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Simulation and Modeling of Laser-Tissue Interactions Based on a Liposome-Dye System

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Abstract: This work presents an overview of the use of liposomes for targeted delivery of photosensitizers to tumors for Photodynamic Therapy (PDT). It assesses the results of a quantitative model to explain the interaction of short-pulsed lasers (in the nanosecond and picosecond domains) with a liposome-dye complex in terms of a localized photo-induced thermal mechanism. Incorporation of an organic dye (sulforhodamine) within lipid vesicles has been investigated in conjunction with the effect of laser irradiation on the integrity of the liposome-dye complex. The variation of the absorption coefficient as a function of wavelength for dye-encapsulated liposomes before and after laser-induced release of dye was studied and modeled. The commercial software *Mathematica* was used to develop a Gaussian model for the energy absorption by the liposome-dye complex. Dye release from 3 μm - liposome encapsulating 25 mM aqueous solution of sulforhodamine dye was studied using 8 ns laser pulses at the second harmonic of the Nd:YAG laser (at 532 nm) and compared with dye release employing 25 ps - laser pulses. In addition, the temperature-dependence of the dye release has been included in the photo-thermal model.

Keywords: Liposome, PDT, laser, dye, sulforhodamine, tumor, photosensitizer, singlet oxygen.

1 Introduction

The use of lipid vesicles as carriers for introducing biologically active materials into cells *in vitro* and *in vivo* is an exciting and rapidly growing area of research in biology and medicine [Gregoriadis and Allison (1980); Feigner (1993); Nelson, Borland, Allbritton and Sims (2007)]. Liposomes (see Fig.1) are artificial tiny vesicles (bubbles) formed with lipid concentric bilayers possessing aqueous compartments. They are obtained from a broad variety of lipid amphiphiles, the most common of which are the phospholipids. In nature, phospholipids are components of cell membranes. Phospholipids are molecules that have both a head and a tail group. In the presence of water, the hydrophilic heads are attracted to the aqueous side and align to form a surface facing the water. The hydrophobic tails are repelled by water, and align to form a surface away from the water. In a cell, one layer of heads faces outside of the cell, attracted to the water in the environment; another layer of heads faces inside the cell, attracted by the water inside the cell. The hydrocarbon tails of one layer face the hydrocarbon tails of the other layer, and the combined structure forms a bilayer [Torchilin (2006)]. When the phospholipids are in the presence of an aqueous solution, they organize in such a way as to minimize the interaction between their hydrocarbon chains and the water molecules. Interestingly drug is enclosed in the aqueous interior of the liposome (e.g. Fig. 1). Liposomes can be formulated for drug delivery through the intravenous or transdermal route for therapeutic use. They can be nano- and micron-sized particles that possess a broad spectrum of useful characteristics, such as prolonged half-life in blood plasma, which allows for their accumulation in pathological areas with compromised vasculature, intracellular penetration suitable for targeted delivery of drugs or magnetic resonance contrast agents [Torchilin (2006)]. Since dye encapsulated liposomes permit targeted delivery of

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photosensitizers to tumors and other pathological sites, it is important to study the effects of laser irradiation on liposomal dye complexes. In view of the structural similarities of liposomes to membranes of biological cells, the response of such a system to laser pulses has important ramifications for PDT applications.

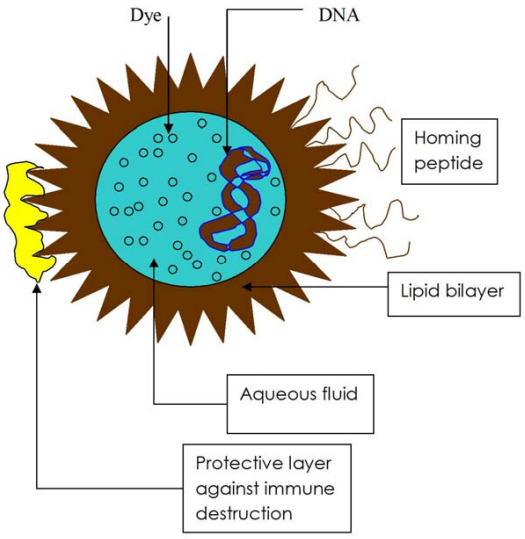

Figure 1: Schematic of a liposome.

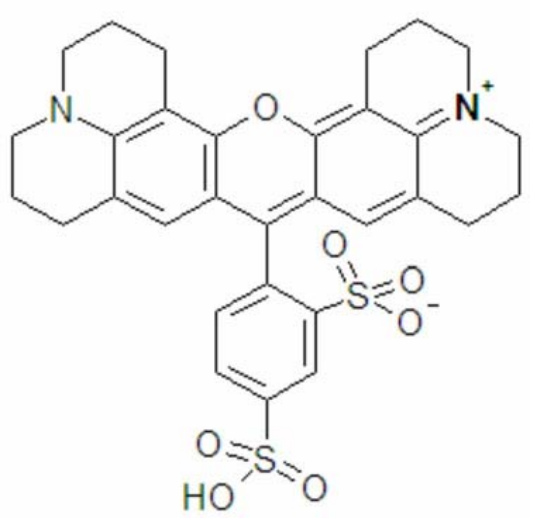

Figure 2: Structural formula of Sulforhodamine SR 101 with molar mass 606.71 g/mol and molecular formula $C_{31}H_{30}S_2N_2O_7$.

2 Literature Review

There have been significant advances in the use of liposomes for targeted delivery of photosensitizers to tumors for PDT applications. We provide here a brief account of the different aspects of PDT as they relate to the use of liposome delivery systems. In PDT, the choice of dye as a sensitizer is very important. Fig. 2 shows the molecular structure of sulforhodamine 101, which is a water soluble dye used in the present study. Some dyes are water-soluble (hydrophilic), whereas others are oil-soluble (lipophilic). Water-soluble dyes are usually charged molecules and do not cross cell membranes, whereas oil-soluble dyes cross cell membranes. Water soluble dyes encapsulated in liposomes can enter the cell through endocytosis [Straubinger, Papahadjopoulos, and Hong (1990); Tempone, Perez, Rath, Vilarinho, Mortara, and Franco de Andrade (2004)]. The chief idea in PDT is to load the tumor with dye molecules, which can absorb radiation at the appropriate wave length to generate excited dye molecules. Transfer of energy from the excited dye molecules to ground state triplet oxygen produces the highly reactive singlet oxygen species, which cause damage to biologically important molecules in the cell. The depth of penetration of tissue by light is enhanced at longer wavelengths and it is important that the wavelength(s) used should also be absorbed by the dye molecules. One problem with intravenous injection of the free dye solution is that the dye will most likely bind with serum proteins and will not reach the tumor. Another problem is that charged water-soluble dye molecules are less likely to cross cell membranes and accumulate inside tumors. Since liposomes can enter the cell *via* endocytosis, it is beneficial to encapsulate the dye into the liposomes. However, liposome may not last long enough in circulation and may be excreted before appreciable accumulation in the tumor is achieved. Extensive research has led to the development of specialized tumor targeted liposomes with improved plasma half-life and dye encapsulation efficiency. Water-soluble dye molecules encapsulated in liposomes are useful only if they reach the tumor and are subsequently released in-

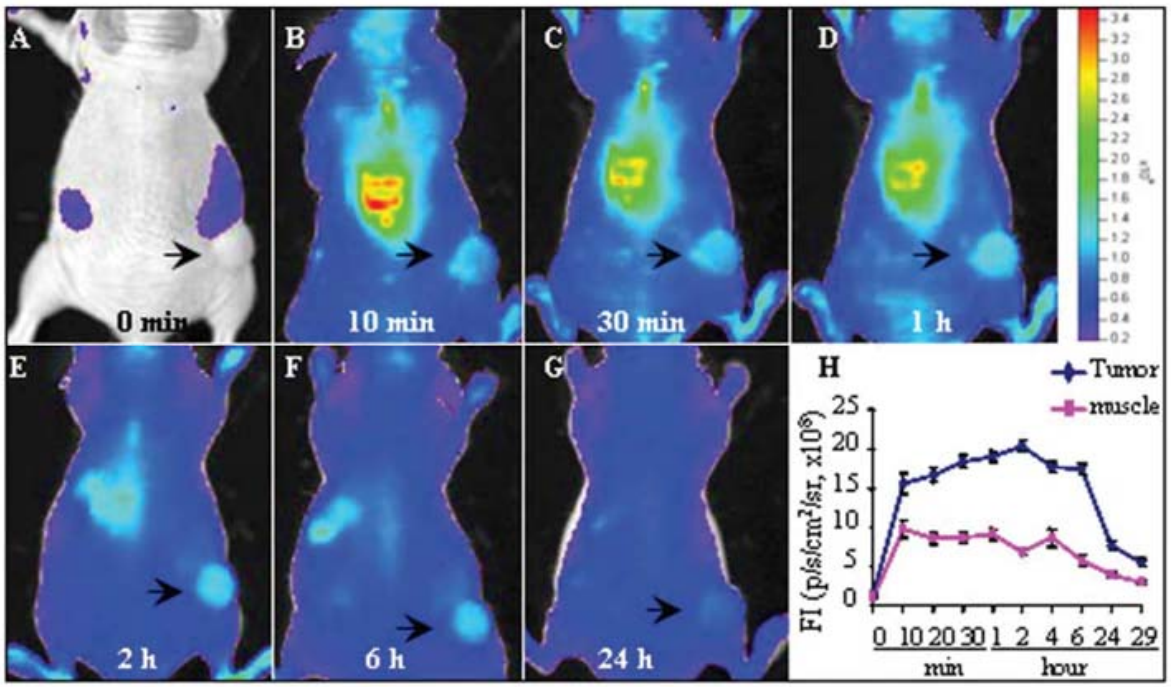

Figure 3: Optical imaging of the tumor after intravenous administration of transferrin-liposome-nitrobenzoxadiazole-contrast agent (TfNIR- LipNBD-CA) showing preferential accumulation of fluorescent signal in tumors (A–G). H shows the plot of signal intensity versus time obtained from the tumor and the contralateral muscle. The signal intensity is expressed in p/s/cm^2/sr. [Ref. Shan, L.; Wang, S.; Sridhar R.; Bhujwalla, Z. M.; Wang, P. (2007): Dual Probe with Fluorescent and Magnetic Properties for Imaging Solid Tumor Xenografts. *Molecular Imaging*, vol. 6, no. 2, pp. 85-95. Reproduced from Molecular Imaging, 2007, with permission.]

side the tumor. Ideally, the goal is to introduce the dye inside the tumor; however this may not always happen. In fact, what is needed is for dye to be inside the tumor vasculature, so that the latter is destroyed by PDT. It is also important to point out that in case of a hydrophobic dye, there is a tendency for the dye molecules to aggregate upon release from the liposomes. Aggregated dye molecules are not very efficient photosensitizers. Thus, the choice of the dye is critical in obtaining high quantum efficiency for PDT applications. A better practice is to irradiate with light or laser radiation when liposomes have accumulated in the tumor and the dye has been released. When laser light impinges on the dye, singlet oxygen may be formed – if oxygen is present. Singlet oxygen is highly reactive and damages unsaturated lipids, proteins, DNA and RNA; it also triggers the immune system response that attacks the tumor. Liposomes labeled with a near infrared fluorescent dye have been utilized for targeted delivery of a magnetic resonance contrast agent for *in vivo* imaging of solid tumors in mice [L. Shan, S. Wang, R. Sridhar, Z.M. Bhujwalla and P. Wang (2007)] and the results are summarized in Fig. 3. The fluorescence signal was detectable as early as 10 minutes after injecting liposomes via the tail vein, and the signal intensity reached a maximum after about 2 hours and decreased gradually thereafter.

Photodynamic therapy (PDT) is a minimally invasive cancer treatment modality that can be used for treating superficial tumors including brain tumors [Juarranz, Jaén, Sanz-Rodríguez, Cuevas and González. (2008)]. Advantage of PDT over other conventional cancer treatments is its low

systemic toxicity and its ability to selectively destroy tumors accessible to light. Therefore, PDT is being used for the treatment of endoscopically accessible tumors, such as lung, bladder, gastrointestinal and gynaecological neoplasms, and also in dermatology for the treatment of non-melanoma skin cancers (basal cell carcinoma) and precancerous diseases (actinic keratosis). In PDT, a sensitizer, light, and oxygen are used to bring about photochemically-induced cell death. The mechanism of cytotoxicity involves generation of singlet oxygen and free radicals. Although selective retention of a sensitizer by malignant tissue is observed *in vivo*, the mechanisms for this sensitizer targeting require further investigation. The first-generation sensitizers are porphyrin-based and vary in lipophilicity and hydrophilicity. Targeting of the vasculature seems to be a prominent feature of the cytotoxic effect of these sensitizers *in vivo*, with resulting necrosis. Treatment depth varies with the wavelength of light that activates the sensitizer used, and the second-generation sensitizers are activated at longer wavelengths, allowing for a 30% increase in treatment depths. The selectivity of targeting can be increased when the sensitizer is delivered with the use of liposomes or monoclonal antibodies specific for tumor antigens [Pass (1993)].

A wide range of molecules have been tested as photosensitizers for photochemical eradication of cancer cells. There is ongoing research to improve the safety and efficacy of PDT for intracranial tumors. Photosensitizer, light and oxygen are the cornerstones of PDT. Singlet oxygen is the major reactive species that is produced when conditions are optimized with respect to these three variables. In the ideal scenario for PDT, the sensitizer will selectively accumulate in the tumor. Excitation of the sensitizer by visible light of 600 nm or higher wavelength (because of better penetration of tissues by light in this range) in the presence of oxygen results in the formation of singlet oxygen as the primary product along with other reactive oxygen species and free radicals as secondary products. The antitumor effects of PDT are attributed to singlet oxygen, which is cytotoxic to normal and neoplastic cells and damages tumor vasculature. There is the additional possibility that singlet oxygen triggers the immune response against tumor cells [Hilf (2007); Gracanin, Hawkins, Pattison and Davies (2009); Kalyanaraman, Feix, Siebert, Thomas and Girotti (1987)].

3 Materials and Methods

In the present work, we have developed a quantitative model to explain the interaction of short-pulsed lasers (in the nanosecond and picosecond regimes) with a liposome-dye complex in terms of a localized photo-induced thermal mechanism. These quantitative studies can be extended to understand the action of short-pulsed lasers on biological tissues in terms of a mechanistic equation-based model by either adapting commercially available finite-element software or developing the required source code and performing the necessary computer simulations and numerical analysis. Feasibility studies and validation of the numerical and theoretical approaches can be done through predictions and assessment of appropriate optical photo-chemical and thermal parameters available in the open scientific literature and via our measurements on the liposome-dye complexes. Liposomes may contain small amounts of other molecules. Though liposomes can vary in size from low micrometer range to tens of micrometers, unilamellar liposomes, as pictured here, are typically in the lower size range with various targeting ligands attached to their surface allowing for their surface-attachment and accumulation in pathological areas for treatment of disease.

Details regarding the liposome-dye preparation and laser irradiation at 532 nm using 8 ns and 25 ps pulses have been given elsewhere [Vander-Meulen, Spears and Misra (1993); Misra, Holt and Misra (1993); Misra and Singh (2003)]. Fluorescence from laser-disrupted liposomes passed orthogonally through a monochromator and was detected by a photomultiplier tube (PMT) used in tandem with a pico-ammeter (Fig. 4.) Output from the picoammeter was relayed to an A/D converter and fed to a microcomputer for signal processing, data storage and subsequent analysis.

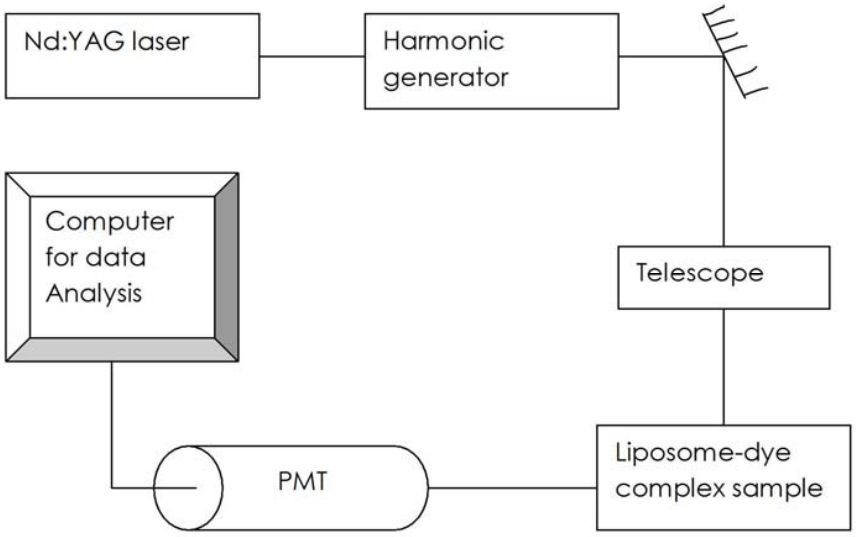

Figure 4: Block diagram of experimental set up to study laser irradiation of liposome-dye complex.

4 Results and discussion

Fig. 5. shows the energy level diagram of a typical organic dye molecule (such as sulforhodamine 101 shown in Fig. 2). Various energy transfer processes - including collisional quenching - are shown in Fig. 5. Absorption, fluorescence, and phosphorescence processes occur between the singlet ground state and the excited singlet and triplet states following irradiation of the dye molecules. Other processes and relationships between various decay rates, namely the observed fluorescence decay rate (K_f), the radiative decay rate (K_r), and the collisional quenching rate (K_q), are also indicated in Fig. 5.

4.1 Quenching due to collisional process

In PDT, a photosensitizer is administered to the patient. The tissue to be treated is exposed to light suitable for exciting the photosensitizer from the ground singlet state (S_0) to an excited singlet state (S_1), which in turn undergoes intersystem crossing to a long-lived excited triplet state (T_1). An important chemical species present in tissue with a ground triplet state is the molecular oxygen, which when in close proximity to the photosensitizer causes an energy transfer that enables the photosensitizer to relax back to its ground singlet state, and create an excited singlet state oxygen molecule ($^1O_2$). Singlet oxygen aggressively and rapidly reacts with nearby biomolecules and causes cell killing through apoptosis or necrosis. PDT is useful for the treatment of certain skin diseases and cancer.

Table 1 summarizes the different values of the absorption obtained with 20 mM sulforhodamine encapsulated in the liposome before and after thermal release.

The samples used for studying energy absorption were not homogeneous. This precludes the application of Beer-Lambert-Bouguer law. The concentration of aqueous dye solution was 20 mM. This solution was encapsulated in liposomes. The liposome preparation in buffer was suspended in a quartz cuvette. The actual path length through the dye solution depends on the liposome concentration and the diameter of the encapsulated region. Therefore, no attempt was made to determine the extinction coefficient.

Data in Table 1 were obtained from Fig. 6., which shows the absorption spectra of the dye-liposome complexes recorded with a UV-VIS spectrometer. It showed a prominent peak at 585 nm and a shoulder at about 545 nm. The shift in spectra after irradiation is due to the formation of dimers

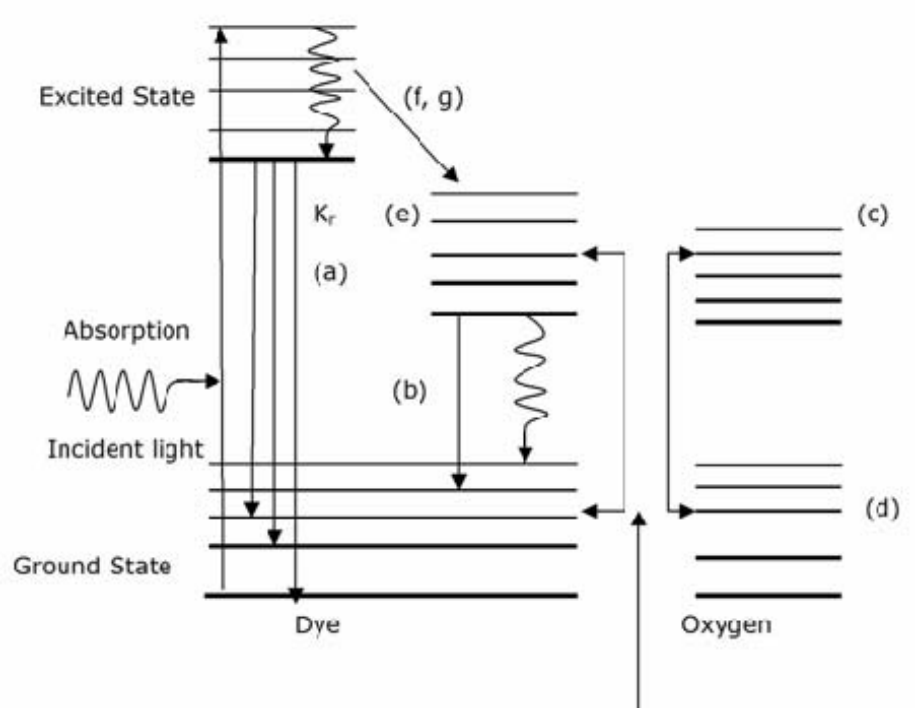

Figure 5: Energy-level diagram for a typical organic dye molecule. Legend: (a) Fluorescence; (b) Phosphorescence; (c) $^1O_2$(Singlet Oxygen); (d) $^3O_2$(Triplet Oxygen); (e) Excited state T_1 (triplet); (f) Radiationless decay; (g) K_x (Electronic energy may be carried away by the medium as thermal energy).

of the dye molecules, while the strong transition arises due to the dye monomers. The curve with circle data points is due to the absorption spectrum of 20 mM sulforhodamine encapsulated in liposomes suspended in aqueous buffer, while the curve with square data points is the spectrum of the dye following thermal release. For dye concentrations above 1 mM, the sulforhodamine encapsulated in liposome exhibits the dimer absorption band around 545 nm, which shows strong quenching of fluorescence. The ratio of the dimer to monomer absorbance increases with rising internal dye concentrations (from about 0.33 for dilute solutions (less than 1 mM) to 0.7 for 1mM sulforhodamine in liposome. The commercial *Mathematica* software was used to obtain a Multiple Gaussian model for the energy absorption by the liposome-dye complex. The Multiple Gaussian model has been used in the past for modeling absorption spectra [Bannister and Wood (1972); Zucchelli, Dainese, Jennings, Breton, Garlaschi, and Bassi (1994)]. We note the strong correlation (r$\sim$1) between the experimental data (the circles and the squares) and the fitted plot (solid lines).

Fig. 7. shows the absorption energy states during energy emission relative to the change in states from a wavelength of 532 nm to a wavelength of 600 nm. Different energy densities and their corresponding percent release (see Table 2) can be observed (see Fig. 8 and Fig. 9). Fig. 9 gives a comparison of the dye release following irradiation with 8 ns and 25 ps laser pulses (both at 532 nm), respectively. With the pulse frequency associated with 8 ns the dye percent release is almost constant. The curve is a line almost parallel to the x-axis, which here is the energy density axis. For a pulse frequency associated with 25 ps one observes the rise in the dye percent release, which gave rise to a sigmoid curve.

Fig. 8 illustrates the laser-induced release of 35 mM sulforhodamine dye from $3\mu m$ liposomes us-

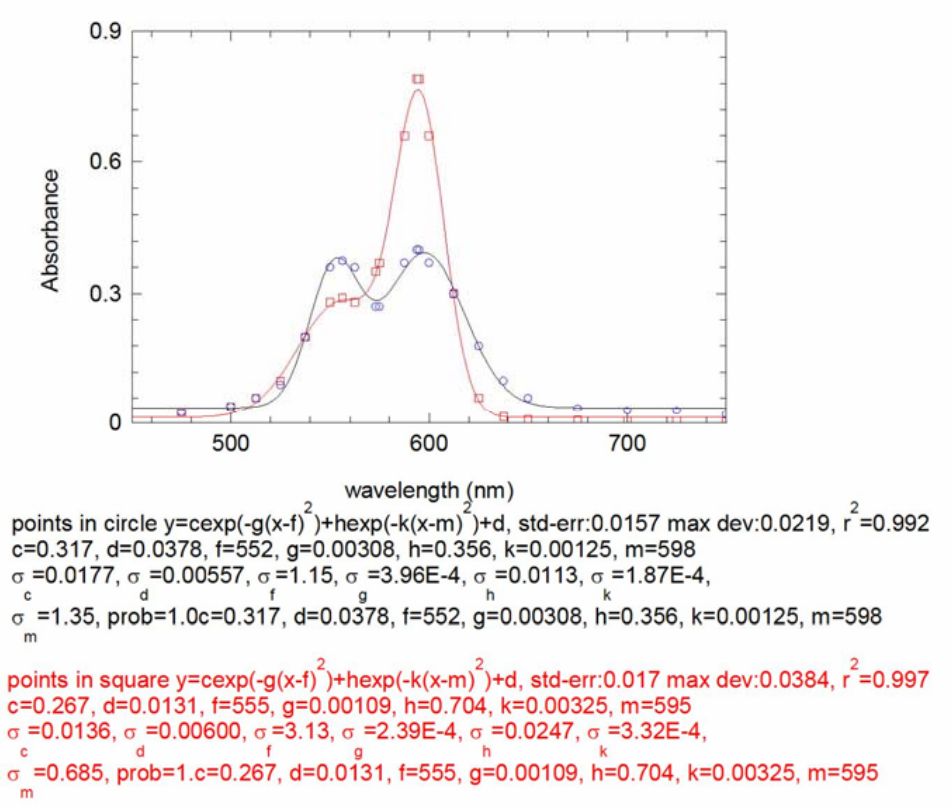

Figure 6: Absorption of energy by the liposome-dye complex as a function of wavelength fitted with a multiple-Gaussian distribution.

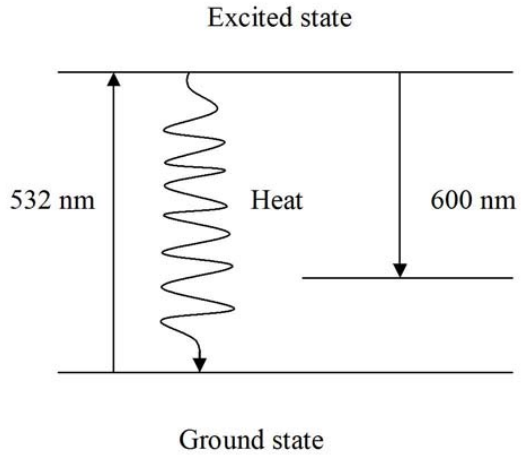

Figure 7: Emission of light and thermal energy by the liposome-dye complex.

ing 8 ns pulses at 532 nm. The function Q representing the percent release of dye in relation to increase in energy density helps model the laser-induced release of dye using the Mathematica version 7.0 software.

We also note that increased temperature influences the enhancement of the dye release. As the temperature increases from 8^oC to 25^oC (see Fig. 10.), the percentage of dye released also increases, reaching a value in excess of 90% at an energy density of 2 J/cm^2.

5 Thermal and mathematical modeling

5.1 Liposome excitation and associated laser parameters

For the liposome excitation with the 532 nm laser, each photon carries an energy equivalent of $E_0 = 3.7 \times 10^{-19} J$. If m is the mass of the intravesicular water (within, say the$3\mu m$-diameter liposome) - estimated to be about $1.8 \times 10^{-12} g$, ΔT is the change in temperature caused by the pho-

Table 1: Absorption (arbitrary units) of light as a function of wavelength for 20 mM sulforhodamine dye encapsulated in the liposome and the absorption following the thermally induced release of dye from liposomes.

Wavelength (nm)	Absorption	Absorption after release
475	0.025	0.025
500	0.04	0.04
512.5	0.06	0.06
525	0.09	0.1
537.5	0.2	0.2
550	0.36	0.28
556.25	0.375	0.29
562.5	0.36	0.28
573	0.27	0.35
575	0.27	0.37
587.5	0.37	0.66
593.75	0.4	0.79
595	0.4	0.79
600	0.37	0.66
612.5	0.3	0.3
625	0.18	0.06
637.5	0.1	0.015
650	0.06	0.008
675	0.035	0.006
700	0.03	0.005
725	0.03	0.005
750	0.02	0.004

ton absorption, and $c_p = 4.18 J/g.^oC$ the specific heat of water at constant pressure, then the thermal energy absorbed at $25\,^\circ C$ is approximated by $E = mc_p\Delta T = 1.9\times 10^{-10} J$. If n is the number of photons produced via 532 nm laser excitation, then we know that
$n = \frac{E}{E_0} = \frac{1.9\times 10^{-10} J}{3.7\times 10^{-19} J/photon} = 5.13\times 10^8 photons$. As a result, it is worthwhile to confirm that at $25\,^\circ C$ about $5.13\times 10^8 photons$ are estimated to be absorbed and converted to heat.

For 8 ns laser pulse energies < 80 mJ at 532 nm, with say $1.5\,\mu m$-diameter liposomes, and $1 J/cm^2$pulse energy density:

1. Absorption cross section for dye: $\sigma_{abs} = 1\times 10^{-16} cm^2$
2. Incident laser intensity $I_0 = 1 J/cm^2$
3. Energy absorbed by dye: $I_0\,\sigma_{abs} = 1 J/cm^2 \times 1\times 10^{-16} cm^2 = 1\times 10^{-16} J$
4. Number of photons cycled per dye molecule is estimated to be $n = \frac{1\times 10^{-16} J}{3.7\times 10^{-19} J/photon} \approx 250 photons$

5.2 Gaussian and Exponential Curve Fitting

In Fig. 6., the graph is fitted with a pair of Gaussian functions: $y = F(x) = c\exp[-g(x-f)^2] + h\exp[-k(x-m)^2] + d$. It is interesting to observe that $\lim_{x\to+\infty} F(x) = d$ is chosen, such that as x tends to infinity, so that the fitting function $F(x)$ does not tend to zero, but instead to a real number different from zero. The maximum of the first Gaussian $c\exp[-g(x-f)^2]$ occurs at $x = f$ and the one for the second Gaussian $h\exp[-k(x-m)^2$ occurs at $x = m$. The standard deviation has been obtained for each constant parameter used in the fitting function. In generating Figs. 8, 9, and10, the same procedure has been used. The curves were fitted by the exponential functions and the standard deviations determined for each constant parameter involved in the fitting function.

Table 2: Percent release of 35 mM sulforhodamine dye from 3 μm liposomes with 8 ns laser pulses at 532 nm

Energy density (J/cm^2)	**Percent dye release**
0	0
0.25	5
0.50	15
0.75	45
1.00	67
1.25	75
1.50	76
1.75	78
2.00	79

6 Conclusions

Modeling and simulation of laser-tissue interactions is a crucial step towards a better understand-

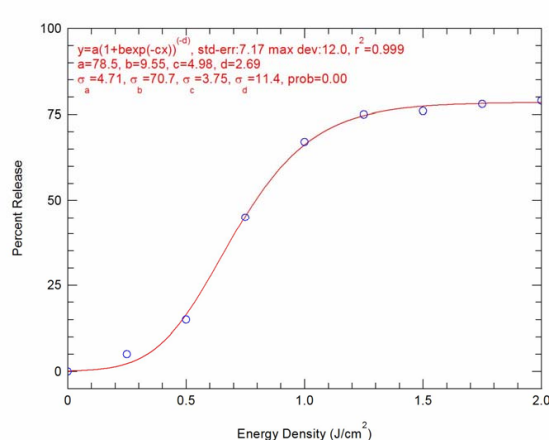

Figure 8: Laser-induced release of 35 mM sulforhodamine dye from $3\mu m$ liposomes using 8 ns laser pulses at 532 nm.

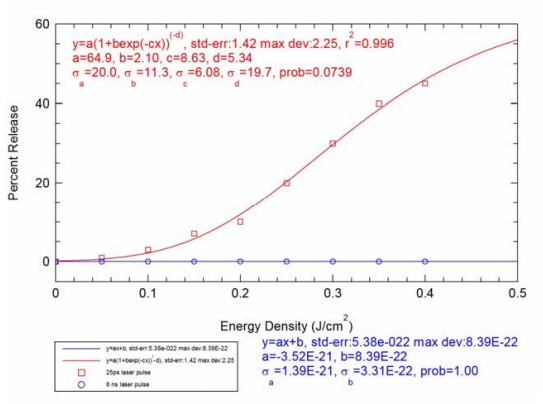

Figure 9: Comparison dye release between 8 ns (circles) and 25 ps (squares) laser pulses at 532 nm modeled using *Mathematica.*

ing of the effects lasers have when interacting with cellular membranes of animal/human origin, as well as artificial membranes that mimic cellular material. In this work we have encapsulated sulforhodamine dye as a photosensitizer within liposomes to form liposome-dye complexes. The interaction of lasers with liposomal formulations of sensitizers is fairly complex. If the free photosensitizer is present inside the tumor cell when light absorption occurs, then the singlet oxygen will be produced inside the cell and causes cell damage. If light absorption occurs when the sensitizer is still encapsulated within the liposome, sin-

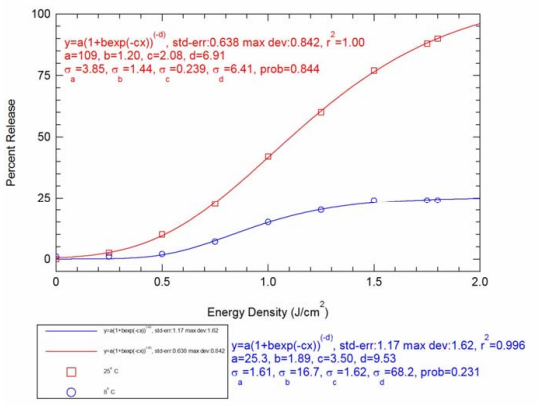

Figure 10: Temperature-dependence of dye release upon 532 nm laser irradiation of liposomes loaded with 50 mM dye (SR 101) in the $3\mu m$ diameter aqueous core simulated using *Mathematica*.

glet oxygen may be produced - but localized damage to the liposome membrane may occur without causing significant damage to the cell itself. Therefore, it is important to know the kinetics of release of the photosensitizer from the liposome in the absence of light absorption and also after exposure to the laser radiation. At present, it is not quite clear if the breakdown products of the liposome membrane, resulting from the attack of singlet oxygen on unsaturated lipids and cholesterol, affect the photosensitizing properties of the pure photosensitizer. Additional experimentation and modeling are required to elucidate such phenomena.

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