Quantitative analysis at the molecular level of laser-neural tissue interactions using a liposome model system

David L. VanderMeulen

Chicago Institute for NeuroSurgery & NeuroResearch
Chicago, Illinois 60614

Prabhakar Misra and Jason Michael

Howard University, Department of Physics & Astronomy
Washington, D.C. 20059

Kenneth G. Spears and Mustafa Khoka

Northwestern University, Department of Chemistry
Evanston, Illinois 60208

ABSTRACT

The research has focused on understanding the effects of irradiating liposomes containing organic dyes with nanosecond and picosecond pulses at 532 nm from Nd:YAG lasers. Time-correlated photon counting and phase-resolved fluorescence spectroscopy techniques have been employed to obtain multicomponent fluorescence lifetimes for the dye-liposome system. A quantitative model based on the experimental data supports a photo-induced thermal mechanism for release of liposome contents.

1. INTRODUCTION

Liposomes are membrane systems that simulate vesicles, organelles and cells. Organic dyes can be encapsulated within the interior volume or bound to the membrane bilayer of liposomes[1,2]. Irradiation of the dye-liposome system induces release of the liposome contents[3,4]. Our efforts have attempted to understand how laser energy in the form of high energy pulses can be absorbed and then converted into release of dye molecules. The fundamental molecular processes involve energy transfer from both electronic and vibrational states. Such pulsed, laser-induced localized heating of liposomes[5] containing a dye become especially meaningful when other release mechanisms, e.g. endocytosis, are ineffective. Potential applications of the technique range from surgical procedures to drug delivery for the treatment of malignant tumors[6-9]. A clear understanding of the mechanics of release of liposome contents can also be applied to the assessment of laser-induced damage to cells.

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2. MATERIALS AND METHODS

Sulforhodamine 640 (equivalent to sulforhodamine 101) was obtained from Exciton; while L-α-phosphatidylcholine (DPPC) and dicetyl phosphate (DCP) were obtained from Sigma. Liposomes were prepared by a modified solvent evaporation-rehydration technique[10]. A mixture of DPPC/DCP (90:10, mol %) was dissolved in chloroform and methanol. Equal volumes of the lipid solution and the dye dissolved in 20 mM Tris buffer (pH 7.7) were rehydrated in a water bath at 55 °C. After equilibration to room temperature, the excess unincorporated dye was removed with Sephadex G-25 columns (Pharmacia PD-10) equilibrated with Tris buffer. Polycarbonate filters (Nucleopore) were used to obtain liposomes of specific size distributions.

Absorption spectra of the liposome-dye system measured with a Perkin-Elmer 330 UV-VIS Spectrophotometer showed an absorption peak at 585 nm due to the monomer together with a shoulder at 545 nm due to the dimer.

Filtered liposomes after dilution in Tris buffer were transferred to capillary tubes for pulsed laser excitation along the long axis of the tubes. Pulses of variable energy densities from frequency-doubled Nd:YAG lasers at 532 nm and of widths 8 ns and 25 ps, respectively, were used to irradiate the liposomes. The emission intensities from the disrupted liposomes following transverse excitation were measured with a fluorescence spectrometer at right angles to the exciting beam. The detection electronics was interfaced to a microcomputer for signal processing and data analysis. For each irradiated sample, the percentage release of dye was determined by comparing the fluorescence enhancement following liposome breakage with the maximum value obtained after additional heating in a water bath maintained above 55 °C.

A frequency-doubled cw mode-locked Nd:YAG laser was used to pump a Spectra Physics Model 3500 dye laser to provide the excitation beam for the time-correlated photon counting experiments. The rest of the apparatus was similar to the one described elsewhere[11] except that we used an ITT 4129 microchannel plate detector with a faster constant fraction discriminator (Tennelec TC454). The response function of the system obtained by detecting light from a scattering solution was typically 70 ps, FWHM.

3. RESULTS AND DISCUSSION

In order to understand the effects of thermal diffusion on liposome breakage (decomposition and release of contents), irradiation experiments were done with both ns and ps laser pulses. Table I below shows a comparison of the extent of liposome breakage using ns and ps laser pulses.

TABLE I

A COMPARISON OF THE EXTENT OF LIPOSOME BREAKAGE WITH NANOSECOND AND PICOSECOND PULSES

DYE CONCENTRATION (mM)	PULSE-WIDTH	ENERGY DENSITY (J/cm^2)	% RELEASE
20	8 ns	1.60	29
20	25 ps	0.32	29
50	8 ns	1.40	73
50	25 ps	0.28	73

For 20 mM sulforhodamine in 2 μm diameter liposomes, 29 % dye release was obtained with a 25 ps pulse of energy density 0.32 J/cm^2, compared to a 8 ns pulse of energy density 1.6 J/cm^2. This five-fold reduction in energy density requirement for a 25 ps pulse to obtain a given percentage release of dye as compared to the 8 ns pulse was confirmed by measuring releases for 50 mM dye in 2 μm liposomes using ns and ps pulses. These results confirm the expectation that ps pulses deposit thermal energy with substantially reduced diffusion losses as compared to ns pulses.

Fluorescence lifetime measurements were made to gain insight into the distribution of the dye molecules in the membrane bilayer and the interior of the liposome[12,13]. The time-correlated photon counting technique was used to obtain data on quenched (encapsulated) and unquenched (released) dye. These results were corroborated by independent measurements using the phase-resolved fluorescence method for the free dye lifetime and the membrane-bound component.

Figure 1 below is an illustration of the fluorescence lifetime least-squares fit for bare sulforhodamine dye (τ_f=4.21 ns) and for 50 mM dye-encapsulated liposomes (τ_1=3.439 ns, τ_2=0.193 ns and τ_3=0.054 ns).

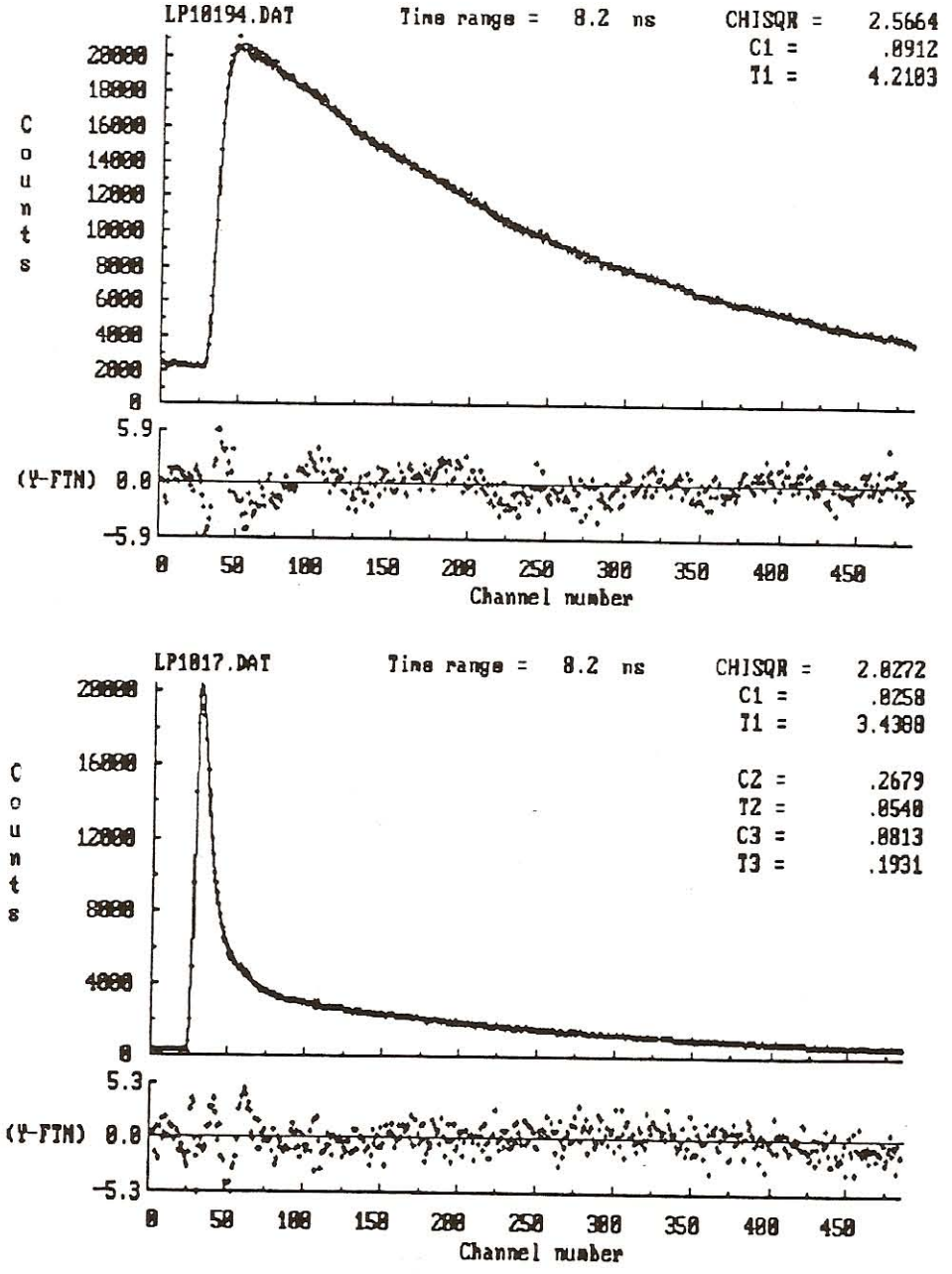

Fig. 1. Fluorescence lifetime least-squares fit for (a) bare sulforhodamine dye and (b) 50 mM dye-encapsulated liposomes.

The data were obtained using the time-correlated photon counting technique employing 585 nm excitation. Figure 2 below is a bar graph of the fluorescence lifetime data showing a comparison of the percentages of the three components for 20, 35 and 50 mM sulforhodamine dye in liposomes. Such a comparison reflects the relative contributions from dimerization (shortest lifetime τ_3 component), partial-quenching (intermediate lifetime τ_2) and membrane-intercalation (longest lifetime τ_1) processes involving the dye molecules.

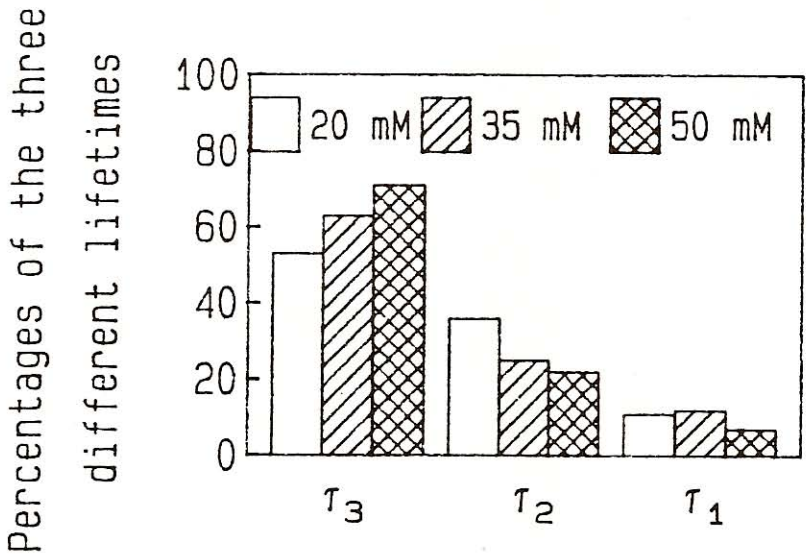

Fig. 2. A comparison of the relative percentages of the three lifetime components for 20, 35 and 50 mM sulforhodamine dye in liposomes.

At higher dye concentrations, the shortest lifetime component is the major fractional component. For 50 mM encapsulated dye, τ_3=0.054 ns gives a value for the quantum yield of fluorescence QY=(0.054/4.21)=0.013, since τ_f=4.21 ns for free sulforhodamine dye.

Owing to the presence of two separate absorption maxima near 545 nm and 585 nm for the dye-liposome system, both 532 nm and 585 nm excitations were used for fluorescence lifetime measurements. The results indicate little difference in either the absolute lifetime values of the three components or their relative percentages.

Figure 3 below shows a comparison of the results from the two separate excitations in the yellow and green regions of the electromagnetic spectrum.

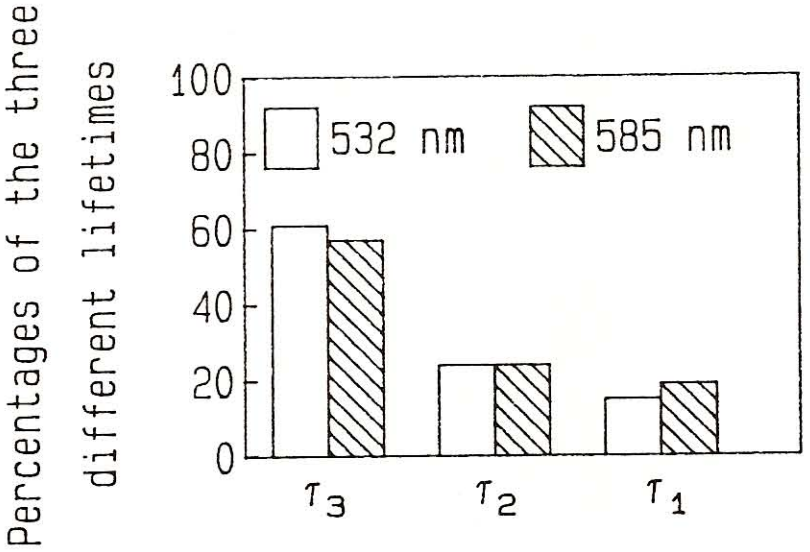

Fig. 3. A comparison of the relative percentages of the three lifetime components for 20 mM dye in liposomes with 532 nm and 585 nm excitation.

In view of the fact that the dimer (which gives the 545 nm absorption band) is non-fluorescent, it is expected that the dye molecules promptly relax back to the same singlet ground state level regardless of whether the excitation wavelength is 532 nm or 585 nm.

For 532 nm laser excitation, each photon carries an energy of 3.7×10^{-19} J. The thermal energy of deposition E can be calculated from the equation $E = nc_p dT$, where n is the mass of intravesicular water, c_p =4.18 J/(g)(°C) is the specific heat of water and dT is the temperature change following photon absorption. For 2 μm diameter liposomes, using $n = 4.19 \times 10^{-12}$ g, there is a temperature rise of 2.7 °C of the liposome, assuming an absorption of one photon per dye molecule. This computation indicates that to raise the average local temperature of the system from 25 to 50 °C ($>T_c$ = 41 °C) there is a need for cycling about 10 photons per dye molecule in the case of 50 mM dye. A similar calculation for 20 mM dye indicates a cycling rate of 25 photons per dye molecule.

In view of the fact that the excitation at 532 nm is within the dimer absorption band and the dye is largely self-quenched, a fast relaxation is expected and has also been measured. During the 8 ns pulse, there are (8/0.054)=148 cycles possible provided the laser power and absorption strengths are of adequate magnitude. Using a fluorescence lifetime component τ_3=0.054 nsec, an absorption cross section $\sigma = 1 \times 10^{-16}$ cm^2 and a photon energy of 3.7×10^{-19} J, the saturation power density is 6.85×10^7 W/cm^2. A laser energy density of 1 J/cm^2 for a 8 ns pulse translates to a power density of 1.25×10^8 W/cm^2, whereby multiple absorptions can be achieved per dye molecule. Energy losses due to thermal diffusion must be taken into account. Simulating the diffusion effects by using the speed of sound as 1.5×10^5 cm/s and a representative liposome diameter of 1.5 μm yields for the characteristic diffusion time a value of 1×10^9 s, whereby the 8 ns pulse would be too long for efficient heating. It follows that the number of photons per dye molecule required for the anticipated diffusion time is less than the maximum possible number for the laser-liposome-dye system. Assuming that the above reasoning establishes an upper limit for the computed values, a localized heating mechanism is the best explanation of the liposome breakage data based on the available results.

Our results are very encouraging because they allow one to relate laser-associated parameters to membrane-model systems like liposomes and allow extension to related studies involving laser irradiation of tissue. It also paves the way for the development of a comprehensive quantitative model for the conversion of laser excitation energy to heat via absorption by dye molecules and other species in liposomes.

4. ACKNOWLEDGMENTS

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