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Simultaneous Two-Photon Excitation of Photodynamic Therapy Agents*

E.A. Wachter[a,b], W.P. Partridge[a], W.G. Fisher[a,b], H.C. Dees[b], and M.G. Petersen[c]

[a]Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831
[b]Photogen, Inc., 7327 Oak Ridge Highway, Knoxville, Tennessee 37931
[c]The University of Tennessee College of Veterinary Medicine,
Department of Small Animal Clinical Sciences, Knoxville, Tennessee 37901

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DISCLAIMER

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[a] Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831
[b] Photogen, Inc., 7327 Oak Ridge Highway, Knoxville, Tennessee 37931
[c] The University of Tennessee College of Veterinary Medicine, Department of Small Animal Clinical Sciences, Knoxville, Tennessee 37901

ABSTRACT

The spectroscopic and photochemical properties of several photosensitive compounds are compared using conventional single-photon excitation (SPE) and simultaneous two-photon excitation (TPE). TPE is achieved using a mode-locked titanium:sapphire laser, the near infrared output of which allows direct promotion of non-resonant TPE. Excitation spectra and excited state properties of both type I and type II photodynamic therapy (PDT) agents are examined. In general, while SPE and TPE selection rules may be somewhat different, the excited state photochemical properties are equivalent for both modes of excitation. In vitro promotion of a two-photon photodynamic effect is demonstrated using bacterial and human breast cancer models. These results suggest that use of TPE may be beneficial for PDT, since the technique allows replacement of visible or ultraviolet excitation with non-damaging near infrared light. Further, a comparison of possible excitation sources for TPE indicates that the titanium:sapphire laser is exceptionally well suited for non-linear excitation of PDT agents in biological systems due to its extremely short pulse width and high repetition rate; these features combine to effect efficient PDT activation with minimal potential for non-specific biological damage.

Keywords: Photodynamic therapy, PDT, two-photon excitation, TPE, photochemistry, spectroscopy, titanium:sapphire.

1. INTRODUCTION

A broad variety of photosensitive pharmaceutical compounds have been developed that may be activated using light in the visible to near infrared (NIR) spectral regions.[1,2] For treatment of disease, these compounds are typically administered, either systemically or directly to diseased tissue, then the diseased area is subsequently irradiated with light suitable for activation of the photodynamic process, yielding photodynamic therapy (PDT). This irradiation may be effected using a variety of optical sources, ranging from arc lamps to light emitting diodes to lasers, and the irradiation process itself may be performed via direct illumination of the surface of the patient or using a catheter to illuminate internal locations. Selection of an appropriate wavelength for activation is primarily controlled by two parameters: the intrinsic excitation spectrum of the PDT agent, and the intrinsic optical properties of tissue.[3] Consequently, the unique combinations of optical properties for PDT agent and of various tissues has lead to the development of specific regimens for treatment of certain diseases. For example, various psoralen derivatives exhibit strong type I PDT response (conversion of the PDT agent into a cytotoxic product) upon activation using ultraviolet light (ie, 350-400 nm). Since skin strongly absorbs light in this band (Figure 1), psoralens have come to be used for treatment of a number of superficial skin afflictions, such as psoriasis. In contrast, porphyrin derivatives typically exhibit type II PDT response (photocatalytic production of a cytotoxic product, such as singlet oxygen) upon activation using red or NIR light (ie, 600-750 nm). Since tissue does not significantly absorb light at these wavelengths, this family of agents have come to be used for treatment of a number of subsurface afflictions, including metastatic cancer of the liver and breast.

Further author information –
E.A.W. (correspondence): Email: wachterea@ornl.gov; Telephone: 423-574-6248; Fax: 423-576-2078

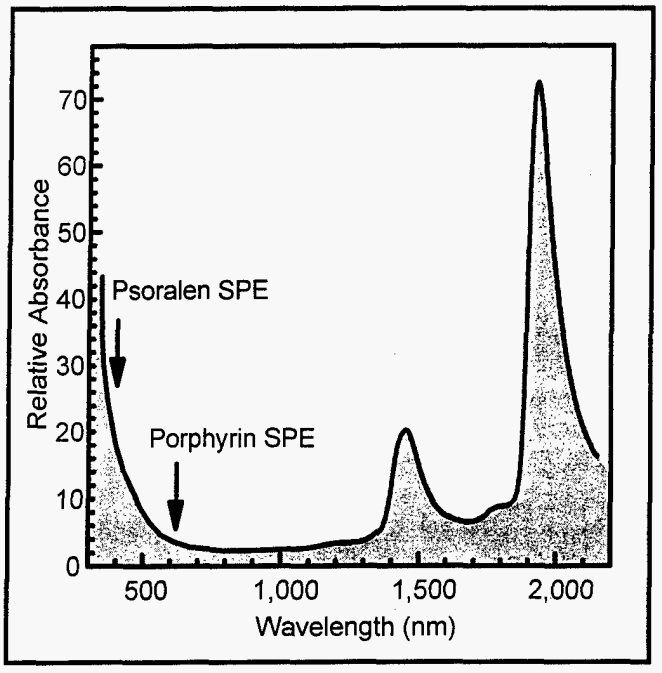

Figure 1. Example absorbance properties for human tissue at UV to NIR wavelengths. Activation of psoralens using single-photon excitation (SPE) occurs principally in the UV, limiting application to topical lesions due to strong absorption of this light by skin and sub-dermal tissue. Highly penetrating red or NIR light used in conjunction with porphyrins allows activation of these agents in deeper lesions, facilitating use in the treatment of subsurface malignancies.

In an effort to increase the range of indications and effectiveness of PDT, considerable investment has been made in development of photosensitive agents that provide efficient activation at wavelengths above 600 nm.[4] As a result of this trend, the current generation of PDT agents, such as 8-methoxypsoralen (or 8-MOP, which is commonly activated using UVA light from a mercury lamp) and Photofrin II (which is commonly activated using a minor absorbance band at 630 nm) may eventually be supplanted by agents offering relatively large SPE cross-sections in the NIR, including benzoporphyrin derivative mono-acid (BPD-MA) and tin etiopurpurin (SnET2).

As an alternative to development of new agents having longer activation wavelengths, new excitation methods might be used to allow activation of existing agents using NIR light. For example, the mode-locked titanium:sapphire laser represents an excellent source for direct activation of chromophors using simultaneous two-photon excitation (TPE).[5,6] Specifically, by use of non-resonant TPE methods, PDT agents that normally require a single photon of UV or visible light for activation might be effectively activated using two NIR photons (Figure 2).[7,8] Typical activation of a type I or type II PDT agent occurs upon absorption of energy imparted by a single photon of light ($h\nu$), promoting the agent from the ground state (S_0) to a higher allowed electronic state (such as S_1). Once in this excited state, the agent may relax via a number of processes, including fluorescence (Fl) or intersystem crossing (IX) to the triplet state (T). Once in the triplet state, further relaxation can occur via phosphorescence (Ph). Depending upon the properties of the excited state, the agent may also decompose or react directly with a cell to elicit cell death (type I PDT) or undergo collisional transfer of energy to triplet oxyen ($^3O_2$) to form singlet oxygen ($^1O_2$); photocatalytic production of this highly cytotoxic form of oxygen is believed to be the primary mechanism underlying type II PDT.[9] Under suitable excitation conditions, the initial excitation step may also be effected via a non-resonant two-photon transition from S_0 to S_1 that is mediated by a transient virtual level (V). Such a transition has been proposed for the hematoporphyrins[10-14] and recently demonstrated in biological systems using psoralens.[8] The energy diagram for this non-resonant process (Figure 2) indicates that concerted interaction of two low energy photons ($h\nu/2$) with the PDT agent can impart sufficient energy to promote the allowed transition from S_0 to S_1. Since the virtual energy level accessed in this process is quantum mechanically disallowed, V has an exceedingly short lifetime ($\tau << 10$ fs, as predicted by the Heisenberg uncertainty principle). Thus, to become significant relative to spontaneous re-emission (via scatter), both photons must interact in an essentially simultaneous manner with the PDT agent in order to successfully promote it from S_0 to S_1. If this occurs, the molecule should exhibit photochemical and photophysical behavior that is identical to that resulting from SPE.

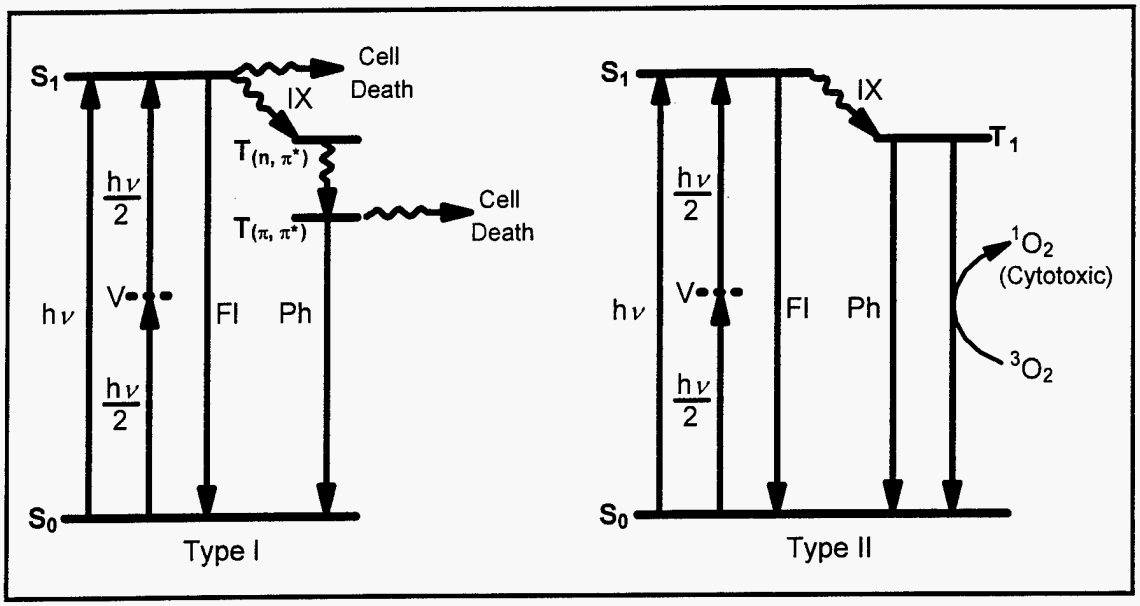

Figure 2. Simplified Jablonski energy level diagrams for type I and type II PDT agents. Promotion of an agent molecule from the ground state (S_0) to an excited state (S_1) may occur upon absorption of a single photon of light (hν), as required in SPE. Excitation to an excited state may also occur upon absorption of energy imparted by two lower energy photons (hν/2); under non-resonant conditions, this process is called TPE.

2. TPE USING THE MODE-LOCKED TITANIUM:SAPPHIRE LASER

The energy level diagrams shown in Figure 2 imply that it should be possible to excite visible or UV active PDT agents using NIR light. For example, activation of psoralens should be possible via TPE using 700-800 nm light (ie, at twice the wavelength, or half the energy, of that commonly used for SPE). Furthermore, TPE should enable effective excitation of the porphyrin family of agents using NIR light at wavelengths well beyond the minor SPE bands commonly accessed between 600-750 nm. Thus, conventional UV-visible agents and the red-NIR agents could acquire additional utility through use of TPE at NIR wavelengths that are not significantly absorbed by skin or tissue. Fortuitously, the wavelength tuning range of the mode-locked titanium:sapphire laser appears to encompass the range of energies required for TPE of a majority of such PDT agents, making this laser an attractive candidate for PDT using TPE methods.

Relative TPE Cross-Section. Note that in comparison with SPE, simultaneous TPE generally has a relatively small cross-section due to the low probability that two photons will simultaneously interact with a molecule during the extremely brief lifetime of the virtual energy level (typical TPE cross-sections for a given molecule may be 10^5–10^7 smaller than equivalent SPE cross-sections). This probability may be dramatically enhanced through the use of a source having a high instantaneous irradiance (such as the mode-locked titanium:sapphire laser), since efficiency of TPE is proportional to instantaneous irradiance squared (or alternately to the product of average and peak powers). For example, by applying a modest average power in the form of a train of mode-locked, fs-duration pulses, the efficiency of TPE can be brought very close to that of SPE. Common commercially available mode-locked titanium:sapphire lasers offer pulse repetition frequencies in the range of 70–90 MHz and pulse widths <200 fs, providing peak powers near 100 kW at average powers near 1 W. Hence, the mode-locked titanium:sapphire laser is capable of exciting 10^5 more TPE events than a continuous wave laser having equivalent average power.

Selection Rules in TPE. Differences in the quantum mechanical selection rules governing SPE and TPE mean that excitation spectra may be similar or quite different, depending on the symmetry properties of the molecule. For

example, Lytle has shown that SPE and TPE spectra for various polycyclic aromatic hydrocarbons can be vastly different.[15] In centrosymmetric molecules, the two photons must interact to raise the molecule to an excited electronic state having like parity.[16] If the ground electronic state is ***g*** (*gerade*, or even with respect to inversion), TPE must occur to excited states of ***g*** symmetry; this is the exact opposite of SPE selection rules. Conversely, in non-centrosymmetric molecules, similar selection rules will apply for SPE and TPE. This is illustrated in Figure 3 for 8-MOP (a non-centrosymmetric psoralen derivative) and HP-IX (a centrosymmetric porphyrin derivative). Both examples of SPE data (shown as dashed lines) are characteristic of those reported in the literature. For the non-centrosymmetric psoralen, the SPE and TPE data are very similar over the spectral range investigated. In contrast, for the highly centrosymmetric porphyrin, the selection rules are clearly different (for instance, the Soret band at 400 nm appears to be disallowed in TPE). These results are not surprising, and demonstrate the critical value of TPE excitation spectra in evaluating the applicability of potential agents for TPE-based PDT. Despite the possibility of differences in selection rules, it is important to note that the properties of the excited state (including fluorescence, intersystem crossing to a triplet state and eventual PDT response) are unaffected by the excitation mechanism[8] while the selection rules determine the probability of a specific transition, it is the intrinsic properties of the excited state that determine the ultimate spectroscopic and photochemical properties of the excited molecule.

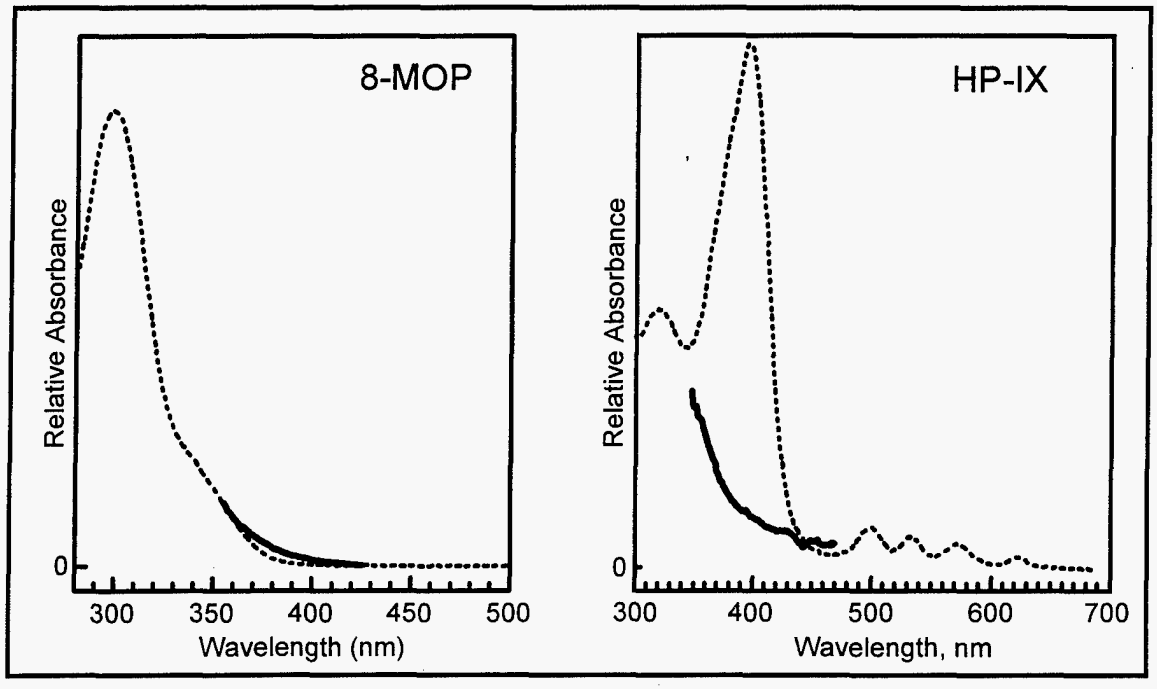

Figure 3. Comparison of relative SPE and TPE excitation properties for 8-MOP and HP-IX. SPE data shown as dashed line, TPE data as solid line. Note that TPE data has been plotted at ½ actual excitation wavelength to facilitate comparison at effective excitation wavelengths.

Action Spectra. In biological systems, it is the action spectrum of the PDT agent that ultimately determines wavelength selection for a given therapeutic regimen. The action spectrum is typically the result of a convolution of the agent's intrinsic excitation spectrum (either SPE or TPE) with the spectrum of the biological system. Since TPE allows agents to be excited using NIR light, the impact of the optical properties of the biological system on the PDT action spectrum should be significantly reduced. Moreover, as a consequence of reported similarities between SPE absorption spectra and action spectra for psoralens,[17] it appears likely that TPE excitation spectra (such as those shown in Figure 3) will be generally representative of the ultimate action spectra for simultaneous two-photon activated PDT.

Safety. The data shown in Figure 3 suggest that it should be possible to use TPE to activate many PDT agents with 700 to 1000-nm light provided by the mode-locked titanium:sapphire laser. However, in addition to spectral properties of the agent, it is critical that the potential effects of the mode-locked beam on tissue be considered. Clearly the absence of tissue absorption features in the NIR should greatly reduce potential for hyperthermal effects when using NIR excitation. However, due to the pulsed nature of a mode-locked beam, potential for optically induced tissue damage may be an important concern. Figure 4 shows tissue damage threshold as a function of irradiance and pulse width.[18,19] Commonly available Q-switched lasers (such as Nd:YAG lasers) yield relatively low damage thresholds as a consequence of avalanche ionization and sequential multiphoton processes that favor ablation and other non-specific damage mechanisms over TPE. In contrast, the fs-duration pulses provided by the mode-locked titanium:sapphire laser are relatively safe because these ultrashort pulses cannot support the ps- to ns-processes responsible for initiation of collisional ionization and other avalanche damage mechanisms. Specifically, the irradiance from an unfocused mode-locked titanium:sapphire laser beam is approximately six orders of magnitude below the tissue damage threshold; even if tightly focused (using a 0.25 N.A. microscope objective), a beam with a 500 mW average power is still well below the damage threshold. Notably, even an unfocused beam from a regeneratively amplified titanium:sapphire laser is below the damage threshold.

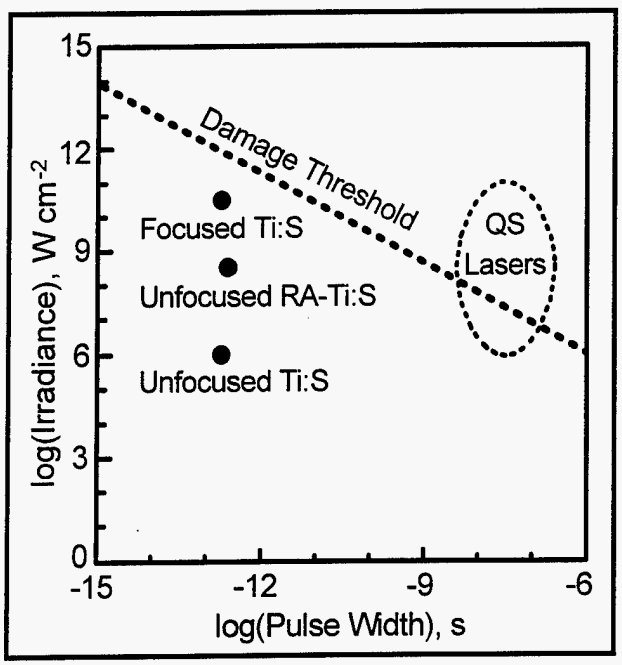

Figure 4. Tissue damage threshold (dashed line) as a function of irradiance and pulse width (adapted from Niemz[18] and Hammer et al.[19]). Q-switched (QS) lasers having ns-duration pulses yield relatively low damage thresholds that favor ablation. The fs-duration pulses provided by mode-locked titanium:sapphire lasers (Ti:S) are too short to support initiation of these damage mechanisms. This trend is maintained for high irradiance ultrafast lasers, such as the regeneratively amplified titanium:sapphire laser (RA-Ti:S).

We have confirmed this margin of safety in studies using various fresh tissue specimens exposed to a stationary collimated beam of 730–920 nm light from a mode-locked titanium:sapphire laser, as well as to a rastered focused beam (0.4 N.A.) from this same source. No optically induced tissue damage was noted in specimens of porcine skin and striated muscle, nor in equine, bovine and canine liver and striated muscle.

The effects of ultrashort NIR pulsed laser light have been further characterized by evaluating the thermal changes induced in various tissue specimens upon prolonged exposure to a laser beam. Figure 5 shows typical thermal profiles for a specimen of striated bovine muscle irradiated with 820 nm, 122 fs pulses from a mode-locked titanium sapphire laser. In these measurements, the beam was focused at the surface of the specimen (0.4 N.A.) and rastered in a square pattern adjacent to a thermocouple located immediately below the tissue surface. Five different intensities were used: 0.40, 0.92, 1.5, 2.0, and 2.5 J/cm^2. By fitting temperature as a function of time, T(t), to an equation of the form:

$$T(t) = T_{\max} + \delta T e^{-t/\tau} \tag{1}$$

where T_{max} is the equilibrium temperature for $t \rightarrow \infty$, δT is the absolute temperature rise from initial temperature (at $t = 0$), and τ is the thermal time constant of the specimen, it was possible to model the thermal response of the specimen to the incident light. Figure 6 shows that the thermal rise as a function of intensity was linear for the mode-locked beam. Identical results were obtained for quasi-CW light at 820 nm (non-mode-locked operation of the titanium:sapphire laser). Similar results were also obtained for continuous wave irradiation using 630 nm light from a Kiton Red dye laser. Equivalent behavior for the mode-locked and non-mode-locked excitation modes at 820 nm demonstrates that thermal response of tissue to a high repetition rate beam is independent of pulse properties. The lower temperature rise observed for 820 nm excitation relative to that observed for 630 nm light agrees with differences in tissue absorptivity at these two wavelengths, and further supports the relative safety of the NIR source for activation of PDT agents.

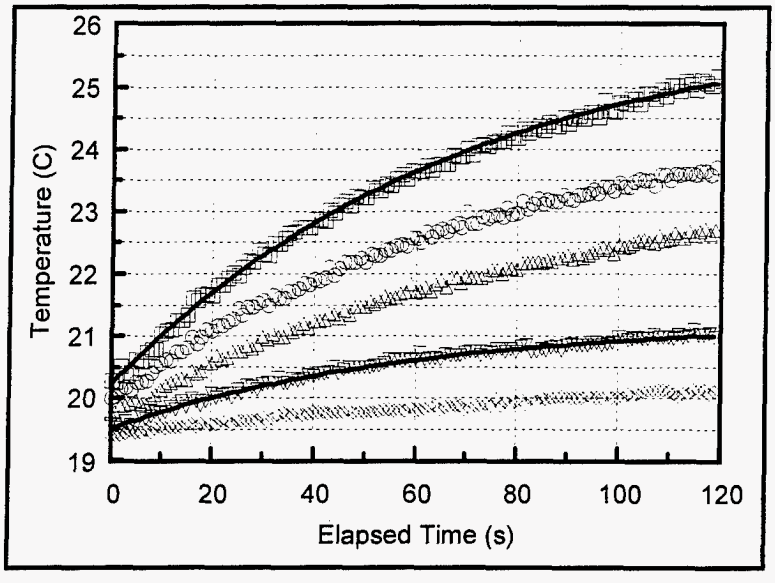

Figure 6. Typical thermal profiles for a specimen of striated bovine muscle irradiated with 820 nm, 122 fs pulses from a mode-locked titanium sapphire laser.

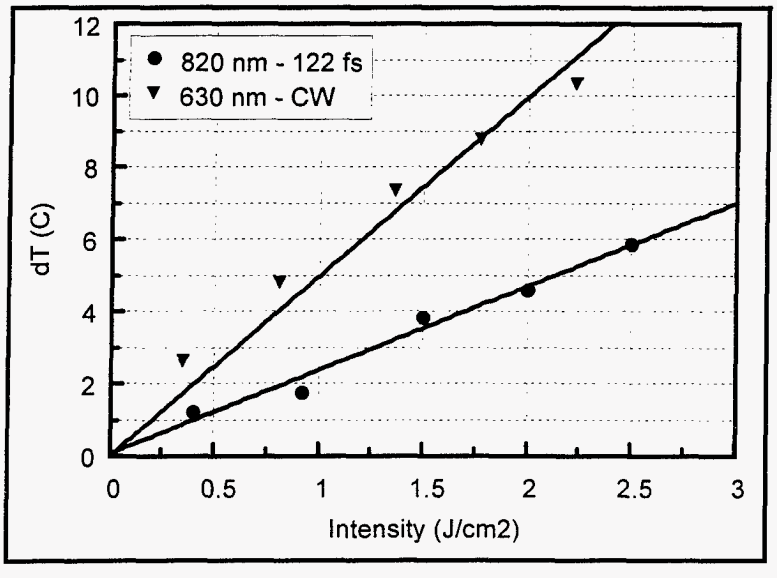

Figure 5. Thermal rise as a function of intensity for mode-locked beam (● 820 nm, 122 fs pulse width) and for continuous wave irradiation using 630 nm light from a Kiton Red dye laser (▼).

Biological Testing. The bacterium *Salmonella typhimurium* was used with 4'-aminomethyl-4,5',8-trimethylpsoralen (AMT, a psoralen derivative) to demonstrate safety and efficacy of the TPE-based PDT process.[8] The experimental protocol was based on the standard Ames II mutagenicity test. Samples were illuminated using 730 nm light having a pulse width of approximately 20 fs. Results of these tests are summarized in Table 1.

Table 1. Results of in vitro testing of TPE using *Salmonella typhimurium* and AMT.

Test Condition	Test Results
Negative Control (No Light, No AMT)	No Effect
TPE Alone (No AMT)	No Effect
AMT Alone (No Light)	No Effect
AMT + Light (Non-Mode-Locked)	No Effect
AMT + Light (Mode-Locked)	Cell Death
Positive Control	Mutation

These results indicate that the mode-locked NIR light used for TPE is not mutagenic, and that the combination of a UV-active photosensitizer with mode-locked NIR light produces the expected PDT effect (cells are killed). Irradiation of samples containing AMT with non-mode-locked NIR light confirm that it is TPE that is responsible for the PDT effect, since no effect is noted on the tester bacteria. Similar results have been obtained using 8-MOP with *S. typhimurium*, along with other combinations of photosensitizer and bacterial strain. Furthermore, preliminary tests using MCF-7 and T-47D human breast carcinoma cell lines with HP-IX appear to show comparable trends. These bacterial and cancer results together provide strong support of both safety and efficacy of TPE-based PDT.

3. CONCLUSIONS

While further work is clearly needed (such as in vivo animal demonstrations), the results provided in this paper support the assertion that the fundamental properties of the excited state of a PDT agent will be identical whether excitation occurs via SPE or TPE. Because TPE appears to occur on a sub-fs time frame, the mode-locked titanium:sapphire laser is well suited for efficient promotion of a molecule from the ground state to an electronically excited state. Further, because the pulse energies produced by this laser are well below tissue damage thresholds, this source provides a wide margin of safety for activation of PDT agents present in biological systems. Moreover, the wide NIR tuning range of this laser makes it well suited for TPE of a wide variety of PDT agents with negligible potential for interference from tissue absorption. This unique combination of features should afford a successful route to development of new PDT regimens offering increased safety and therapeutic flexibility, especially for subsurface applications or where increased control over the point of treatment is desired.

While the current generation of mode-locked titanium:sapphire laser is primarily suited for laboratory testing and evaluation of new PDT modalities, rapid progress in the development of mode-locked solid-state lasers suggests that in the near future inexpensive, turn-key sources will become available that will be capable of meeting the unique requirements posed by the medical suite. We believe that such lasers will become increasingly important for therapeutic two-photon activation of PDT using psoralens, porphyrins, and many other classes of PDT agent.

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