A Theoretical Formalism for Aggregation of Peroxidized Lipids and Plasma Membrane Stability During Photolysis

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ABSTRACT The objective of this investigation was to examine, from a theoretical perspective, the mechanism underlying the lysis of plasma membranes by photoinduced, chemically mediated damage such as is found in photolysis. Toward this end, a model is presented which relates the membrane lifetime to the thermodynamic parameters of the membrane components based upon the kinetic theory of aggregate formation. The formalism includes a standard birth/death process for the formation of damaged membrane components (i.e., peroxidized lipids) as well as a terminating condensation process for the formation of aggregates of peroxidized plasma membrane lipids. Our theory predicts that 1) the membrane lifetime is inversely correlated with predicted rate of membrane damage; 2) an upper limit on the duration of membrane damage exists, above which the mean and variance of the membrane lifetime is independent of further membrane damage; and 3) both the mean and variance of the time of membrane lifetime distribution are correlated with the number of sites that may be damaged to form a single membrane defect. The model provides a framework to optimize the lysis of cell membranes by photodynamic therapy.

INTRODUCTION

Photolysis, or photodynamic therapy, is a method that uses phototoxins (i.e., singlet oxygen) (Brault et al., 1988; Rakestraw et al., 1992) generated by illumination of photosensitizers [i.e., erythrosin B (Yonuschot, 1991), bilirubin, and protoporphyrin (Girotti and Deziel, 1983), and tin chlorin e6 (Brault et al., 1988; Rakestraw et al., 1992; Girotti, 1979; Lu et al., 1992; Orenstein and Kostenich, 1996)] to kill cells. A variety of tumors refractory to conventional therapies have been treated by photodynamic therapy (e.g., Orenstein and Kostenich, 1996; Heier et al., 1995). To improve the clinical efficacy of photodynamic therapy, attention has been directed toward improvement of photosensitizers (Bachor et al., 1995; Fan et al., 1996; Michelsen et al., 1996; Phillips et al., 1994), as well as strategies for photosensitizer and phototoxin delivery (Alleman et al., 1996; Bachor et al., 1994; Hisazumi et al., 1995; Yarmush et al., 1993). To improve the understanding of the cellular and molecular level processes important in photolysis, a limited number of experimental studies have been conducted (e.g., Yonuschot, 1991; Girotti and Deziel, 1983; Deziel and Girotti, 1980a, b; 1982; Thorpe et al., 1995).

In a model system of resealed erythrocyte ghosts (with the photosensitizers bilirubin and protoporphyrin) it was observed that an early event in the photolysis of cells was the permeation of the plasma membrane to small ions (Girotti and Deziel, 1983; Deziel and Girotti, 1980a, b; 1982). In thymocytes (with the photosensitizer erythrosin B), it was shown that the permeability of the plasma membrane to calcium occurred before the entry of ethidium bromide (Yonuschot, 1991). Later events included the permeation to larger molecules such as glucose-6-phosphate and molecules at least as large as the trisaccharides (Girotti and Deziel, 1983). In a single cell system (using malignant melanoma cells), it was shown that the membranes of laser-illuminated cells in the presence of the photosensitizer tin chlorin e6 destabilized at a single location and resulted in punctate membrane rupture (Thorpe et al., 1995).

The stability characteristics of lipid bilayer membranes, similar to the plasma membrane, have been well-characterized (Kashchiev and Exerowa, 1980; Penev and Exerowa, 1981; Penev et al., 1987). In these investigations, classical nucleation theory was used to describe the lifetime of the membrane presuming that a finite, but large, number of holes existed within the membrane. By using classical nucleation theory it was shown that the predicted mean rate of the formation of aggregates of these holes correlated with the mean rupture time of the Newtonian black films (Kashchiev and Exerowa, 1980; Penev and Exerowa, 1981; Penev et al., 1987). In evaluating the lifetime of Newtonian black films under α-particle irradiation, the radiation damage was assumed to not accumulate in the membrane and the interaction of an α-particle with a critical defect within the membrane was a Poisson process (Penev and Exerowa, 1981). In photolysis, the photosensitizer produces a phototoxin, upon illumination, which presumably acts to damage the membrane (see Rakestraw et al., 1992; Lu et al., 1992; Thorpe et al., 1995). The rate of plasma membrane damage may be controlled by manipulating the rate of delivery of phototoxin production as well as the time duration of phototoxin delivery (Thorpe et al., 1995). However, theoretical studies aimed at clarifying these cellular and molecular processes have yet to be presented.

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We present a model in which two parallel processes occur to influence the membrane stability: first, defects are formed by the action of the phototoxin upon the plasma membrane; second, the defects diffuse within the membrane to the surface of a growing defect aggregate. We presume that the membrane ruptures when a single aggregate containing a critical number of defects is formed (cf. Kashchiev and Exerowa, 1980; Penev et al., 1987). The model permits detailed study of the dynamics of the rupture event, and provides a framework to optimize lysis of plasma membranes based upon the rate and time duration of damage by singlet oxygen.

PEROXIDIZED LIPID FORMATION IN CELL MEMBRANES

Experimental evidence indicates that plasma membranes rupture as a result of simultaneous exposure to the photosensitizer tin chlorin e6 (SnCe6) and 630-nm laser illumination (Thorpe et al., 1995). When exposed to 630-nm laser illumination SnCe6 is excited to the triplet state, ^{3}SnCe6, which in turn excites molecular oxygen to the singlet state, $^1\Delta_g$ by decaying to the ground state (Thorpe et al., 1995; Boudin, 1930; Reid, 1958). Carbon–carbon double bonds in lipids are labile to peroxidation, as are proteins, by singlet oxygen (Boudin, 1930; Reid, 1958; Anderson and Thompson, 1992; Girotti, 1990; Gollnick, 1968; Hartman et al., 1988; van Kuijk et al., 1987). We presume that two parallel processes may be operational to cause the membrane rupture as a result of phototoxin production: first, membrane components are damaged because of direct peroxidation mediated by the singlet oxygen; second, damaged lipids that are hydrophilic (see, for example van Kuijk et al., 1987) condense to form a phase separate from the continuous phase of undamaged membrane lipids. The combination of these two processes is presumed to lead to small, mechanically weak regions of the membrane which are particularly susceptible to rupture, and thus result in cell lysis. In the binary system of peroxidized and nonperoxidized lipids, the lipid type in abundance (denoted $\mathcal{A}$), is assumed to form a continuous phase, while the lean lipid type (denoted $\mathcal{B}$), is assumed to form a disperse phase of aggregates (Frenkel, 1946). The distribution of type $\mathcal{B}$ aggregate sizes will depend upon the total free energy difference for a single type $\mathcal{B}$ lipid free and dispersed in the type $\mathcal{A}$ phase and in the condensed type $\mathcal{B}$ phase. If the total chemical potential difference has a maximum with respect to the number of lipids in the aggregate, then when the first aggregate forms in the membrane corresponding to this maximum, membrane rupture is presumed to occur. To examine the partitioning of the peroxidized lipids in the membrane we require a relationship between singlet oxygen production and the thermodynamic potential for a single type $\mathcal{B}$ phase lipid.

Singlet oxygen production

The concentration of singlet oxygen, $[^1\Delta_g]$ (z), in the photodynamic boundary layer of thickness, δ, near the membrane may be obtained from the kinetics of singlet oxygen production (Rakestraw et al., 1992) as

$$[^1\Delta_g](z) = \frac{\phi_{^1\Delta_g}[\mathrm{SnCe6}]\lambda \mathrm{P_L}\alpha_\Delta}{hc}\left[\left\{\frac{1}{k_d}+\frac{\delta^2}{2\mathcal{D}_{O_2}}\right\}\frac{z}{\delta}-\frac{z^2}{2\mathcal{D}_{O_2}}\right] \quad (1)$$

where $[^1\Delta_g]$ (z) $\{[\doteq]\{\mathcal{M}\}\}$ is the concentration of singlet oxygen as a function of distance z $\{[\doteq]\{\mathrm{nm}\}\}$ from the membrane surface. The surface of the membrane corresponds to $z = 0$. $\mathcal{D}_{O_2} = (3 \times 10^{-5}\ \mathrm{cm^2/s})$ is the diffusivity of oxygen in water (Lindig and Rodgers, 1981) and [SnCe6] is the concentration of the photosensitizer in the medium. The laser power density is $\mathrm{P_L}$ in units of mW/cm^2, $k_d = 2.0 \times 10^5\ \mathrm{s^{-1}}$ is the collective rate constant for singlet oxygen decay in phosphate buffered saline, and $\phi_{^1\Delta_g} = 0.85$ $^1\Delta_g$/photon is the singlet oxygen quantum yield for SnCe6 irradiated with 630 nm light (Rakestraw et al., 1992). Planck's constant and the speed of light are respectively h and c. The SnCe6 molar absorbance cross section at 630 nm wavelength is $\alpha_\Delta = 2.0$ Å^2 (Rakestraw et al., 1992). The thickness of the photodynamic boundary layer was estimated to be $\delta = \sim$100 nm, and depends upon the distance a singlet oxygen molecule can diffuse before reacting with a substrate, encountering a quenching agent, or decaying from a variety of radiative and nonradiative processes (Thorpe et al., 1995). The $^1\Delta_g$ diffusional distance was estimated from the $^1\Delta_g$ lifetime of 3 μs in DPBS (Rodgers and Snowden, 1982). Since we are interested in analyzing the functional relationships between singlet oxygen flux to the membrane and dynamic photolysis events, the singlet oxygen flux was evaluated based upon a photodynamic boundary thickness of $\delta = 100$ nm.

Based upon the concentration of singlet oxygen given by Eq. 1, the flux of singlet oxygen to the membrane surface at $z = 0$, $\mathcal{N}_{^1\Delta_g}$, $\{[\doteq]\{^1\Delta_g$ molecules/Å^2-s$\}\}$ can be obtained from

$$\mathcal{N}_{^1\Delta_g} = \frac{\phi_{^1\Delta_g}[\mathrm{SnCe6}]\lambda \mathrm{P_L}\alpha_\Delta}{hc}\left[\frac{2\mathcal{D}_{O_2}+k_d\delta^2}{2k_d\delta}\right] \quad (2)$$

All the parameters in Eq. 2, except the thickness of the photodynamic boundary layer, may be experimentally determined. Equation 1 provides the rational basis for grouping the experimental variables SnCe6 and $\mathrm{P_L}$ into a single number $\mathcal{N}_{^1\Delta_g} \propto$ SnCe6 $\times$ $\mathrm{P_L}$, (e.g., Rakestraw et al., 1992; Thorpe et al., 1995). Therefore $\mathcal{N}_{^1\Delta_g}$ provides a convenient vehicle for presentation of results.

Peroxidized lipid aggregate formation in plasma membranes

In the following development, which is based on the classical theory of aggregating systems (Frenkel, 1946), the

plasma membrane is considered to be a two-dimensional fluid composed of circular disks of radius a_0, (which we take to be the mean projected radius of the lipid headgroup), representing the projection of the undamaged lipids onto the membrane surface as shown in panel *a* of Fig. 1. Singlet oxygen acts upon the undamaged lipids, converting them to the damaged form (Fig. 1 *b*). These damaged lipids diffuse laterally in the membrane and condense to form aggregates (Fig. 1 *c*). When the aggregate reaches a sufficiently large size, it is presumed to be the site of punctate membrane rupture (Fig. 1 *d*).

Define a site as that location, in the plasma membrane, where singlet oxygen acts (the plasma membrane lipids are presumed to have more than a single site at which the singlet oxygen may act). For simplicity, the photoinduced plasma membrane damage is presumed to sequentially occur at multiple sites within an area equivalent to the lipid headgroup. Let $P_0(t)$ $\{[\doteq]\{\text{sites}/\text{Å}^2\}\}$ denote the total number density of nonperoxidized sites per lipid headgroup area. The initial condition for the $P_0(t)$ is $P_0(t)|_{t=0} = 1/A_0$, and $A_0 = 153.39$ Å^2 is the mean projected area of a lipid headgroup onto the plasma membrane surface. Let f be the number of peroxidized sites in each lipid headgroup area, and $P_f(t)$ $\{[\doteq]\{\text{sites}/\text{Å}^2\}\}$ be the number density of lipids with f peroxidized sites. The maximum number of sites which may be peroxidized per lipid is f^+. All lipids with fewer than f^+ peroxidized sites are type $\mathcal{A}$ lipids, which immediately become type $\mathcal{B}$ lipids when exactly f^+ sites became peroxidized. Then the rate of formation of lipids with f peroxidized sites is given by

$$\frac{dP_f(t)}{dt} = \begin{cases} -\mathcal{N}_{^1\Delta_g}P_0(t) + \mathcal{R}P_1(t) & \text{for } f = 1, \\ \mathcal{N}_{^1\Delta_g}P_{f-1}(t) - \mathcal{N}_{^1\Delta_g}P_f(t) - \mathcal{R}fP_f(t) & \\ \quad + \mathcal{R}(f+1)P_{f+1}(t) & \text{for } f \in [1, f^+] \end{cases} \tag{3}$$

where $\mathcal{N}_{^1\Delta_g}$ is the predicted flux of singlet oxygen to the membrane given by Eq. 2 and $\mathcal{R}$ $\{[\doteq]\{s^{-1}\}\}$ is the repair rate due to active cellular metabolism. By carefully selecting the unit surface area of membrane, the number density of peroxidized and nonperoxidized sites may be normalized such that the initial conditions on these quantities are $P_0(t)|_{t=0} = 1.0$ and $P_f(t)|_{t=0} = 0.0$ $\forall f \geq 1$. While Eq. 3 has been solved for $P_f(t)$ in the $f \geq 0$, $t > 0$ quadrant of the (f, t) plane (Penev and Exerowa, 1981; Penev et al., 1987), an analytical solution has not been previously discussed for the case $f \in [1, f^+]$. In this study, the sequence of equations given in Eq. 3 will be solved numerically; of particular interest is the time evolution of the number of lipids with f^+ peroxidized sites. We now require the formalism describing the condensation of lipids with f^+ peroxidized sites into aggregates.

Let $p_g(t)$ $\{[\doteq]\{\text{particle}/\text{Å}^2\}\}$ be the number density of aggregates containing g peroxidized lipids in the membrane at time t. Then an aggregate of size zero, with number density $p_0(t)$, represents a membrane devoid of aggregates of lipids; it may, however, contain lipids that have been damaged as described in Eq. 3. The aggregate number densities $p_g(t)$ may be normalized (by multiplying by the projected area of a lipid headgroup onto the plasma membrane to obtain the nonnormalized cumulative probability functions) such that $p_0(t)|_{t=0} = 1.0$; however, it is not necessarily true that $\Sigma_{g=0}p_g(t) = 1$ $\forall t \geq 0$ (see Bailey, 1964; Feller, 1968a, b). The system of equations for $p_g(t)$ may be obtained by examining the chemical potential difference, for a single peroxidized lipid, between the disperse type $\mathcal{A}$ phase and the aggregated type $\mathcal{B}$ phase.

In percolating systems, it is easy to demonstrate that the perimeter of a cluster varies approximately linearly with the number of occupied sites (the probability of site occupation in a square lattice is p, and no mutual influence exists between neighboring sites on the lattice) (Stauffer and Aharony, 1992). In fact, the perimeter, $C(g)$, varies approximately as

$$C(g) \sim g\frac{1-p}{p} + Dg^{\zeta}, g \to \infty \tag{4}$$

where D is a constant and ζ is a fractal dimension. In the case under investigation in this work, interactions between type $\mathcal{A}$ and type $\mathcal{B}$ molecules as well as between type $\mathcal{B}$ molecules are presumed to drive the aggregation process. Also, it is expected that the type $\mathcal{B}$ molecules will be several orders of magnitude fewer in number than the type $\mathcal{A}$ molecules, and hence the concentration of type $\mathcal{B}$ molecules will be far below the percolation threshold. Therefore, the conditions for percolation and the linearity between cluster

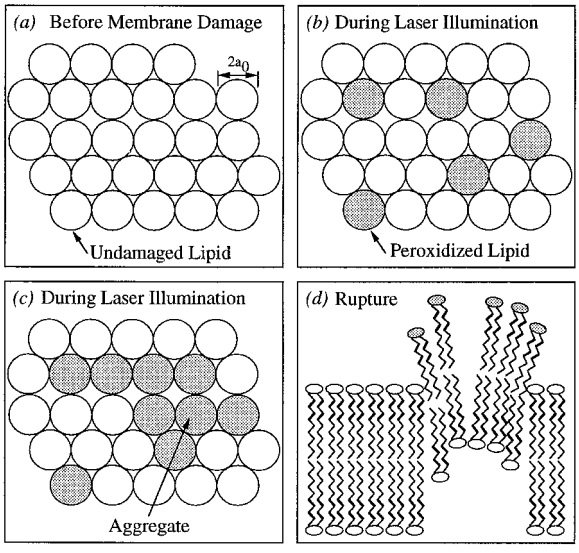

FIGURE 1 (*a*)–(*c*) The membrane is viewed normal to its surface; in (*d*) the membrane is viewed parallel to its surface. In (*a*) no defects exist in the membrane before the initiation of photoinduced, chemically mediated damage. The initiation of membrane damage converts a fraction of the membrane components into defects, as shown in (*b*); the defects form aggregates (*c*). The aggregate is the site of membrane rupture as shown in (*d*).

perimeter and the number of molecules in the cluster are not satisfied in this investigation. We presume that the cluster shape most likely to be observed in the membrane composed of type $\mathcal{A}$ and $\mathcal{B}$ molecules will be the one that minimizes the total free energy of formation of the cluster. Simple analysis indicates that the most frequently observed shape will then be circular.

The surface free energy between the aggregated lipid phase and the continuous phase of nonperoxidized lipids is defined to be σ (Gibbs, 1928; Tolman, 1949). Furthermore, define $\Delta\mu$ to be the chemical potential difference between these two phases (Sigsbee, 1969; Ford, 1996). The total free energy change for forming an aggregate of size g from g uncondensed molecules is presumed to be linearly dependent upon the aggregate size with proportionality constant $\Delta\mu$. Furthermore, the work due to forming a circular boundary between the aggregate of size g and the surrounding monomers is $\sigma\sqrt{g}$ (see Weaver, 1994). Then the change in total free energy of the membrane peroxidized lipids due to condensation of g monomeric peroxidized lipids to an aggregate of size g is

$$\Delta\Gamma_g = g\Delta\mu + \sigma\sqrt{g}. \tag{5}$$

When $\Delta\mu < 0$ (that is, the peroxidized lipid prefers to be in the condensed type $\mathcal{B}$ phase rather disperse in the type $\mathcal{A}$ phase), then $\Delta\Gamma_g$ will exhibit a maximum at $g = g^\dagger$. In classical nucleation theory, $g^\dagger$ is called the "critical" aggregate size and is given by

$$g^\dagger = [(\sigma/2\Delta\mu)^2]_I, \tag{6}$$

where the notation $[\ ]_I$ indicates that an integer not less than the quantity in the square brackets is used. To determine the rate at which peroxidized lipid aggregates of size g are formed, the theory developed by Frenkel (1946), and solved for a special case of condensation (Kashchiev, 1969), is employed. Given the total free energy of forming an aggregate of size g, in Eq. 5 the Frenkel-Zeldovich theory (Frenkel, 1946; Kashchiev, 1969) yields the equilibrium number density of aggregates, N_g. From simple arguments (Frenkel, 1946) it can be shown that the number of peroxidized lipids striking the surface of an aggregate is proportional to

$$\beta_g = p_1(t)\sqrt{\frac{k_B T}{2\pi m}}\exp(-\mathcal{U}/k_B T) \tag{7}$$

where m {[≐]{g/lipid}} is the mass of the peroxidized lipid (assumed to be equal to the mass of a single lipid), k_B is Boltzmann's constant, T {[≐]{K}} is the temperature, and $\mathcal{U}$ {[≐]{$k_B T$}} is the mean field potential energy on the peroxidized lipid. Let I_g be the difference between the rate of gaining and losing a single peroxidized lipid from the surface of an aggregate of size g, which depends linearly upon β_g and the number of peroxidized lipids on the surface of the aggregate. Since an aggregate of size g may be formed by the addition of a single peroxidized lipid to an aggregate of size $g - 1$ as well as by the loss of a single peroxidized lipid from an aggregate of size $g + 1$, then the rate of change in the formation of an aggregate of size g is simply (Frenkel, 1946)

$$\frac{\partial p_g(t)}{\partial t} = I_g(t) - I_{g+1}(t),\ g \geq 2. \tag{8}$$

Normalizing $p_g(t)$ by dividing by the projected area of a single lipid converts the number densities to cumulative probability functions, which would be particularly useful in analyzing experimental photolysis results.

The number of size zero aggregates, $p_0(t)$, is obtained from Eq. 3 as $p_0(t) = P_0(t)$. The number of size 1 aggregates ($p_1(t)$), however, cannot be obtained directly from Eq. 3, but must account for all peroxidized lipids which constitute the aggregates. Recalling that sites on a single lipid become sequentially damaged because of the action of singlet oxygen, until a total of f^+ are damaged (see Eq. 3), then the total number density of peroxidized lipids is $P_{f^+}(t)$. Therefore, the number density of monomeric peroxidized lipids with f^+ damaged sites (those not associated with an aggregate) is

$$p_1(t) = P_{f^+}(t) - \sum_{g=2} g p_g(t)\ \forall t > 0. \tag{9}$$

In using classical nucleation theory to describe the process of forming aggregates of size g from monomeric peroxidized lipids, an infinite background of peroxidized lipids would necessarily be assumed (see Frenkel, 1946; Kashchiev, 1969; Turnbull, 1949; Turnbull and Fisher, 1949), and when an aggregate of size $g^\dagger$ was formed, it would be spontaneously removed from the system and replaced by $g^\dagger$ monomeric peroxidized lipids (Frenkel, 1946). In this study, when an aggregate of size $g^\dagger$ is formed the system terminates (the membrane ruptures). Because of the limited number of condensable, peroxidized lipids in the system we do not expect that an aggregate of critical size will spontaneously grow to form the bulk condensed state; the aggregate of terminal size, $g^\dagger$, simply represents a mechanically unstable location in the membrane, which is the location at which the rupture is presumed to occur. The use of the conditions that $p_0(t) = P_0(t)$, $p_{g^\dagger+1}(t) = 0$ along with Eq. 9 as boundary conditions for Eq. 8 therefore represents a significant variance from application of Eq. 8 in classical nucleation theory. Under certain conditions, an analytical solution to Eq. 8 may be obtained (Kashchiev, 1969). However, an analytical solution to Eq. 8 with the boundary condition Eq. 9 is intractable, and therefore we solve Eq. 8 using numerical techniques (Wilemski, 1995; Wilemski and Wyslouzil, 1995a, b) for the number densities of size g aggregates, $p_g(t)$, where $g \in [1, g^\dagger]$. Equation 9 with Eq. 8 and 5 constitute the required formalism which relates the nonequilibrium distribution of peroxidized lipid aggregates, $\{p_g(t)\}$, to the thermodynamic properties of the undamaged/damaged membrane component system. The connection between the singlet oxygen flux and cumulative distribution of membrane rupture times remains to be determined.

Plasma membrane rupture

A formalism is now in hand relating the formation of peroxidized lipids and the thermodynamic potential for aggregate formation. The experimental data typically consist of cumulative membrane rupture time probability distributions, $F_E(t)$ (see, for example, Thorpe et al., 1995); therefore, a relationship is required between the formation of peroxidized lipids and the time of plasma membrane rupture. The key assumption in this investigation is that when a single aggregate (of peroxidized lipids) of size $g^\dagger$ appears in the membrane, then the membrane "ruptures" (Canaday et al., 1994). Let t_D be the time at which the first aggregate of size $g^\dagger$ occurs in the membrane. Therefore, t_D coincides with the time at which the membrane is observed to rupture. In other words, t_D is the first passage time (Feller, 1968a, b) of the system to the rupture state. The experimentally observed distribution of the first passage times to the rupture state is $F_E(t)$, where

$$F_E(t) = \Pr\{t_D \leq t | [\text{SnCe6}], P_L\}. \tag{10}$$

Since $p_{g^\dagger}(t)$, appropriately normalized, is the probability of observing an aggregate of size $g^\dagger$ at time t, then the theoretical distribution of first passage times to the appearance of an aggregate of size $g^\dagger$ is

$$F_T(t) = \Pr\{t_D \leq t | [\text{SnCe6}], P_L\} = p_{g^\dagger}(t)/M \tag{11}$$

where $p_{g^\dagger}(t)$ satisfies the differential-difference equation given by Eq. 8 subject to the condition that $p_{g^\dagger}(t)|_{t=0} = 0$ and M is an appropriate normalization (defined in detail below). Consequently, the relationship among the singlet oxygen flux to the membrane, the total thermodynamic potential for lipids in the membrane, and membrane rupture is $F_E(t) = F_T(t)$. The means of these two distributions are denoted $\langle t_D \rangle_E$ and $\langle t_D \rangle_T$, respectively, while the respective variances are $\text{Var}\{t_D\}_E$ and $\text{Var}\{t_D\}_T$.

Recall that the number density of undamaged sites, $P_0(t)$, (given by Eq. 3), was normalized such that it, as well as $p_0(t)$, is unity at the initiation membrane damage by singlet oxygen. The normalization used was the mean projected surface area, A_0 $\{[\doteq]\{\text{Å}^2\}\}$, of a single lipid. However, the normalization for $p_{g^\dagger}(t)$ to form $F_T(t)$ is not so straightforward. If we were to use the infinite time limit of $p_{g^\dagger}(t)$ as the normalization for $p_{g^\dagger}(t)$ to form $F_T(t)$ ($M = \lim_{t\to\infty} p_{g^\dagger}(t)$) then the infinite time limit of $F_T(t)$ would be independent of $\mathcal{N}_{^1\Delta_g}$ and duration of laser illumination, which is contrary to experimental evidence. Indeed, the basic assumptions of the model imply that the $F_E(t)$ must depend upon the number of damaged lipids required to rupture the membrane (Canaday et al., 1994). Neither the number of peroxidation steps required to create a monomeric peroxidized lipid nor the total number of these lipids required to rupture the membrane is known. Therefore, we cannot a priori determine the appropriate normalization for $p_{g^\dagger}(t)$ to yield $F_T(t)$. However, careful examination of experimental data will illuminate these unknown quantities.

Consider a system in which the number of monomeric peroxidized lipids at any time t is $p_1(t)$, and the number density of aggregates of size $g > 1$ is $p_g(t)$. The total number of peroxidized lipids in the system is

$$p_{1,\text{Total}}(t) = p_1(t) + \sum_{g=2}^{g^\dagger} g p_g(t). \tag{12}$$

If at some time $t = \tau$, the total number of peroxidized lipids was held fixed at $p_{1,\text{Total}}(\tau)$, then the system would relax to equilibrium with the number density of aggregates of size g, $g \geq 2$, being N_g (Frenkel, 1946), and the total number of single peroxidized lipids not associated with an aggregate is $N_1 = p_{1,\text{Total}}(\tau) - \Sigma_{g\geq 2} g N_g$. Let each single, monomeric peroxidized lipid and each aggregate constitute a single particle. The number of particles in the system at equilibrium is then $F = N_1 + \Sigma_{g\geq 2} N_g$. To derive the normalization for $p_{g^\dagger}(t)$ in Eq. 11, consider the infinite time limit of $p_g(t)$, which is N_g (for $g < g^\dagger$), including the case where $\Delta\mu > 0$. If $\Sigma_{g=2} N_g \ll N_1$, then $F \sim N_1$, giving (Courtney, 1961; Ford, 1996)

$$p_g(t) \sim N_1 \exp\left\{\frac{-\Delta\Gamma}{k_B T}\right\}. \tag{13}$$

Now, $N_1 = P_{f^\dagger}(t) - \Sigma_{g=2} g p_g(t)$, so the number of condensable molecules in the system at any given time is approximated by

$$p_1(t) \sim 1 - \exp\{-\mathcal{N}_{^1\Delta_g} t\}. \tag{14}$$

We may then write that

$$p_g(t) \sim [1 - \exp\{-\mathcal{N}_{^1\Delta_g} t\}] \exp\left\{\frac{-\Delta\Gamma}{k_B T}\right\}. \tag{15}$$

Therefore, $\mathcal{N}_{^1\Delta_g}$ influences $p_g(t)$ and hence the first passage time to an aggregate of size $g^\dagger$.

The normalization for $p_{g^\dagger}$ in Eq. 11 is necessarily a function of the total number of lipids that must be peroxidized to rupture the membrane. If fewer lipids are produced, then the probability that all the membranes in a population of cells will rupture will be less than unity. If more lipids are peroxidized, then all the membranes in a population of cells will rupture. We now present an argument showing that M in Eq. 11 can be obtained directly from the analysis of experimental plasma membrane rupture times. Let t_{ill} be the time duration of peroxidized lipid production, and denote the total observation time by t_{obs} ($t_{ill} \leq t_{obs}$); peroxidation of lipids occurs only during the time interval $[0, t_{ill}]$ and no peroxidation occurs after t_{ill}. Suppose a singlet oxygen flux, $\mathcal{N}_{^1\Delta_g}$, (sufficiently large that $\langle t_D \rangle \ll t_{obs}$) is used such that $F_E(t) = 1$ for some $t < t_{obs}$ with $t_{ill} = t_{obs}$. This means that all membranes have an aggregate of size $g^\dagger$ (all membranes will rupture) some time before the total observation time, and peroxidized lipid production continues to occur for the duration of the total observation time. Clearly, those peroxidized lipids formed after the membranes have ruptured play no role in the rupture process. Furthermore, under the

conditions that $t_{ill} = t_{obs}$, the number of monomeric peroxidized lipids in the membrane is in great excess of the number required to rupture all of the membranes in a population of cells. The current model predicts that for any $\mathcal{N}_{^1\Delta_g} > 0$, there exists a $t_{ill} \ll \infty$ (denoted t'_{ill}) such that $\lim_{t\to\infty}\{F_E(t) - 1\} = \epsilon$, ϵ infinitesimally small, independent of t_{obs}. In other words, for any finite damage rate to the membrane, we can find an illumination time in which all of the cell membranes are ruptured without the formation of excess peroxidized lipids. Let the total number of peroxidized lipids for the combination $(t'_{ill}, \mathcal{N}_{^1\Delta_g})$ be $p_{1,Total}(t)|_{t=t'_{ill}}$. For any t'_{ill}, increasing $\mathcal{N}_{^1\Delta_g}$ will only have the effect of decreasing the mean cell rupture time (see Eq. 15); it will not increase the total number of peroxidized lipids required to produce an aggregate of size $g^\dagger$ in all membranes. Also, for any given $\mathcal{N}_{^1\Delta_g}$, varying t_{ill} such that it is greater than t'_{ill} will not increase the total number of peroxidized lipids to the extent that there is an effect upon either $g^\dagger$ or $p_{g^\dagger}(t)$. Increasing t_{ill} above t'_{ill} or $\mathcal{N}_{^1\Delta_g}$ above that required to produce $p_{1,Total}(t)|_{t=t'_{ill}}$ peroxidized lipids only serves to yield excess peroxidized lipids above that required to rupture the membrane. Therefore, precisely $p_{1,Total}(t)|_{t=t'_{ill}}$ peroxidized lipids are required to rupture the membrane and the $p_{g^\dagger}(t)$ associated with this $p_{1,Total}(t)|_{t=t'_{ill}}$ is the appropriate normalization, M, to be used in Eq. 11. From a practical viewpoint, determining $\mathcal{N}_{^1\Delta_g}$ such that the condition that $\lim_{t\to\infty}\{F_E(t) - 1\} = \epsilon$ is satisfied may be very difficult because of the length of time required for the experiments. An alternative route is required.

As a result of physical limitations, such as SnCe6 toxicity, laser power deposition levels, and available molecular oxygen to participate in the peroxidation process, a maximum $\mathcal{N}_{^1\Delta_g}$ exists. At this maximum, sequential experiments in which t_{ill} is reduced incrementally from t_{obs}, such that $\langle t_D\rangle \ll t_{obs}$, will yield a $t_{ill} = t'_{ill}$ such that $\lim_{t\to t_{obs}}\{F_E(t) - 1\} = \epsilon$, ϵ infinitesimally small; that is, all membranes are ruptured before t_{obs}. Presumably at the maximum possible $\mathcal{N}_{^1\Delta_g}$ and illumination time $t_{ill} = t_{obs}$ ($\langle t_D\rangle \ll t_{obs}$), a large excess of peroxidized lipids will be formed. Reducing t_{ill} will yield fewer peroxidized lipids until $t_{ill} = t'_{ill}$ is reached, at which point no excess peroxidized lipids are formed. When $t_{ill} = t'_{ill}$ the number of peroxidized lipids will be precisely $p_{1,Total}(t)|_{t=t'_{ill}}$.

This experiment then yields exactly the proper normalization for $p_{g^\dagger}(t)$ to obtain $F_T(t)$ as

$$M = \lim_{t\to\infty} p_{g^\dagger}|_{M^+}, \tag{16}$$

where

$$M^+ = \operatorname*{lub}_{\left\{t_{ill},\, \int_0^{t'_{ill}} \mathcal{N}_{^1\Delta_g}(\tau)d\tau\right\}} \{\lim_{t\to\infty} p_{1,Total}(t) | \lim_{t\to t_{obs}}\{F_E(t) - 1\} = \epsilon\} \tag{17}$$

with ϵ vanishingly small. Recognize, of course, that our singlet oxygen flux (here we imply an expanded definition of the singlet oxygen flux depending upon the number of nonperoxidized sites available on the membrane) is nonzero only as long as there are sites remaining to be damaged. Now that the normalization for $p_{g^\dagger}(t)$ has been obtained, we turn our attention to f^+.

The number of sites that must be damaged by singlet oxygen to form a monomeric peroxidized lipid is f^+. Because peroxidized lipid formation depends upon the composition of the membrane, then f^+ is only a property of the membrane and it is independent of $\mathcal{N}_{^1\Delta_g}$, t_{ill}, and the triad $\{\mathcal{U}, \Delta\mu, \sigma\}$. The distribution $p_1(t)$ is dependent upon f^+ (recall that $p_1(t) = P_{f^+}(t)$). Since the number density of aggregates of size $g^\dagger$ is a function of both time and the temporal distribution of peroxidized lipids ($p_{g^\dagger}(t) = p_{g^\dagger}(t, p_1(t))$), then $p_{g^\dagger}(t)$ depends upon f^+. Consider Eq. 3 and ignore the repair rates. For finite f^+, we easily obtain that

$$P_{f^+}(t) = 1 - \exp(-\mathcal{N}_{^1\Delta_g}(t)t) \sum_{i=0}^{f^+-1} \frac{1}{i!} \mathcal{N}^i_{^1\Delta_g}(t)t^i. \tag{18}$$

The mean of this distribution (for constant singlet oxygen flux), $\langle t\rangle_{f^+}$, is $\langle t\rangle_{f^+} = f^+/\mathcal{N}_{^1\Delta_g}$, also the variance is $f^+/\mathcal{N}^2_{^1\Delta g}(t)$. While increasing f^+ has the same effect upon the mean of the distribution given in Eq. 18 as does decreasing the predicted singlet oxygen flux, the same cannot be said regarding the respective influence of f^+ and $\mathcal{N}_{^1\Delta_g}$ upon the variance of this distribution. Although the predicted singlet oxygen flux, given in Eq. 2, may be in error, accounting for this error by shifting the value for $\mathcal{N}_{^1\Delta_g}$ in Eq. 3 may not correct discrepancies between the observed distributions, $F_E(t)$, and the theoretical distributions, $F_T(t)$. Therefore, since $p_{g^\dagger}(t)$ depends upon both $\mathcal{N}_{^1\Delta_g}$ and f^+, via Eq. 3, then we may determine f^+ by observing $F_E(t)$ as a function of $\mathcal{N}_{^1\Delta_g}$ when $\lim_{t\to\infty} F_E(t) = 1$.

Define p_1^+ as the total number of peroxidized lipids in the membrane at rupture; that is, at the time that an aggregate of size $g^\dagger$ is formed ($p_1^+ = p_{1,Total}(t)|_{t=t_D}$). Consider a membrane in which a total of p_1^+ peroxidized lipids is injected at time zero. At equilibrium, the number density of aggregates of size g may be obtained using the Boltzmann distribution given in Eq. 13, with $N_1 \equiv p_1^+$. Now, precisely one aggregate of size $g^\dagger$ will be formed. Therefore, p_1^+ depends only upon the Gibbs free energy difference for a peroxidized lipid between phases $\mathcal{A}$ and $\mathcal{B}$. This Gibbs free energy is only a property of the membrane, hence p_1^+ is also only a property of the membrane. p_1^+ is independent of t_{ill}, $\mathcal{N}_{^1\Delta_g}$, and f^+; furthermore, it is independent of $\mathcal{U}$. We propose then that p_1^+ must depend upon the ratio of the two thermodynamic parameters $\Delta\mu$ and σ. The normalization for $p_{g^\dagger}(t)$ to form $F_T(t)$ and the number of sites required to create a monomeric peroxidized lipid may each be obtained from observations on the experimentally observed rupture distribution obtained by varying specific experimental conditions. That is, the normalization for $p_{g^\dagger}(t)$ is obtained in experiments in which t_{ill} is varied, while the number of sites per lipid may be obtained from experiments in which $\mathcal{N}_{^1\Delta_g}$ is varied. The definition of M and f^+ implicitly defines $\mathcal{N}_{^1\Delta_g}(t)$, or alternatively the total number of peroxidized lipids required to rupture the membrane, for all peroxidized

lipids in excess of p_1^+ contribute nothing to the rupture process. Unfortunately, our problem is yet to be resolved since we have yet to specify the procedure by which $\mathcal{U}$ may be obtained from experimental data.

The cross section of an aggregate for a peroxidized lipid, (β_g given by Eq. 7), is a function of the number density of monomer peroxidized lipids, the mean field potential, and the temperature. For all experimental parameters fixed, two alternative methods present themselves for determining β_g: first, by varying $\mathcal{N}_{^1\Delta_g}$, $p_1(t)$ may be altered, thus giving an indication of the value of $\mathcal{U}$; second, by varying the temperature we obtain information on the influence of β_g, via $\mathcal{U}$, upon the rupture time distributions. Since f^+ and $\mathcal{U}$ are completely uncorrelated, then no overspecification in the regression is encountered in obtaining both quantities from experiments in which $\mathcal{N}_{^1\Delta_g}$ is altered while $t_{ill} = t_{obs}$. The remaining parameters in the model are σ and $\Delta\mu$, and hence $g^\dagger$, which may be obtained by regression of the model, defined by Eq. 11, against the experimental membrane rupture distributions.

RESULTS

The objective of this investigation was to establish a theoretical formalism for examining the membrane lifetime distributions obtained as a result of photolysis experiments in single cell systems. We recognize that the chemical potential difference for a lipid between the $\mathcal{A}$ phase and $\mathcal{B}$ phase is a function of the supersaturation (Sigsbee, 1969). Also, the surface tension is necessarily a function of the cluster size (Tolman, 1949). Since we are interested in examining the influence of the various parameters, in the current theory, (including f^+, p_1^+, $\mathcal{U}$, and $g^\dagger$, which is obtained from the ratio of the surface tension and the chemical potential) on the rupture distributions, and since including the functional details of the chemical potential and surface tension will only serve to obscure our case, then we choose, for the sake of simplicity, to momentarily set aside the exact details of these two parameters and instead presume them constant. Equivalence between $F_E(t)$, given by Eq. 10, and $F_T(t)$, given by Eq. 11, represents this relationship based upon the thermodynamic properties of peroxidized lipids within the membrane, given by the triad $\{\mathcal{U}, \Delta\mu, \sigma\}$, and the generation of these peroxidized lipids by the action of singlet oxygen upon the membrane. The number density of aggregates of size $g^\dagger$, which are presumed to be the location of punctate membrane rupture, is obtained as the solution of the set of equations given in Eq. 8. Since the theory presumes that precisely one aggregate of size $g^\dagger$ is sufficient to rupture the membrane, then $p_{g^\dagger}(t)$, properly normalized as defined in Eq. 16, gives the membrane lifetime distribution, $F_T(t)$. In the following, we investigate the influence of the thermodynamic parameters $\{\mathcal{U}, \Delta\mu, \sigma\}$ and the rate of peroxidized lipid formation upon the membrane lifetime distributions.

In the system of interest, the number of monomeric peroxidized lipids is neither uniform with time, nor infinite [as is presumed in classical nucleation theory (Frenkel, 1946; Kashchiev, 1969)], but are created by the action of singlet oxygen. To study the time course of the generation of monomeric peroxidized lipids and their effect upon the distribution of selected aggregate sizes, we conducted a computational experiment in which $\mathcal{N}_{^1\Delta_g} = 0.409\ ^1\Delta_g$ molecules/nm^2-s, 3 min illumination time, and the triplet $\{\mathcal{U}, \Delta\mu, \sigma\}$ takes the values $\{26.7\ k_BT, 0.1257\ k_BT, 1.365\ k_BT\}$, which corresponds to $g^\dagger = 30$. These quantities were selected because they gave a mean membrane lifetime similar to those previously reported (Thorpe et al., 1995). In Fig. 2 the number density (scaled by $A_0 = 153.39$ Å^2) of monomeric peroxidized lipids and aggregates of size $g = 10$ and $g^\dagger = 30$ is presented as a function of time for the current model and for the classical nucleation theory (see Frenkel, 1946; Kashchiev, 1969). The current model assumes the boundary condition given by Eq. 9 instead of an infinite and uniform background of peroxidized lipids, as in the case of classical nucleation theory. The results for the current model are scaled by 1×10^3 and 1×10^7 for aggregates of size $g = 10$ and $g^\dagger = 30$, respectively. The classical nucleation theory results are scaled by 10 and 100 for aggregates of size $g = 10$ and $g^\dagger = 30$, respectively. Even though the time duration for the generation of monomeric peroxidized lipids is 3 min, the number density of these particles ($g = 1$) exhibits a maximum at 78 s, indicating that a substantial fraction of the monomers are rapidly sequestered into aggregates. Also observe from Fig. 2 that the number of monomeric peroxidized lipids is approximately at its equilibrium value before ~10% of the total number of aggregates of size $g^\dagger$ have been formed. Over the observation time, the number density of monomers reaches a steady state quantity, as does the number density of aggregates of size $g = 10$ and $g^\dagger = 30$. Since the asymptotic value of the number density of aggregates containing 30 peroxidized lipids ($g^\dagger = 30$) is 7.2×10^{-8}, then for the membrane to have exactly one aggregate of size 30 (the membrane reaches the rupture state) a total of 1.39×10^7 ($=p_1^+$) monomeric peroxidized lipids must be formed. The classical nucleation theory results indicate that the number density of monomeric peroxidized lipids decreases monotonically from unity, and the number of aggregates of size 10 as well as size 30 require much longer times to reach their respective asymptotic values than the current theory. Classical nucleation theory predicts that the asymptotic value of $A_0\, p_{g^\dagger}(t)$ is 1.27×10^{-5}, requiring only 7.8×10^4 peroxidized lipids to rupture the membrane, a surprisingly small number. Since $p_0(t)|_{t=0} = 0$, and $p_1(t)|_{t=0} = 1$, then the major shortcoming in applying classical nucleation theory to the analysis of membrane rupture distributions in photolysis is that the predicted dynamics of aggregate formation is independent of $\mathcal{N}_{^1\Delta_g}$, which is contrary to experimental evidence (Thorpe et al., 1995). We therefore conclude, then, that classical nucleation theory is unlikely to adequately represent the process dynamics that may occur in photolysis.

The total number of peroxidized lipids required to generate exactly one aggregate of size $g^\dagger$ in the membrane plays

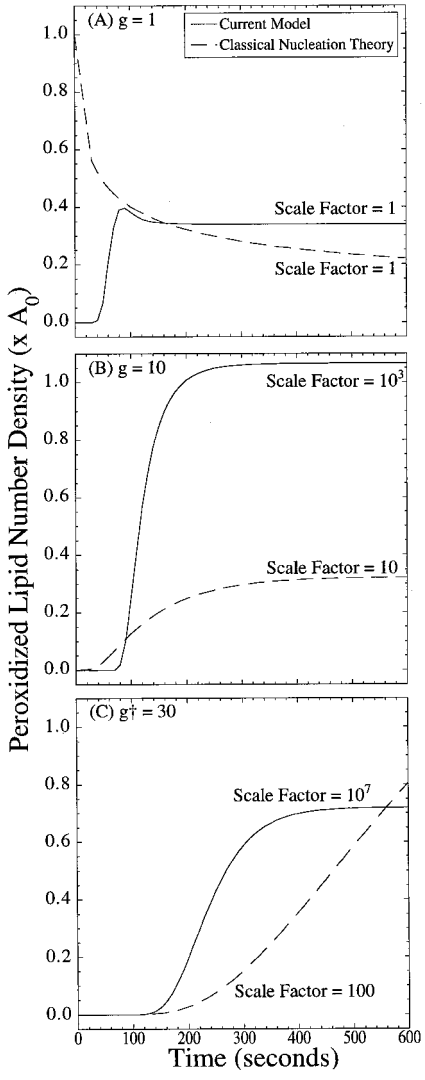

FIGURE 2 The number densities of monomeric defects (*A*), size 10 (*B*), and size 30 (*C*) aggregates as computed using classical nucleation theory and the current model for photoinduced membrane damage. The results for the current model are scaled by 1×10^3 in (*B*) and 1×10^7 in (*C*) and the results for the classical nucleation theory scaled by 10 in (*B*) and 100 in (*C*) for aggregates of size $g = 10$ and $g^\dagger = 30$, respectively. The model parameters used in these calculations are $\{\mathcal{U}, \Delta\mu, \sigma\} = \{26.7\ k_BT, 0.1257\ k_BT, 1.365\ k_BT\}$ with singlet oxygen flux, $\mathcal{N}_{^1\Delta_g} = 0.409\ ^1\Delta_g$ molecules/nm^2-s and 3 min illumination time. Under these conditions the current theory predicts that 1.39×10^7 ($=p_1^+$) monomeric defects must be formed for a single aggregate of size $g^\dagger = 30$ to appear in the membrane. Classical nucleation theory predicts a monotonically decreasing number of monomeric defects, and the number densities of size 10 and 30 aggregates are particularly high.

an important role in the comparison of theoretical and experimental results (see, for example, Penev et al., 1987) through Eq. 10 and 11, and therefore needs to be carefully determined. We have identified p_1^+ as an equilibrium property of the membrane dependent upon the Gibbs free energy difference, for a peroxidized lipid, between aggregated and nonaggregated peroxidized lipids. The Gibbs free energy depends upon the chemical potential difference, $\Delta\mu$, and the surface tension, σ, between a peroxidized lipid in the type $\mathcal{A}$ phase and type $\mathcal{B}$ phase. To examine the influence of the relative value of $\Delta\mu$ and σ upon p_1^+, we systematically varied their ratio (that is, $g^\dagger$ as given in Eq. 5) and computed the resultant value for p_1^+ presuming that $f^+ = 1$. The results, shown in Fig. 3, indicate that a positive correlation exists between $g^\dagger$ and p_1^+. Varying $f^+ \in [2, 30]$ yielded p_1^+ versus $g^\dagger$ results, identical to those obtained for $f^+ = 1$. To further investigate the sensitivity of p_1^+ to membrane thermodynamic parameters a study was performed in which the rate of peroxidized lipids entering the aggregate of size $g \leq g^\dagger = 30$ was varied. This was accomplished by varying the mean field potential $\mathcal{U}$ in Eq. 7 over the range $\pm 10\%$ of the value used for Fig. 3 (see Table 1). It is clear from these results that p_1^+ is independent of both the number of damaged sites per peroxidized lipid and the rate at which the peroxidized lipids strike the surface of a growing aggregate. The interpretation for this theoretical observation is that the number of peroxidized lipids required to rupture the membrane, $p_1^+ = p_{1,\text{Total}}(t)\big|_{t=t'_{\text{ill}}}$, does not depend upon how they were formed (one or multiple damage sites), nor does it depend upon how fast they condense ($\mathcal{U}$). It simply depends upon the total amount of work required to form the aggregate of size $g^\dagger$ from $g^\dagger$ individual peroxidized lipids.

Three parameters in the current model may be experimentally manipulated to influence the membrane survival. Two of these of interest are $\mathcal{N}_{^1\Delta_g}$ and t_{ill}. Presuming that the total number of peroxidized lipids required to have exactly a single aggregate of size $g^\dagger = 30$ is $p_1^+ = 1.39 \times 10^7$, for $t_{\text{ill}} = 3$ min, then the probability of observing a single

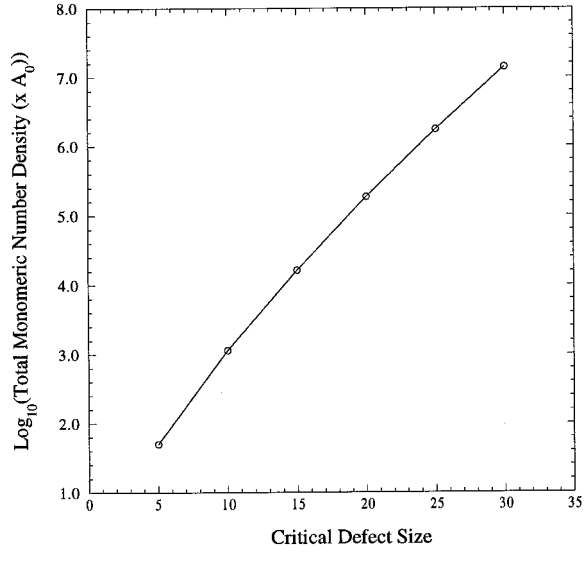

FIGURE 3 The influence of the aggregate of size $g^\dagger$ (and therefore the ratio of σ and $\Delta\mu$) upon the total number of peroxidized lipids which must be formed in the membrane. These simulation results indicate the extent of photoinduced membrane damage which must occur before a size $g^\dagger$ aggregate forms and thus leads to an unstable plasma membrane. The conditions used for these predictions were $\mathcal{N}_{^1\Delta_g} = 0.409\ ^1\Delta_g$ molecules/nm^2-s, 3 min illumination time, and the parameter pair $\{\mathcal{U}, \Delta\mu\} = \{26.7\ k_BT, 0.1257\ k_BT\}$. The surface tension, σ, was varied to yield the desired value of $g^\dagger$.

TABLE 1 The influence of variations in the mean field potential, $\mathcal{U}$, upon the temporal distribution of aggregates of size $g^\dagger$

$\mathcal{U}$ Deviation	Velocity Å/s	$p_1^+ \times (1.0 \times 10^7)$	$\langle t_D \rangle_T$	St. Dev. $\{t_D\}_T$
90%	9.263	7.0149	81.156	9.276
95%	2.437	7.0149	117.380	19.729
100%	0.642	7.0099	245.320	67.603
105%	0.169	7.1141	729.094	249.260
110%	0.044	7.1235	2259.931	923.832

aggregate of size $g^\dagger = 30$ (the first passage time to an aggregate of size $g^\dagger$) may be readily calculated. To examine the influence of the predicted singlet oxygen flux upon the first passage time to an aggregate of size $g^\dagger = 30$, $F_T(t)$, we varied $\mathcal{N}_{^1\Delta_g}$ over a relatively large range. In Fig. 4 we present the simulation results for $F_T(t)$ versus time when $\mathcal{N}_{^1\Delta_g}$ is assumed to be 0.069, 0.139, 0.208, and 0.417 $^1\Delta_g$ molecules/nm^2-s. The results for the classical nucleation theory are also given with the abscissa scaled by 1/10 for convenience. The parameter triplet $\{\mathcal{U}, \Delta\mu, \sigma\}$, was assumed to be $\{26.7\ k_BT, 0.1257\ k_BT, 1.365\ k_BT\}$, which corresponds to $g^\dagger = 30$. When the total number of peroxidized lipids at the end of the observation time (which is sufficiently large) is larger than p_1^+ ($\lim_{t\to\infty} p_{1,Total}(t) \geq p_1^+$), then the probability is unity that a membrane will have exactly one aggregate of size $g^\dagger = 30$; that is, all membranes will rupture. When $p_{1,Total}(t) < p_1^+$, then the probability of having an aggregate of size $g^\dagger = 30$ is less than unity and not all membranes will rupture. Since the results presented in Fig. 4 are for variations in the rate of membrane damage, then $p_{1,Total}(t)$ (and hence $p_{g^\dagger}(t)$) is dependent upon the singlet oxygen flux. Since the total number of peroxidized lipid formation is important, then $\mathcal{N}_{^1\Delta_g}$ and t_{ill} may be combined to a single parameter as $\int_0^{t_{ill}} \mathcal{N}_{^1\Delta_g}(\tau)d\tau$. For the parameters used here, the classical nucleation theory yields a mean time to an aggregate of size $g^\dagger$, which is approximately twice the value obtained from the maximum singlet oxygen flux case studied in the current theory, while the variance is greater by a factor of approximately four.

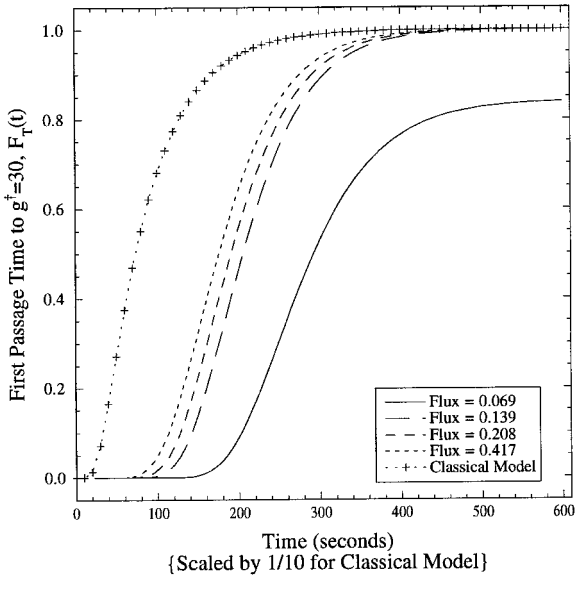

FIGURE 4 Classical and current theoretical predictions on the time at which an aggregate of size $g^\dagger = 30$ will appear in the membrane (the first passage time). The abscissa has been scaled by a factor of 1/10 for the classical nucleation theory results to permit comparison with the current model predictions. The current model predicts a dependence of the first passage time upon the rate of photoinduced membrane damage, and that if the total amount of photoinduced damage is not sufficiently large (that is, if a total of p_1^+ peroxidized lipids are not created in the membrane), then the probability that a membrane will have exactly one aggregate of size $g^\dagger$ is less than unity.

The number of membrane sites that may be damaged per lipid headgroup area, f^+, is independent of the value of $g^\dagger$. The mean and variance of $P_{f^+}(t)$, and hence $p_1(t)$, depends upon f^+. To determine the relative influence of f^+ and $g^\dagger$ upon the first passage time to an aggregate of size $g^\dagger$, we performed simulations in which both f^+ and $g^\dagger$ were systematically varied. Presented in panel *A* of Fig. 5 is the mean first passage time to the formation of an aggregate of size $g^\dagger$ as a function of $g^\dagger$ as well as f^+. The conditions used for these predictions were $\mathcal{N}_{^1\Delta_g} = 0.409$ $^1\Delta_g$ molecules/nm^2-s, 3 min illumination time, and the parameter pair $\{\mathcal{U}, \Delta\mu\} = \{26.7\ k_BT, 0.1257\ k_BT\}$. The surface tension, σ, was varied to yield the desired value of $g^\dagger$. These simulation results clearly indicate the expected linear dependence of the mean time to formation of a size $g^\dagger$ aggregate upon the number of sites per peroxidized lipid. Also, these results indicate an approximately quadratic dependence upon the critical aggregate size. Since classical nucleation theory presumes that $p_1(t) = 1$, then it does not predict a dependence of the mean time of formation of aggregates of size $g^\dagger$ upon the number of sites per peroxidized lipid. From these results we conclude that increasing f^+ has the net effect of shifting the mean of the critical peroxidized lipid distribution to higher time points. Since the mean of the temporal distribution of size $g^\dagger$ aggregates is sensitive to both f^+ and $g^\dagger$, a study was conducted concerning the variance of this distribution. Given in panel *B* of Fig. 5 is the standard deviation of the distribution of size $g^\dagger$ as functions of the number of sites per peroxidized lipid and the size of the aggregate. Clearly, the standard deviation is dependent upon $g^\dagger$ but is weakly dependent upon f^+. From the results presented in Fig. 5 we conclude that the mean of the distribution of aggregates of size $g^\dagger$ is strongly dependent upon the mean time at which peroxidized lipid are formed (larger f^+ implies longer mean time of peroxidized lipid formation); however, when the bulk of the peroxidized lipids are formed (compare Fig. 2) the formation of aggregates of size $g^\dagger$ is controlled by the number density of the peroxidized lipid, not the number of sites per peroxidized lipid.

Fig. 4 suggests that the mean time to the occurrence of an aggregate of size $g^\dagger$ is also dependent upon the rate, through the singlet oxygen flux, of damage to the membrane. Since the number density of size $g^\dagger$ aggregates, $p_{g^\dagger}(t)$, depends, in

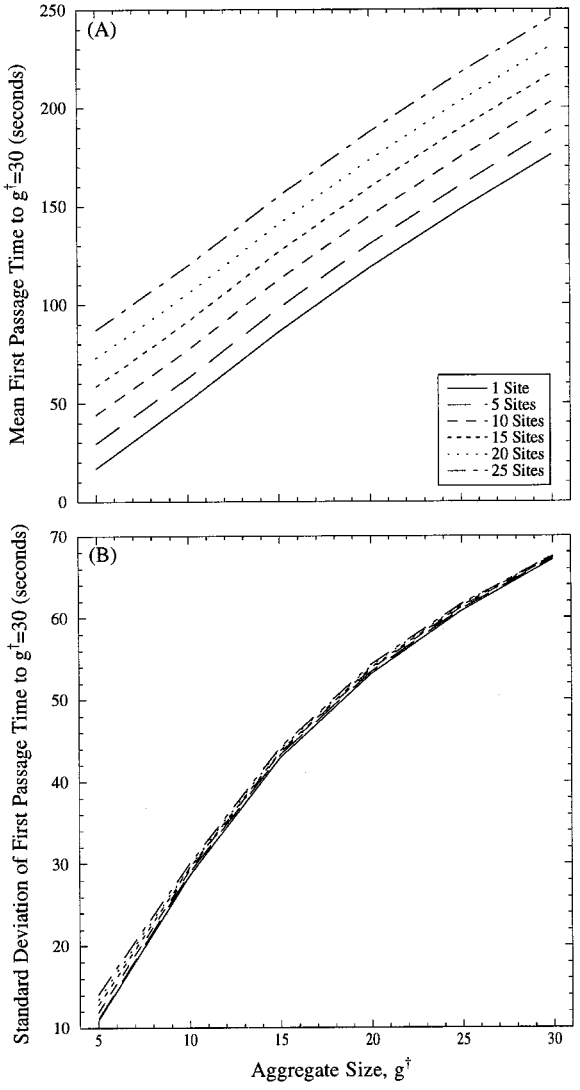

FIGURE 5 The mean (*A*) and standard deviation (*B*) of the first passage time to the formation of an aggregate of size $g^{\dagger}$ versus $g^{\dagger}$, where the number of sites per lipid is varied from one to 25. The minimum number of sites per defect yields the minimum mean time to the formation of aggregates of size $g^{\dagger}$. The standard deviation of the first passage time distribution is only weakly dependent upon the number of damaged sites required to form a single peroxidized lipid. The conditions used for these predictions were $\mathcal{N}_{^1\Delta_g} = 0.409\ ^1\Delta_g$ molecules/nm^2-s, 3 min illumination time, and the parameter pair $\{\mathcal{U}, \Delta\mu\} = \{26.7\ k_BT, 0.1257\ k_BT\}$. The surface tension, σ, was varied to yield the desired value of $g^{\dagger}$.

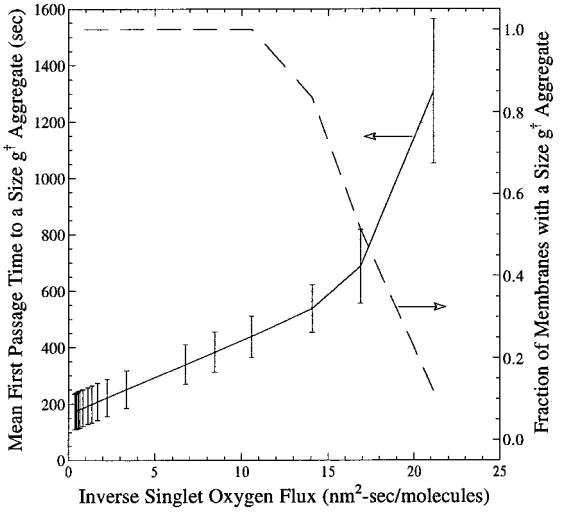

FIGURE 6 Mean and variance of the theoretical membrane rupture time distribution for continuous membrane damage and 25 damaged sites for each monomeric peroxidized. Also plotted is the predicted fraction of membranes ruptured against the inverse singlet oxygen flux to the membrane. The model predicts that all membranes are ruptured for singlet oxygen flux $> \sim 0.083\ ^1\Delta_g$ molecules/nm^2-s.

part, upon the singlet oxygen flux to the membrane, we conducted a sensitivity experiment to probe the nature of this dependence. Shown in Fig. 6 is the mean and variance of the first passage time to a single size $g^{\dagger}$ aggregate as a function of inverse singlet oxygen flux. The mean and variance of $F_T(t)$ are calculated using the standard integral definition, $\langle t_D \rangle = \int_0^{\infty} t\ dF_T(t)$ and $\mathrm{Var}\{t_D\} = \langle t_D^2 \rangle - \langle t_D \rangle^2$, where $\langle t_D^2 \rangle = \int_0^{\infty} t^2\ dF_T(t)$, respectively. Also shown is the total fraction of membranes which contain exactly one aggregate of size $g^{\dagger}$ within the observation time, $t_{obs} = 30$ min, due to an illumination time of $t_{ill} = 3$ min with 25 peroxidation sites per peroxidized lipid. When the fraction of membranes with exactly one size $g^{\dagger}$ aggregate is unity, $\lim_{t\to\infty} F_T(t) = 1$, then the mean rupture time and the singlet oxygen flux are inversely correlated with probability unity. While $\lim_{t\to\infty} F_T(t) = 1$ the variance of the distribution is weakly correlated with the singlet oxygen flux. From Figs. 4 and 6 we conclude the following: the probability of observing an aggregate of size $g^{\dagger}$ in a membrane, and hence the time of membrane rupture, is controlled by the ratio of the total number peroxidized lipids in the membrane and p_1^+; that is, if the total number of peroxidized lipids in the membrane is fewer than the total number required to rupture the membrane, then the membrane is not likely to rupture. The total number of peroxidized lipids in the membrane at any given time is controlled by the rate of peroxidized lipid formation given that the repair process is ignored in our treatment. Classical nucleation theory is unable to predict these two dependencies.

To establish the protocol for obtaining both f^+ and $g^{\dagger}$ from experimental observations, we require information about the dependence of the mean passage time to an aggregate of size $g^{\dagger}$ upon the rate at which peroxidized lipids strike the surface of an aggregate. This rate, β given in Eq. 7, is determined in part by the mean field potential, $\mathcal{U}$. The resulting distribution, of aggregates of size $g^{\dagger} = 30$, and variance (shown in Table 1), clearly indicate that the rate at which the peroxidized lipids strike the surface of a growing aggregate strongly influences the temporal distribution of aggregates of size $g^{\dagger}$ in the membrane. However, the net rate at which peroxidized lipids strike the aggregate surface is also dependent upon the number of monomeric peroxidized lipids in the membrane. Therefore, a study is required to determine the relationship among $p_1(t)$, $\mathcal{N}_{^1\Delta_g}$, and f^+.

To determine the number of sites per peroxidized lipid from experimental observations, information about the relative influence of f^+ and $\mathcal{N}_{^1\Delta_g}$ upon the formation of aggregates of size $g^\dagger$ is required. The temporal distribution of monomers in the membrane affects $p_{g^\dagger}(t)$ which, when appropriately normalized, gives the probability of observing exactly one aggregate of size $g^\dagger$ in the membrane. The rate of damage to the membrane strongly influences $p_1(t)$ (see Fig. 6). We therefore performed a computational experiment which allows direct examination of the influence of f^+ upon the first- and second-order moments of the theoretical membrane lifetime distribution. Presented in panel *A* of Fig. 7 is the mean first passage time (the mean of the distribution $F_T(t)$) to the formation of an aggregate of size $g^\dagger = 30$ as a function of the singlet oxygen flux, $\mathcal{N}_{^1\Delta_g}$, and the number of sites that must be damaged to form a single peroxidized lipid when the laser illumination time is 3 min. Simple regression analysis of the mean cell rupture time as a function of inverse singlet oxygen flux gives 163.8 s as the 0th-order term and a linear dependence of the first-order coefficient upon f^+. The 0th-order term is in surprisingly good agreement with the minimum time required to rupture a membrane in a model system (Thorpe et al., 1995). We therefore conclude that f^+ has a strong effect upon both the high and low singlet oxygen flux asymptotes of the $\langle t_D \rangle$ versus $\mathcal{N}_{^1\Delta_g}$ graph. Fig. 7 *B* shows the standard deviation of the first passage time (the standard deviation of the distribution $F_T(t)$) to the formation of an aggregate of size $g^\dagger = 30$ as a function of the singlet oxygen flux, $\mathcal{N}_{^1\Delta_g}$, and the number of sites that must be damaged to form a single peroxidized lipid when the laser illumination time is 3 min. Analysis indicates an approximately linear relationship between the first-order term coefficient and the number of sites. Also, the minimum standard deviation at high singlet oxygen flux is 65.3 s. Since the number of sites per peroxidized lipid influences $p_1(t)$, it in turn influences $p_{g^\dagger}(t)$. We conclude then that f^+ may be obtained from experimental lifetime distributions obtained for variations in the rate of damage to the membrane, $\mathcal{N}_{^1\Delta_g}$.

The formation of peroxidized lipids by the sequential damage of individual sites includes the possibility of a repair mechanism. To study the effect of this repair, which was presumed to be constant, upon the formation of peroxidized lipids, and ultimately upon the rupture distribution, we simulated cell rupture distribution while maintaining the singlet oxygen flux to the plasma membrane constant and varying the repair rate constant. In Fig. 8 is presented the theoretical rupture time distribution for $\mathcal{N}_{^1\Delta_g} = 0.89\ ^1\Delta_g$ molecules/nm^2-s, $t_{ill} = 3$ min for a limited range of repair rate constants. The results are striking in that for rates of

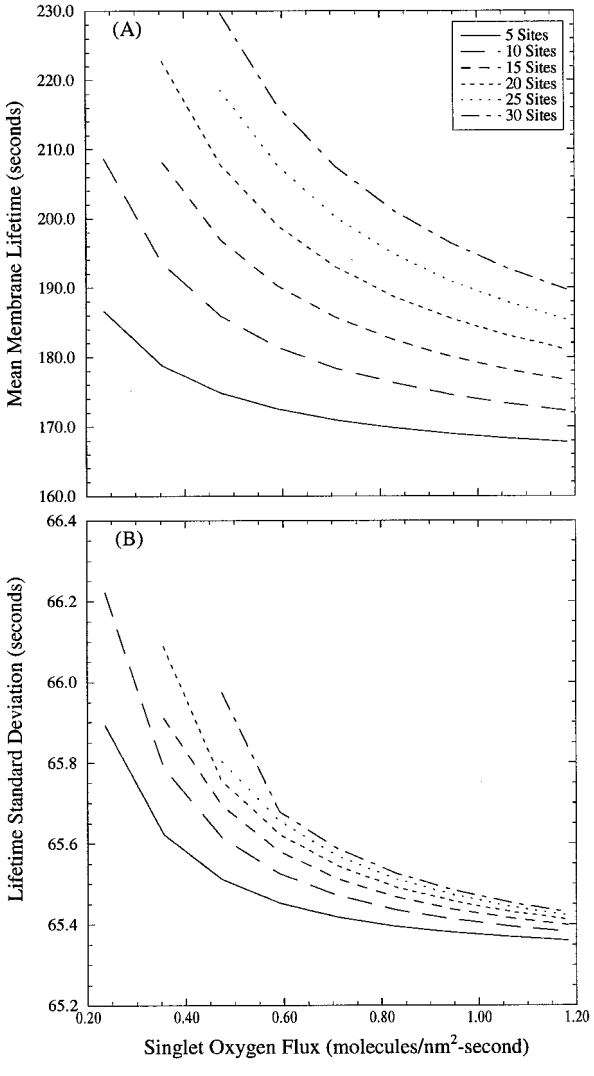

FIGURE 7 The dependence of the mean and standard deviation of the membrane rupture time upon the singlet oxygen flux to the membrane as well as the number of sites which must be damaged to form a single, monomeric peroxidized lipid. The laser illumination time is 3 min.

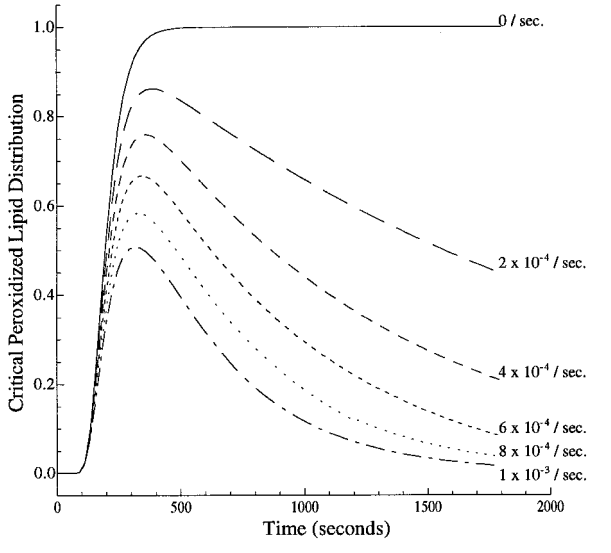

FIGURE 8 The model for the formation of single, monomeric defects includes the repair of the damaged sites in each peroxidized lipid. This figure illustrates the influence of variations in the repair rate upon the theoretical membrane rupture distribution when the total laser illumination time is 3 min and ~0.89 $^1\Delta_g$ molecules/nm^2-s.

repair significantly less than the damage rate, the model predicts a maximum in the rupture time distributions. Furthermore, for repair rates for which $\lim_{t\to\infty} F_T(t) - 1 = \epsilon$, that is for $\mathcal{R} \leq 2 \times 10^{-6}$ sites/lipid-s, (data not shown), there is no significant influence of the repair rate on the mean rupture time. While these results are of considerable theoretical interest, their practical value is indeed limited. We may conclude, however, that the repair rate is probably not linearly dependent upon the number of sites damaged for each peroxidized lipid. This rate may depend in a nonlinear manner upon the number of peroxidized lipids in the membrane as well as the probability of observing a single peroxidized lipid of size $g^\dagger$ in the membrane.

DISCUSSION

We have presented a theoretical formalism that may prove valuable in analyzing experimental membrane rupture time distributions. The input parameters to the model are those parameters which may be experimentally manipulated: the time of laser illumination, t_{ill}; the singlet oxygen flux to the plasma membrane, $\mathcal{N}_{^1\Delta_g}$. The results of the theory include the total number of peroxidized lipids required to rupture the membrane, the size of the aggregate where rupture is presumed to occur, and the distribution of membrane rupture times. The current theory relates the rate of membrane damage to the membrane lifetime through the thermodynamic properties of the peroxidized lipids, which are presumed to be produced via the action of singlet oxygen. The assumption that connects the current theory with the membrane lifetime distributions is that when exactly one aggregate of size $g^\dagger$ is formed in the membrane, the membrane ruptures.

A central feature of the current model is the concept that a lipid has a multiplicity of sites which must be damaged to create a single peroxidized lipid. The inverse dependence of the mean cell rupture time upon the singlet oxygen flux and the linear dependence of the proportionality constant, between rupture time and singlet oxygen flux, upon the number of sites for each lipid makes experimental determination of the number of sites straightforward. That is, the number of sites can be obtained directly from a set of experimental rupture time distributions which have been collected for varying singlet oxygen flux conditions. The inverse relationship between the standard deviation of the rupture time distribution and singlet oxygen flux indicates that this quantity should be carefully considered in obtaining the triad $\{\mathcal{U}, \Delta\mu, \sigma\}$ (or the functional forms for $\Delta\mu$ and σ). That is, ensuring equivalence of the means of the experimental and theoretical distributions is insufficient to yield an adequately accurate estimate of the triad $\{\mathcal{U}, \Delta\mu, \sigma\}$ if the variance is not also considered.

The scaling parameter M for $p_{g^\dagger}(t)$ to form the first passage time to an aggregate of size $g^\dagger$, $F_T(t)$, is important as it provides the key connection between the thermodynamics of aggregate formation and experimentally observed membrane rupture time distributions, $F_E(t)$. The parameter is dependent only upon the nature of the membrane. The reasoning for this is as follows: the ratio of σ and $\Delta\mu$ yields the critical aggregate size $g^\dagger$. The infinite time limit of $p_{g^\dagger}(t)$, for high singlet oxygen flux, is the normalization factor for $p_{g^\dagger}(t)$ to yield $F_T(t)$ as indicated in Eqs. 16 and 17. Therefore, M depends upon $g^\dagger$ and consequently upon $\Delta\mu$ and σ, which are both properties of the plasma membrane. M is not yet accessible by direct experimental investigation; therefore, its value cannot be set a priori to the regression of $F_T(t)$ against $F_E(t)$, but must be as a consequence of this regression. As $g^\dagger$ is obtained as a result of the regression of $F_T(t)$ against $F_E(t)$, then also, $p_{1,\text{Total}}(t)|_{t=t'_{ill}}$, and consequently M is also a result of this regression.

When the lipids in the plasma membrane are damaged, specific intracellular pathways involving phospholipase A_3 and arachadonic acid are upregulated to repair the membranes. In this investigation, the repair rate was presumed to be only a function of the number of sites damaged on the lipids. As a result, the influence of the repair rate upon the rupture time distribution indicates a maximum in the distribution for all repair rates greater than zero. Although physically unrealistic, this information is of some considerable value as it indicates that the repair rate is not only a function of the number of damaged sites, but also must be correlated with the number of aggregates of size $g^\dagger$, as well as the dynamics of the repair process. The repair rate cannot be obtained from the rupture time distributions as there is not a sufficient quantity of information in these distributions to obtain both the number of cites per peroxidized lipid and the repair rate. Although it is of considerable theoretical interest, the repair rate as currently formulated is of limited practical value.

Our finding that a correlation exists between the number density of monomeric peroxidized lipids and the mean rupture time is consistent with the experimental observation that the rupture time in Newtonian black films is correlated with the surfactant concentration (Penev et al., 1987). In Newtonian black films, a higher surfactant concentration implies a lower hole concentration and therefore a more stable film. In the present study, the peroxidized lipids are analogous to the holes discussed in other investigations (Kashchiev and Exerowa, 1980; Penev and Exerowa, 1981; Penev et al., 1987), the number of which determine the stability of the membrane. Furthermore, our results show that a critical number of peroxidized lipids exists below which not all membranes will rupture, which is also consistent with previous investigations (Penev et al., 1987).

The theory presented here differs from classical nucleation theory in several important areas. Among those are: in the current theory, the number density of peroxidized lipids is time-dependent since they are produced during the laser illumination time and are consumed by aggregation; the number of monomeric peroxidized lipids is finite and is not assumed to be significantly larger than the total number of aggregates. In classical nucleation theory the equilibrium

distribution of aggregates of size g is given by

$$N_g = N_1 \exp(-\Delta\Gamma(g)/k_B T) \tag{19}$$

where N_1 is the number of single, monomeric peroxidized lipids in the membrane at equilibrium. Since the driving force to form aggregates (say of size g) is the number density difference between the dynamic and equilibrium distribution of these aggregates, then we require the number of aggregates of size g at some time t when the number of single peroxidized lipids at equilibrium is exactly $p_1(t)$. Since we cannot assume that the total number of aggregates is significantly smaller than the number of monomeric peroxidized lipids, then the required equilibrium density of size g peroxidized lipids (at any time t) as (Courtney, 1961)

$$N_g = F\left(\frac{p_1(t)}{F}\right)^g \exp(-\Delta\Gamma(g)/k_B T) \tag{20}$$

where F is the total number of monomers and aggregates in the system. The membrane lifetime distribution is defined to be proportional to the first passage time to the formation of an aggregate large enough to cause membrane rupture. Neither transient nor steady-state nucleation theory are able to correctly represent a system in which the concentration of aggregating peroxidized lipids is changing with time. Indeed, both classes of nucleation theory base the transition to the rupture state upon an initial concentration of aggregating monomeric species. In the case presented here the initial concentration is identically zero. An essential difference between classical nucleation theory and the current theory for a finite system is that the bimolecular reaction of adding a single peroxidized lipid to an aggregate of size $g^\dagger$, which is a necessary step to form an aggregate of infinite radius, cannot occur in our system. The reason for this is that a total of p_1^+ single, monomeric peroxidized lipids exist in the membrane when the aggregate of size $g^\dagger$ is formed. To form an aggregate of size $g^\dagger + 1$ will require more than p_1^+ (for example, see Eq. 20) peroxidized lipids in the membrane at the time of rupture, a situation which cannot occur since when the membrane has one aggregate of size $g^\dagger$ it ceases to exist. It is for this reason that the term "critical peroxidized lipid size" as used in classical nucleation theory is not entirely appropriate in the context of the system of interest in this investigation.

In applying classical nucleation theory to the analysis of Newtonian black films, the probability of membrane rupture was assumed to be negative exponential in time with the rate constant, depending upon the rate of formation of aggregates of size $g^\dagger$ (Penev and Exerowa, 1981; Penev et al., 1987). Since the first passage time to an aggregate of size $g^\dagger$, and hence to membrane rupture, is obtained directly from the present technique, then further assumptions about the rupture probability are not necessary. An additional result of the current technique is the prediction that the fraction of membranes which eventually exhibit an aggregate of size $g^\dagger$ depends, in a nonlinear fashion, upon both the rate and time duration of membrane damage. The dependence of the fraction of cells ruptured upon illumination time and singlet oxygen flux as predicted by the current theory is easily tested through experiment.

The strength of the current model is that by using it to analyze experimentally observed membrane rupture time distributions, key thermodynamic parameters of the peroxidized lipids in the membrane may be obtained; namely, the triad $\{\mathcal{U}, \Delta\mu, \sigma\}$. With these three parameters, the size of the aggregate at which membrane rupture is presumed to occur, $g^\dagger$, may be predicted. However, even more valuable information may be obtained from the model once the thermodynamics of the membrane have been described. This information includes the number of lipids which must be peroxidized in order to rupture the membrane, $p_{1,\mathrm{Total}}(t)|_{t=t'_{ill}}$, and the time course of the rupture event once the singlet oxygen has damaged the membrane lipids. Future experiments, using *cis*-parinaric acid incorporated into the phospholipids of the plasma membrane (McKenna et al., 1991; Ruggiero and Hudson, 1989; Sklar, 1980) may permit a direct determination of the number of lipids peroxidized by the singlet oxygen. The predictions of the current model may then be directly tested by experimental observation.

Several items present themselves as weaknesses in the current model: first, we presume that the aggregate of size $g^\dagger$ is the site of membrane rupture, while no direct experimental evidence exists to support this assumption; second, the number of sites that may be damaged by the singlet oxygen in each lipid is unknown. However, the effect of multiple sites of damage is valuable in the model, as it incorporates a time delay between the onset of damage to the lipid and its becoming peroxidized. Once peroxidized, the lipid preferentially partitions into the type $\mathcal{B}$ phase to form aggregates. The net effect of the multiplicity of damage sites is then to include the dynamics of membrane events that are important to the rupture event. Future theoretical developments need to probe these events and account for their dynamics in predictions of the membrane rupture during photolysis; the third item which can be viewed as a weakness is that, for simplicity, the membrane is modeled as a monolayer of cylinders, with thickness equal to the bilayer. The interaction between the lipids in each layer, of the bilayer, must certainly influence the dynamics of the rupture process.

A theoretical framework correlating membrane rupture time distributions with the thermodynamics of aggregate formation has been presented. This formalism predicts that the mean membrane rupture time is inversely proportional to the predicted singlet oxygen flux provided that all membranes are ruptured during photolysis; the fraction of membranes ruptured is a function of both the singlet oxygen flux and laser illumination time; and multiple peroxidation sites exist for each peroxidized lipid formed. Furthermore, the theory presented here permits determination of the number of peroxidized lipids which must be formed to cause membrane rupture. All of the quantities obtained from the theory are based upon analysis of the membrane rupture time distributions, which are dependent upon both the singlet

oxygen flux and the laser illumination time. Furthermore, all of these quantities may be verified by independent experimental techniques. The framework presented here may be useful in optimizing clinical application of photodynamic therapy through variations in the singlet oxygen flux to the membrane as well as the laser illumination time.

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