with a lipid delivery system, such as multilamellar vesicles (MLV; i.e. liposomes), allows the drug to be applied in an aqueous environment. It is believed that once in the blood stream, Verteporfin rapidly partitions onto lipoproteins, thus providing a route for localization in areas of cellular and neovascular growth (4–6).

During treatment with PDT, a measurable decrease in fluorescence is observed due to the inactivation or destruction of the photosensitizer through the process of photobleaching. Photobleaching can occur through several excitation pathways, including destruction by singlet oxygen (Eq. 1 and 2) (7–9).

\[
1 \text{VP} + 2h\nu \rightarrow 1\text{VP}^* \quad (1a)
\]

\[
1\text{VP}^* \rightarrow 1\text{VP} + h\nu' \quad (1b)
\]

\[
1\text{VP}^* \rightarrow 1\text{VP} + h\nu' \quad (1c)
\]

\[
3\text{VP}^* \rightarrow 1\text{VP} \quad (1d)
\]

\[
3\text{VP}^* + 1\text{O}_2 \rightarrow 1\text{VP} + 1\text{O}_2^* \quad (1d)
\]

\[
1\text{VP} + 1\text{O}_2^* \rightarrow X \quad \text{(nonfluorescent species)} \quad (1e)
\]

\[
1\text{O}_2^* \rightarrow \text{O}_2 + h\nu'' \quad (1f)
\]

\[
3\text{VP}^* + A \rightarrow \text{VPA} \quad (2a)
\]

\[
1\text{VP}^* + A \rightarrow \text{VPA} \quad (2b)
\]

where VP denotes Verteporfin in its singlet (1VP) and triplet (3VP) states, O₂ denotes molecular oxygen in its natural triplet state (1O₂) or the excited singlet state (3O₂), X is an unknown photoproduction.
The scattering force is balanced by the field gradient and gravitational and the laser beam results in a transfer of momentum. When the particle is near the desired particle and the laser beam, a dipole force termed optical trapping or optical tweezers. Optical trapping occurs when a polarized can be optically immobilized through a process commonly known to partition into lipid environments in the cell, a liposome often used as models for cell membranes, and because Verteporfin is limited excitation volumes for TPE-PDT (10). We believe that by examining the oxygen-dependent and oxygen-independent kinetics of photobleaching, the TPE-PDT efficiency of the photosensitizer, in this case Verteporfin, can be determined.

Because the consumption of oxygen is a marker of dose in PDT, understanding and quantifying TPE-induced photosensitizer bleaching should lead to a model for TPE-PDT dosimetry. Foster and coworkers (10–12) have identified an explicit set of reaction pathways similar to those presented in (Eqs. 1 and 2) here, which can be represented by a set of coupled differential equations. Unfortunately, there is no general analytic solution to these differential equations and thus no simple integrated rate equation can be presented that represents both the consumption of photosensitizer and oxygen. By directly measuring the changing concentration of \( \Delta O_2 \) in a multicell tumor spheroid model system during PDT, Finlay et al. (10) have circumvented this roadblock to assess the factors contributing to the photodynamic dose delivered for TPE-PDT, a facile photobleaching model should be created. The physical model should be applicable to diffraction-limited excitation volumes for TPE-PDT (~1 \( \mu \)m). Liposomes are often used as models for cell membranes, and because Verteporfin is known to partition into lipid environments in the cell, a liposome model was chosen for this study. However, the measurement of TPE photobleaching in a heterogeneous liposome solution is challenging, because of the convolution of photobleaching dynamics and the diffusion dynamics of the liposomes. Both of these will contribute to the measured changes in fluorescence intensity reported by TPE within the interrogation volume (13). Therefore, we have chosen to optically trap liposomes loaded with Verteporfin to immobilize them and thus remove the diffusional contribution to the measured changes in photosensitizer fluorescence intensity.

Liposomes and other small, transparent particles that can be polarized can be optically immobilized through a process commonly termed optical trapping or optical tweezer. Optical trapping occurs when dipole, scattering and gravitational forces are balanced between the desired particle and the laser beam. A dipole force forms from the interaction of a radially and axially induced field gradient upon a particle that can be polarized. These particles are attracted to the strongest part of the induced field, which occurs at the focal plane. The scattering of light from the interaction of the particle and the laser beam results in a transfer of momentum. When the scattering force is balanced by the field gradient and gravitational forces, it is possible to three-dimensionally trap particles (14,15).

In the present work, we describe the results of photobleaching via TPE of Verteporfin loaded into optically trapped liposomes. The photobleaching rate coefficients are compared with those recorded from Verteporfin in live African green monkey kidney (CV-1) cells. We find that there is evidence for local oxygen depletion both in the liposome model and in the live cells, suggesting a common mechanism of photobleaching.

**MATERIALS AND METHODS**

**Two-photon fluorescence set-up.** The TPE-PDT experimental setup has been described previously (16). Briefly, a Zeiss 63X objective, NA 1.4, with a 190 \( \mu \)m working distance was used. Unless otherwise stated, the laser (Spectra Physics Tsunami; Mountain View, CA) power ranged between 40 and 80 mW at the back aperture of the microscope, and the TPE wavelength was set at 780 nm with approximately 100 fsec pulses at an 82 MHz repetition rate. Fluorescence emission is filtered with a broadband notch filter (Q565LP, Chroma; Rockingham, VT) and subsequently detected using an avalanche photodiode detector (Photon Counting Module SPCM CD2882; Perkin-Elmer, Vaudreuil, QC, Canada).

**Liposomal Verteporfin preparation.** Stock solutions of dioleoyl-phosphatidylcholine (DOPC; Avanti Polar Lipids, Inc.; Alabaster, AL) were prepared by dissolving powdered DOPC in a 3:1 chloroform:methanol (OmniSolv; VWR, Mississauga, ON, Canada) solution (10 mg mL\(^{-1}\)). Verteporfin (QLT Inc., Vancouver, BC, Canada) was dissolved in dimethyletheroxide (Sigma, Oakville, ON, Canada) and the concentration (1.0 x 10\(^{-3}\) M) was determined spectrophotometrically (e \( \lambda \) = 32740 L mol\(^{-1}\) cm\(^{-1}\)). The appropriate volume of the DOPC stock solution was transferred to glass vial and the chloroform:methanol mixture was evaporated using nitrogen gas. The lipids were resuspended in phosphate buffer (0.02 M, pH 7.0) and stock Verteporfin to create the final liposomal solution of 0.01 mg mL\(^{-1}\) Verteporfin:1.0 mg mL\(^{-1}\) DOPC. Multilamellar vesicles (MLV) were formed by stirring and vortexing the solution four times in 10 min intervals. Often, the liposomal solution was diluted to decrease the number of MLV in the solution but the same effective ratio of lipid to photosensitizer remained constant. The liposomal solution was transferred to the middle well of a three-welled microscope slide (Fisher, Fairlawn, NJ) and the glass coverslip (Coming Brand No. 1; VWR) was sealed with mounting medium (Cytoseal 280, VWR) preventing atmospheric exposure. All samples including the microscope slides were wrapped in aluminum foil when not in use to protect the Verteporfin from stray light.

**Deoxygenated liposomal Verteporfin preparation.** The liposomal Verteporfin solutions were prepared as described above. A steady stream of nitrogen was flowed over the samples for several minutes and placed in a single equipment-sleeved nitrogen-purged glove bag (Model “S”; Instruments for Research and Industry [IRI]; Terre Haute, IN) for 24 h. Sample slides were prepared in the nitrogen-purged glove bag as above.

**CV-1 cells.** African green monkey kidney cells from a CV-1 line were grown onto glass coverslips in Dulbecco’s modified Eagle medium nutrient mixture F-12 powder (DMEM/F12) (Invitrogen; Grand Island, NY), 1% penicillin (Invitrogen), 0.5% sodium pyruvate (Invitrogen), 0.5% non-essential amino acids (Invitrogen) and 10% fetal bovine serum (FBS) (HyClone, Logan, UT). The cell-coated coverslips were incubated with Verteporfin (250 nM) in a 5% FBS and PBS solution for at least ½ h. Once incubation was complete, the coverslips were removed from the Verteporfin solution and were rinsed several times with 5% FBS to eliminate excess Verteporfin. The coverslip was placed cells-down onto a well slide containing 5% FBS. The slide cover was stabilized with a small amount of vacuum grease. Exposure to light was minimized throughout this procedure by preparing the slides under a red light, and covering slides with aluminum foil whenever light exposure was possible.

**Optical trapping and Verteporfin photobleaching.** MLV were selected for trapping under dim stage-light illumination. Appropriate sized vesicles were selected (400–700 \( \mu \)m) and trapped in the laser path by adjustment of the planar and focus controls. Once trapped, the vesicle was “dragged” a short distance to ensure stable trapping. The trapping laser beam was also used as the excitation laser beam, and the resulting fluorescence from the lipid membrane surface was collected after the stage light was shut off and the fluorescence directed into the detector.

Laser intensities of 40 or 80 mW average laser power (average irradiance of \( 3 \times 10^7 \) W cm\(^{-2}\) and \( 6 \times 10^7 \) W cm\(^{-2}\), respectively) were found to balance the optical and physical forces of trapping most effectively and allow the examination of the changing laser irradiance on the bleaching kinetics. Peak powers in this 100 fsec, 82 MHz, pulsed laser are approximately 5–10 kW. These power ranges also conserve the membrane integrity and allowed photobleaching to occur over an appropriate period of time.
CV-1 cell photobleaching. Photobleaching within single cells was observed using 26 mW average laser power. Laser powers larger than this have been shown to compromise cell viability (17). The TPE focal volume was placed into the cell, but limitations in the microscope capabilities did not allow determination of subcellular location, although measurements were localized in the perinuclear region. A different CV-1 cell was used for each photobleaching run. For the optical setup used here (i.e. excitation wavelength and fluorescence detection filter system), no TPE-autofluorescence was observed from the cells.

RESULTS

Optical trapping of Verteporfin-loaded liposomes

Early on in this study it was found that TPE Verteporfin photobleaching measurements were hampered by the solution heterogeneity of the free-floating lipid vesicles in solution. The large fluorescence intensity spikes found in Fig. 1 are representative of these free-floating liposomes. During these experiments, it was also observed that the optical pressure exerted by the laser beam caused the liposomes to be pulled into and subsequently pushed away from the TPE focal spot, thus suggesting that optical trapping of the liposomes would be possible. A short movie of this phenomenon is available (see photon-pressure.avi in SUPPLEMENTAL MATERIALS), which depicts a liposome moving under the influence of 120 mW, 780 nm pulsed laser light, imaged as described in the Materials and Methods section.

Optical trapping was used to overcome the solution heterogeneity issue by stabilizing a single liposome within the focal plane of the laser beam and simultaneously collecting photobleaching data. A movie of an optically trapped liposome can be viewed (see trapped.avi in SUPPLEMENTAL MATERIALS). Trapping at 120 mW as shown in this movie was possible only for very large liposomes. The smaller vesicles used for the photobleaching studies required lower powers for stable trapping.

Verteporfin photobleaching in liposomes and CV-1 cells

Verteporfin-loaded liposomes were simultaneously optically trapped and TPE photobleaching data were collected using a single laser beam. Representative decay curves for both oxygenated and deoxygenated Verteporfin liposomes and in oxygenated subcellular locations of CV-1 cells are given in Fig. 2A–D. In order to compare the photobleaching behavior and rates between experiments, the decay curves were fitted using the simplest function that modeled the data. In this case it was the sum of exponential decays.

\[ I(t) = I_0 + \sum_{n=1}^{\infty} A_n e^{-k_n t} \]  

where \( I(t) \) is the fluorescence count rate, \( I(\infty) \) is the count rate at time infinity, \( t \) is time, \( A_n \) represents experimental pre-exponential coefficients and \( k_n \) represents the apparent kinetic rate constants. The sum indicates either mono-exponential or biexponential fluorescence photobleaching where appropriate. The parameters were allowed to float until the fit converged as determined by a minimization of \( \chi^2 \). We understand that this mathematical description does not represent the kinetic mechanism when oxygen is present and is used here strictly to compare different bleaching experiments. Because we have no independent measure of oxygen concentration, the approach used by Foster and coworkers (10–12) was not possible to perform in this study. However, for an oxygen-free environment, a more satisfying approach can be taken.

In an oxygen-free environment, it can be argued that a mono-exponential decay is reasonable for loss of sensitizer through photobleaching. Following the method described by Finlay et al. (10), under conditions of constant oxygen concentration a solution for the time-dependent sensitizer concentration can be derived. For the data collected in the present study, the loss of Verteporfin through TPE triplet-mediated (i.e. Eq. 2a) photobleaching is given by the following equation:

\[ [\text{VP}]_c = \left[ \frac{1}{[\text{VP}]_0} + C_2 \right] \exp(C_1 t) - C_2 \]  

where it has been shown that the constant \( C_2 \) is directly proportional to \([1 \text{O}_2]\) (10). Thus, in a deoxygenated solution, \( C_2 \rightarrow 0 \) and (Eq. 4) becomes:

\[ [\text{VP}]_c = [\text{VP}]_0 \exp(-C_1 t) \]  

with

\[ C_1 = \frac{(\delta \Phi_i/\hbar \nu)^2 (k_{TA}[A]) \Phi_i^2}{(k_{TP}[A])} \]  

where \( \delta \) is the TPE cross-section (cm\(^2\) s\(^{-1}\)), \( \Phi_i \) is the triplet quantum yield and \( \Phi_i^2 \) is the square of the irradiance (for TPE, units of W\(^2\) cm\(^{-2}\)). It is assumed that the reactant, A, is in excess, or it does not change significantly in concentration during the photobleaching of VP (or both).

It was found that the oxygenated Verteporfin liposomes and the subcellular CV-1 decay curves were best fit using biexponential decay curves (Fig. 2A,C,D, respectively). The deoxygenated Verteporfin-loaded liposome photobleaching curves were best fit using a single exponential decay curve as shown in Fig. 2B. For trapped liposomes under oxygenated conditions, the fluorescence could be completely bleached (Fig. 2A,C), whereas in CV-1 cells and hypoxic liposomes complete bleaching was never achieved.

The apparent rate constants found by fitting the data using (Eq. 3) are grouped according to the average laser power in Table 1. The individual fast and slow exponential decay rates for oxygenated Verteporfin MLV and CV-1 cells are compared in Fig. 3. These kinetic rates are well grouped but they lack an obvious relation to
each other, except that there is a trend toward larger values of $k_1$ correlated with larger values of $k_2$.

The apparent kinetic rate constants for oxygenated MLV trapped with 40 mW and CV-1 cells photobleached with 26 mW average laser power were scaled to 80 mW by using (Eq. 7):

$$k_N = k_i \times \left( \frac{I_N}{I_i} \right)^2$$

where $k_N$ is the normalized kinetic rate, $k_i$ is the initial rate, $I_N$ is the laser intensity to which the data are normalized and $I_i$ is the laser intensity at which $k_i$ was determined. The intensity ratio is squared because the probability of TPE and photobleaching is directly proportional to the excitation rate, which itself is proportional to the square of the laser intensity.

### Oxygen and Verteporfin diffusion in CV-1 cells

Oxygen and Verteporfin diffusion in CV-1 cells was monitored by photobleaching, allowing the sample to recover for 15 min, and then photobleaching a second time. The decay curves that were obtained from these trials are illustrated in Fig. 4. A fluorescence signal was not recovered, but it can be noted that the fast portion of the decay curve returned in the second photobleaching run. These results suggest immobile Verteporfin and freely diffusing oxygen.

### DISCUSSION

#### Optical trapping

It is clear from Fig. 1 that TPE photobleaching analysis was not possible in cases in which the liposomes were not optically trapped. Under nontrapping conditions, there was a relatively symmetric increase and decrease of fluorescence signal as the liposome diffuses into and subsequently out of the TPE volume. Once the liposomes were trapped, the decay of fluorescence was apparent. Trials were performed in which liposomes without

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**Table 1.** The apparent rate constants for Verteporfin photobleaching in MLV and CV-1 cells.

<table>
<thead>
<tr>
<th>Oxygen present</th>
<th>Laser power (mW)</th>
<th>Fast decay rate</th>
<th>Slow decay rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$k_1$ (s$^{-1}$)</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>MLVs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>80</td>
<td>0.17</td>
<td>0.06</td>
</tr>
<tr>
<td>No</td>
<td>80</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Yes</td>
<td>40</td>
<td>0.16</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Scaled to 80</td>
<td>0.64</td>
<td>0.08</td>
</tr>
<tr>
<td>CV-1 cells</td>
<td>26</td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Scaled to 80</td>
<td>0.47</td>
<td>0.09</td>
</tr>
</tbody>
</table>

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**Figure 3.** Comparison of the kinetic rate constants for oxygenated Verteporfin-loaded MLV at 40 and 80 mW of laser power and CV-1 cells incubated with Verteporfin photobleached with 26 mW power.
Verteporfin were trapped (data not shown) and the characteristic decay curve was not observed. Therefore, simultaneous optical trapping and fluorescence detection is presented as a valid method for determining TPE photobleaching decay rates in a heterogeneous liposome solution.

It was noted that the trapping efficiency of the liposomes was increased as the vesicle diameter was greater than that of the focal waist (∼0.5 μm) of the TPE laser beam. The trapping of larger vesicles required larger excitation intensities. Therefore, medium-sized liposomes (500–700 μm diameter) were used throughout this study. Care was taken to trap vesicles of similar size to minimize the variation in the Verteporfin environment between samples.

**Photobleaching**

TPE photobleaching of Verteporfin was demonstrated in oxygenated and deoxygenated liposome samples as well as in vitro CV-1 cells. The apparent rate constants for these three systems are presented in Table 1. The standard deviation for each of the reported constants is quite large. This can be expected for the liposome solutions, as the size difference between the liposomes was not insignificant. Moreover, Finlay et al. (10) have demonstrated that one-photon excitation bleaching rates for Photofrin depend on sensitizer concentration, oxygen concentration and the concentration of other cellular targets, all of which may differ from liposome to liposome. It was impossible to determine the number of lipid bilayers within each liposome, but for each photobleaching run a single liposome was in the optical trap.

Based on the oxygen-dependent and -independent reaction schemes in (Eqs. 1, 2) and the equations developed by Foster and coworkers (10–12), it is proposed that the TPE-PDT–related photobleaching kinetics for the oxygen-rich samples will follow a non–mono-exponential decay, whereas a mono-exponential decay best represents the data from hypoxic solutions. This trend is evident from the average apparent kinetic decay constants presented in Table 1. For the oxygenated liposomes the difference in the decay rates for 40 mW vs 80 mW average power appears, interestingly, only in the nonscaled, slow component. The fast components are virtually identical. However, the slow rate constants for oxygenated and deoxygenated liposomes are not the same for the same laser power (80 mW). This suggests that while (Eq. 5) may be valid for photobleaching in a deoxygenated environment, it does not directly map into the oxygenated system. This makes sense because (Eq. 5) was developed assuming constant [O₂], which is not the case here.

For the deoxygenated liposome bleaching data the constants k₂ (slow component) from (Eq. 3) and C₁ from (Eq. 6) are equivalent. Therefore, it may be possible to relate this constant to known parameters of Verteporfin. Using the known values of δ (3.3 × 10⁻⁴⁷ cm³ s⁻¹ photon⁻¹) (1), kₘ (4 × 10⁷ s⁻¹), φ₁ (0.79) (18) and φ₂ (1 × 10⁻¹⁴ J cm⁻³), one can estimate k₉ₐ[A] using (Eq. 6). One finds that k₉ₐ[A] ~ 0.05 s⁻¹, which is very small, but consistent with the observed low one-photon excitation Verteporfin photobleaching yield (2 × 10⁻⁵) in the absence of oxygen. (18). Because we cannot directly measure the concentration of the reaction partner, A, it would not be justified to estimate the value of k₉ₐ[A].

The apparent rate constants for TPE photobleaching of Verteporfin in CV-1 cells suggest that the photobleaching decays are slightly different than those found in liposomes. The average power was kept at 26 mW to avoid direct cell damage due to TPE of endogenous photosensitizers (17). The CV-1 cell data are most directly comparable with the 40 mW liposome decays. Both display the non–mono-exponential form, however, in the cells the fast component is considerably slower (0.05 vs 0.16 s⁻¹, cell vs liposome), whereas the slow components are nearly the same (0.006 vs 0.007 s⁻¹). This difference could result from differing oxygenation or differing average sensitizer concentrations, both of which have been shown to affect the photobleaching rates in other systems (10–12).

The slow and fast exponential constants for the oxygenated Verteporfin-loaded MLV and CV-1 cells are plotted in Fig. 3 to determine whether or not there was a correlation between them. Although these kinetic rate constants are clustered, there seems to be no clear correlation, probably owing to the fact that these constants may not be related to all the photobleaching parameters in a straightforward manner.

When we consider the rate constants scaled to have the same number of TPE excitation events using (Eq. 7) (Table 1), then the constants can be explained qualitatively and are consistent with the proposed model for photobleaching. The scaled apparent rate constants for the slow component (kₙ) agree within the standard deviation for the oxygenated liposome experiments. That the apparent slow rate constant for the deoxygenated liposome sample is larger than that for the oxygenated solution is consistent with the fact that some remaining oxygen in the solution would cause triplet lifetimes to shorten, and thus the rate of triplet-mediated bleaching is reduced. The scaled fast apparent rate constants (k₁) suggest faster photobleaching at lower irradiance. Under the conditions of lower irradiance, oxygen consumption is slower and thus the oxygen-dependent regime lasts longer (10). The difference between the scaled k₁ and k₂ values in CV-1 cells and liposomes could still be rationalized by differences in the environment.

**Oxygen and Verteporfin diffusion in CV-1 cells**

We have suggested the fast kinetic portion of the photobleaching plots is related to the oxygen-dependent decay and the slower portion may represent a different, reduced oxygenation regime. In order to test this hypothesis, one could stop the photobleaching...
reaction by blocking the laser beam for a time sufficient to allow rediffusion of oxygen back into the depleted area. It was not possible to do this for trapped liposomes, because blocking the beam released the liposomes from the trap. However, it was possible to undertake a photobleaching recovery study for the CV-1 cells.

Figure 4 displays two sets of photobleaching kinetic data for the same location within a CV-1 cell. The second data set represents the effect of blocking the laser beam for 15 min and then reinitiating TPE photobleaching. As shown in Fig. 4, no recovery of the fluorescence signal was observed for the second run. In 1987, Morlier et al. (4) showed that fluorescence signal recovery after a period of photobleaching rest indicates the movement of photosensitizer within the system. Therefore, we believe these results are indicative either of a stationary photosensitizer population or light-induced reactions that inhibit the diffusion of photosensitizer back into the region of photobleaching. It is also important to note that although the fluorescence signal does not increase, there is a recovery of the fast portion of the photobleaching decay. This shows that there is a diffusible population of oxygen within the lipid membranes, thus supporting our claim that the most oxygen-dependent portion of the photobleaching decay is represented by the region of the most rapid loss of sensitizer.

CONCLUSIONS
It was shown that Verteporfin can be photobleached within an optically trapped liposome system as well as the perinuclear region in CV-1 cells via TPE. It was determined that during this process, systems with oxygen present display a non–mono-exponential trend in the photobleaching kinetics, while systems without oxygen display mono-exponential loss of sensitizer with time. The fast kinetic portion of the biexponential photobleaching decay likely represents oxygen-dependent photodynamics and the slow component may represent photobleaching in an environment with reduced oxygen. The photobleaching behavior of optically trapped liposomes is consistent with that from CV-1 cells, suggesting a trapped liposome is a valid model for evaluating and predicting TPE-PDT phenomena in cells.

SUPPLEMENTAL MATERIAL
The video files can be found at DOI: 10.1562/2005-05-28-RA-549.s1.

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