Laser-Induced Dye Release from Liposomes
A Model for Drug Delivery and Laser Damage

Liposomes — vesicles formed by phospholipid bilayers dispersed in aqueous media — are being used in a variety of biomedical research projects, including liposome-based therapies now in clinical trials (1). Liposomes can serve as a model system for studies of structural similarities to the membranes of biological vesicles and cells. However, few quantitative studies exist that relate the action of lasers on biological tissues to adequate mechanistic models (2-4), including laser surgery (5,6). In this article we will examine the use of lasers and spectroscopic techniques with liposomes containing encapsulated or membrane-associated dyes to investigate, for example, mechanisms of photodynamic release of liposomal contents. A few reports support the selective modification or inactivation in biological systems as a result of laser irradiation of dyes (7,8). Studies with liposomes could further develop selective techniques that target specific receptors or tumor sites or that deliver anticancer, antibiotic, antifungal, antiviral, or other therapeutic and diagnostic agents.

Before liposomes can be considered clinically useful as carriers, however, a few obstacles must be overcome. These include problems associated with encapsulation, stability and clearance, targeting, and release of contents (1,9-11). Approaches under development for efficiently delivering liposomal contents include mechanisms that rely on:

- **an endocytic pathway** (12)
- **pH sensitivity** (13,14)
- **heat sensitivity**, requiring several minutes of exposure to temperatures greater than the transition temperature of the composite lipids (13,15,16)
- **photon sensitivity**, in which light is the primary activator of release (although other properties such as heat sensitivity could be operative).

A variety of photoinduced and laser-related approaches to vesicle lysis have been used with liposomes (17-34). Advantages of a photoinduced mechanism include:

- **controlled timing**; for example, in critical stages of surgical or clinical procedures
- **controlled dosage** (duration and strength)
- **differential dosage**; for example, site specificity or spatial localization of a preselected cellular subset or affected target area
- **lack of critical dependence on endocytosis** of the carrier, a property either not exhibited or well controlled in all target cells.

We have been developing a technique for controlled release using laser excitation and heating of carriers such as liposomes that incorporate dyes. Advantages of this technique compared with other photoinduced methods include:

- **much shorter exposure times**, in many cases requiring one or a few laser pulses (compared to irradiation periods of minutes or hours for other techniques), which would minimize any time-dependent instabilities, diffusion, or clearance-related drawbacks but also enhance this form of treatment in a surgical or clinical environment where much longer periods may be unacceptable
- **the fact that no ultraviolet (UV) radiation is required**, thus eliminating unwanted side effects
- **the ability to use longer-wavelength visible or near-infrared (near-IR) light**, which allows greater penetration depths than does UV.

Recently we were able to show for the first time that the contents of liposomes with encapsulated dyes could be released by direct, liposome-localized heating, which was induced by single laser pulses of nanosecond or picosecond duration (35). We now present results extending our studies with encapsulated dyes, especially comparing data obtained with membrane-associated dyes.

**EXPERIMENTAL**
Diphosphatidyl choline (DPPC), diethanol phosphate (DCP), cholesterol, and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO); sulforhodamine 640 (equivalent to SR 101) was obtained from Exciton (Dayton, OH). Liposomes were prepared by a modified solvent evaporation-rehydration tech-

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nique (36) using a mixture of DPPC/DCP (90:10 mol%) dissolved in chloroform. After rehydration in a 55°C water bath with buffered dye solution followed by cooling to 25°C, excess unincorporated dye was removed with Sephacryl G-25 (Pharmacia PD-10, Pharmacia LKB Biotechnology, Piscataway, NJ) equilibrated with the same buffer (20 mM tris, pH 7.5).

Liposome size distribution was determined by laser-based dynamic (quasi-elastic) light scattering (Nicomp model 370 particle sizing system, Santa Barbara, CA). Software for data processing included both Gaussian and multimode distribution analyses. The average diameter of unfiltered vesicles was 2.2 μm. Polycarbonate membranes (Costar/Nucleopore, Cambridge, MA) filtered smaller-diameter liposomes. Larger-diameter vesicles were selected by low-speed (<1000 × g) centrifugation. Typical standard deviations were ±0.5 μm for 2-μm vesicles and ±1.0 μm for 4-5-μm diameter samples.

Absorption spectra were measured with a Perkin-Elmer 330 UV/Vis spectrophotometer (Norwalk, CT). Emission intensities were measured with a fluorescence spectrometer assembled in our laboratory and interfaced to a microcomputer for signal processing and data analysis.

Samples for laser excitation were diluted with buffer in order to maintain the absorbance at 532 nm, at levels below ~0.06 for an irradiation pathlength of 1 cm. Samples of 50 μL were placed in cylindrical quartz tubes (2-mm i.d., 3-mm o.d.), which were then inserted into a custom-designed holder fitting into a standard 1-cm fluorescence cell. The geometry of the system allowed either pulsed laser irradiation along the long axis of the tube or transverse excitation with 90° fluorescence detection. The space surrounding the tube in the 1-cm cell was filled with deionized water to minimize any scattering losses due to surface refractive index mismatches.

Calibration of the system has shown that the same linear response range was obtained as for a standard fluorimeter. Temperature of the sample was controlled by a circulating water bath connected to an insulated metal housing surrounding the sample cell. The crystal-doubled output of a Q-switched Quanta-Ray DCR Nd:YAG laser (Spectra Physics, Mountain View, CA) provided 8-ns pulses at 532 nm with maximum energy of 75 mJ; lenses were used to focus the beam to a typical spot size of 2.5-μm diameter. For irradiation with a single 25-ps pulse at 532 nm from a Q-switched Quantel/Continuum Corp. Nd:YAG laser (Santa Clara, CA), 20 μL of sample was placed in a 1-mm-diameter tube. The picosecond pulse energy was varied from 0.75 to 7.80 nJ for a typical spot size of 1.6 mm.

Because the fluorescence yield of sulfurhodamine (SR) increases from 1% to 2% when encapsulated at 20–50 mM to 90% in free solution after vesicle breakage, the percentage release of liposome contents was quantitated by monitoring this large increase in emission intensity. The maximum (100%) release was determined by heating the entire tube in a water bath at >55°C, which produced similar results to that of disruption by addition of 0.05% surfactant (Triton X-100). Absorbance changes of methylene blue (MB) samples were measured by dilution of single or pooled irradiated samples with buffer into micro- or semi-microcells.

A frequency-doubled, continuous-wave, mode-locked Nd:YAG laser was used to pump a Spectra Physics model 3500 dye laser to provide the excitation beam for time-correlated photon counting measurements. The remainder of the apparatus was similar to that described previously (37) except that an ITT 4129 microchannel plate detector (Roanoke, VA) with a faster constant fraction discriminator (Tennelec TC454, Oak Ridge, TN) was used.

RESULTS AND DISCUSSION

We have been developing methods for preparing liposomes with dyes encapsulated in the internal volume or intercalated in the bilayer membrane (Figure 1). One goal of these studies has been to release the internal contents by pulsed laser excitation. Demonstration of this effect depends on the controlled preparation of liposomes. We have shown that this preparation depends on several interdependent variables of the liposome-dye-laser system, including composition and size of lipid bilayer, type and concentration of dye, and delivery of laser pulses. As shown in Figure 1, phospholipids form ordered liposome structures in aqueous solutions as the hydrophilic heads act to sequester the hydrophobic tails from water molecules. Depending on their chemical characteristics, dyes or other compounds become encapsulated in the aqueous interior space (for example, sulfurhodamine) or intercalated.

Figure 1. Sulfurhodamine (SR) is encapsulated in the aqueous interior space; methylene blue (MB) is intercalated within the membrane bilayer. The figure is not to scale; the width of the bilayer membrane is ~100-fold thinner than the diameter of the micron-sized vesicles.
within the membrane bilayer (for example, methylene blue).

**Encapsulated dyes.** Effective release of liposome contents was achieved by directly exciting dyes encapsulated in the internal volume. Of many candidates tested, sulfourhamidine has proven to be among the most effective, in part due to a reasonably good match between dye absorption and laser excitation wavelengths. When encapsulated in liposomes at high local concentrations (5-50 mM), the fluorescence was largely self-quenched, and an absorption band near 540 nm due to dimer formation increased dramatically compared to the 590-nm transition (Figure 2). This change was seen in the absorption spectrum but not in the excitation spectrum, indicating that a nonfluorescing complex was formed (38,39).

Disruption of vesicles by detergent or heat treatment above the critical gel-liquid crystal transition temperature (Tc) resulted in dilution of dye, loss of dimer absorption, and a large increase in fluorescence intensity. This provided us with a sensitive method for detecting the laser-induced disruption of liposomes over a wide dynamic range. Our hypothesis is that liposomes can also be released by localized heating upon dye absorption of a laser pulse where most of the excitation energy becomes deactivated by nonradiative pathways (Figure 3), due to either dimer-related quenching (for example, sulfourhamidine) or to low intrinsic fluorescence yield (for example, methylene blue).

We made fluorescence lifetime measurements to gain insight on the distribution of sulfourhamidine molecules in the interior of the liposome and membrane bilayer (35,38,39). The time-correlated photon counting technique (585- or 512-nm excitation) provided data on quenched (encapsulated) and unquenched (released) dye. Some results were corroborated independently by measurements using the phase-resolved fluorescence method for the free dye lifetime (4.2 ns) and the membrane-bound component (3.2 ns).

Fluorescence decays for liposomes were complex, requiring at least three exponential least-squares component fitting for solutions containing 20-50 mM sulfourhamidine inside 2-μm liposomes. Figure 4 compares the percentages of the components for 0.05, 20, 35, and 50 mM dye in liposomes, with measured values for the corresponding lifetimes also displayed. These data reflect the relative contributions from weak membrane-associated complexes (longest lifetime T1 component), partial quenching (intermediate T2 component), and complete quenching (shortest T3 component). The T1 component is the major fractional component at higher dye concentrations. More detailed interpretation of the liposome-dye lifetimes has been presented, in which quenching is assumed to be due to Förster energy transfer (35,39). The combined data, including results for thermal breakeage with picosecond pulses (discussed below), are consistent with the shortest decay arising from efficient quenching at picosecond or even sub-picosecond rates.

Excitation by pulsed laser radiation at 532 nm was primarily within the nonfluorescent dimer band of sulfourhamidine resulting in localized heating (Figures 2, 3). Results in Figure 5 show that a single 8-ns laser pulse at 532 nm produced significant release of liposome contents, dependent on size, internal dye concentration, and pulse energy density; nearly 100% release was achieved with 4.5-μm diameter vesicles containing 50 mM dye when the energy density exceeded 0.5 J/cm².

Diffusion losses of thermal energy deposited by laser excitation were significant within the width of the pulse. Because the rate of thermal diffusion at 25 °C in water was close to the rate of photon propagation (1.5 × 10⁷ m/s), energy could diffuse about 12 μm in 8 ns. Diffusion losses were thus substantial for micron or submicron particles, which was consistent with the clear enhancement in efficiency of release observed as the diameter was increased (with 20 mM dye, for example; 25% for 2-μm and 50% for 5-μm vesicles).

If the mechanistic basis for the data is indeed that of thermal release, delivery of laser excitation energy with shorter pulses should significantly decrease the effect of diffusion. In fact, we found that at least five times lower energy density was required to produce the same extent of release in 2-μm liposomes (20 mM encapsulated dye) with 25-ns pulses (0.3 J/cm²) compared to 8-ns pulses (1.6 J/cm²).

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cles containing 50 mM dye produced a downward shift in the curve similar to that obtained by reducing the internal dye from 50 to 20 mM (Figure 5, open squares and open circles). In addition, we also tested the effect of cholesterol, because it is known to stabilize phospholipid membranes, producing broadened melting curves and shifting the effective $T_c$ toward higher temperature with low (<33%) levels of incorporation (36). We used liposomes containing 16 mM encapsulated sulfonohoradamine with excitation conditions that produced at least 90% release for DPPC/DCP membranes (88:12 mol/mol). The extent of release was reduced to 30% when 15% cholesterol (mol/mol) was incorporated into the bilayer (data not shown).

**Model for vesicle release.** A study of malachite green linked to antibody carriers provided support for alteration of proteins by dye-mediated localized heating (37). The heating of myocardial cells stained with Janus green B was blocked by pulse laser excitation at 530 nm, which was accounted for by thermal photoinactivation (22). In order to test the feasibility of a localized heating mechanism in our system and take into account the experimental data, we initiated the development of a quantitative model for the conversion of laser excitation energy to heat through dye absorption. Since excitation at 532 nm is within the absorption band of the dimer, which is self-quenched at a nominal rate of 1 ps or faster, multiphoton cycling per dimer is easily possible, provided the laser power and the cross-section for absorption by the dye are large enough. The nominal maximum number of absorption cycles is estimated by dividing the pulse duration by the relaxation rate; for example, in a 25-ps pulse with a 1-ps relaxation rate, one could cycle 25 photons if the power were sufficient.

The minimum power density necessary to achieve the maximum number of absorption cycles can be approximated by calculating the power needed to saturate the absorption event during the lifetime of the transition. Thermal diffusion during the laser pulse can reduce the temperature rise for ns pulses. Calculations for these quantities have been presented in detail (35). They indicate that both picosecond and nanosecond pulses can provide about 25 absorption events in some of these experiments, explaining the high efficiency of liposome disruption we observed for single laser pulses. Even without a complete mathematical model incorporating detailed diffusion terms, our estimates showed that a photoinduced heating mechanism is the most likely explanation for liposome breakage and release of contents.

**Membrane-associated dyes.** The use of membrane-associated dyes may be advantageous because they require a smaller dye/lipid ratio, potentially resulting in less interference with the internal volume useful for encapsulation of the molecular cargo. Initially, the nonfluorescent triphenylmethane dyes were selected because they have a high efficiency for converting the absorbed excitation energy to heat (3); typically, we have measured the fluorescent lifetime to be 20 ps or less for dyes of this type. The brilliant blue G (or R) triphenylmethane dyes were intercalated in the bilayer membrane, apparently facilitated by the nonpolar "tail" portion of the structure, but the level of binding (10 mmol/mmol lipid, or 100:1 [mol lipid]/[mol dye]) was insufficient to produce significant localized heating and liposome release. Terbium or calcium ions were encapsulated as internal markers to monitor release of contents as measured by formation of luminescent complexes with dipicolinic acid (for Tb) or with Quin-2 (for Ca).

However, methylene blue proved to be the most successful candidate to date and is especially favorable because it has a history of approved human applications (40–43). Moreover, MB can be incorporated into liposomes at levels as high as ~10:1 (mol lipid)/(mol dye). A major shift in MB absorbance occurs when the hydrophobicity of the solvent is increased (655 nm in CHCl$_3$, 560 nm in CCl$_4$; see Figure 6). When MB is associated with the liposome membrane, there is a similar shift in the maximum of absorption from 665 nm (buffer) to 580 nm (Figure 7). These results are consistent with the introduction of MB into liposomes primarily in membrane-associated form. The release of MB from the vesicle can be quantitatively monitored by the release of an internal marker or by the changes in MB absorbance. For example, we were able to construct standard curves based on the fivefold linear increase in absorbance at 665 nm for liposome mixtures prepared to yield 0%–100% net release (not shown). Laser excitation of MB-liposomes at 532 nm resulted in a very effective release (Figure 7). Thus, with at least an order of magnitude less dye than that used with encapsulated SR (comparing [mol dye]/[mol lipid]), photoinduced release
of MB liposomes by localized thermal destabilization of the vesicle bilayer was very efficient. Clearly, the effectiveness of a single pulse depends on not only the degree of dye incorporation, but also the molar extinction at that wavelength (532 nm).

In order to understand why 100% dye release was not obtained from liposomes exposed to a single laser pulse, we irradiated liposomes containing 20 mM SR with multiple, sequential pulses at sufficient intervals to allow interpulse cooling. Two separate energy densities of the laser pulse provided two different starting values for the efficiency of dye release produced by a single pulse. Despite the greater dye release with additional pulses, the same release tended to level off after approximately five pulses; a similar response was produced upon sequential pulsing of MB-liposomes (Figure 8). For example, the efficiency per pulse decreased from 25% to 7% for irradiation of SR-liposomes with 1.7 J cm⁻² pulses, and from 7.5% to 2% for 0.8 J cm⁻² pulses. Liposome preparation used in these studies exhibited a distribution of sizes, and thermal diffusion effects could be used to interpret this data. According to our hypothesis, the only population of liposomes undergoing breakage were those vesicles exceeding a certain minimum size; thermal diffusion was expected to cool smaller sizes.

SUMMARY AND FUTURE DIRECTIONS
Our studies of laser irradiation of liposomes have demonstrated the feasibility of rapidly releasing thermally the internal contents of the vesicles by excitation of incorporated dyes. Potential applications range from targeted release of drugs to localized photothermal release of photosensitizers followed by photothermal destruction of tumor tissue. It may be feasible to establish a protocol that includes the initial release of sensitizing dye (for example, methylene blue) from the targeted liposome at the site by pulsed laser excitation, followed by subsequent acoustic irradiation to produce photothermal tumor destruction.

Of special concern is the extent to which the excitation parameters of this technique are compatible with practical usage in areas of biomedical interest. For example, although observed effects were strictly dependent on the presence of a specific (exogenous dye) absorber, in most of the experiments reported thus far, transient heating by single laser pulses was achieved with relatively high laser power density (near 1 J cm⁻²). Although this may be compatible with certain applications (such as elimination of undesirable tissue in well-defined exposed areas), in general, potential interference by nonzero background absorption will have to be taken into account.

However, even simple analysis of the data obtained thus far with a nonoptimized system suggests that a reduction in laser power density of at least 1–2 orders of magnitude would provide adequate release through 1) better matching of laser output and dye absorption and 2) use of a protocol of statistically graded release. This estimate is based on the methylene blue experiments, in which the absorbance at 532 nm was approximately eight-fold lower at the peak and where laser power was reduced, but significant release was measured with approximately five times less laser power than the maximum used (and thus, five times greater sensitivity).

We recognize that many factors contribute to an effective release mechanism, and it would seem prudent to systematically establish the optimum limits of the method by considering the most critical parameters. The quantitative basis of these parameters remains to be determined by

- developing a more extensive thermal model that accounts for absorption kinetics, activation energies, and thermal diffusion, which can also be related to general quantitative models for the thermal response to laser irradiation of tissue (5,6)
- measuring the kinetics of decomposition and release, because the most significant factors affecting release by transient heating are not well understood. For example, the activation energy (E) can be obtained from measurements of the rate (k) at different
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