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THE TWO-PHOTON INDUCED FLUORESCENCE OF THE TUMOR LOCALIZING PHOTOSENSITIZER HEMATOPORPHYRIN DERIVATIVE VIA 1064 NM PHOTONS FROM A 20 NS Q-SWITCHED ND-YAG LASER

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We demonstrate the direct 1064 nm two-photon excitation of hematoporphyrin derivative (HPD), a complex mixture of photosensitizing porphyrins which is selectively retained in tumor tissue and used in cancer photochemotherapy. Although 1064 nm is outside of the one-photon HPD absorption spectrum, two-photon induced fluorescence from HPD was observed following excitation by the 20 ns output of an amplified, Q-switched Nd-YAG laser at peak power levels of 0.1 to 3 GW/cm^2. Evidence for the successful two-photon excitation to vibrational levels of the S_1 state consists of the observation of the known HPD fluorescence spectrum exhibiting peaks at approximately 615 and 675 nm, with the observed two-photon induced fluorescence intensity exhibiting a quadratic dependence on the excitation laser intensity as required for a direct two-photon process. More generally, these results suggest the possibility for the achievement of photosensitized oxidations utilizing photons of lower energy than that required for single photon excitation, offering the potential for both greater selectivity and a reduction in competing photochemical processes.

The study of photosensitized reactions is a vigorously pursued area in chemical and biological research and is becoming of major importance in clinical medicine. Of particular interest in this latter context is hematoporphyrin derivative, a complex mixture of photosensitizing porphyrins which binds with increased avidity to tumor tissue (1-4). Following photoexcitation in either the ultraviolet or visible, HPD exhibits both fluorescence and the formation of singlet molecular oxygen via energy transfer from the HPD triplet. It is the significant HPD quantum yield of singlet oxygen formation which is thought to mediate tumor destruction. In aqueous solution HPD exhibits absorption maxima at approximately 370, 504, 538, 566 and 612 nm

Abbreviations: HPD = hematoporphyrin derivative; S_n = nth excited singlet state; $^3O_2$ = triplet (groundstate) molecular oxygen.

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(S_1). There is no significant HPD absorption from 780 through 1100 nm. Following photoexcitation in either the visible or ultraviolet there is substantial fluorescence in the red, originating from vibrationally relaxed S_1 with peaks at approximately 615 and 675 nm.

The experiments described herein demonstrate the two-photon absorption of HPD at 1064 nm. This is an excitation wavelength lying outside the HPD one-photon absorption spectrum, resulting in fluorescence maxima at approximately 615 and 675 nm and exhibiting a quadratic dependence on laser intensity.

MATERIALS AND METHODS[5]

HPD and Photofrin II (stated to be a further purified preparation of HPD) were obtained from Photofrin Medical, Inc. in aqueous solutions containing 5 mg per ml and 2.5 mg per ml respectively. Further dilutions were made in 10 mM potassium phosphate buffer pH 7.4 to a final HPD concentration of 5.4 µg per ml. This concentration was used in all experiments. All spectra were taken at ambient temperature utilizing quartz cuvettes. Absorption spectra were run on a Varian Cary 2300 spectrophotometer with buffer alone in the reference cell. Fluorescence spectra were run on an Aminco SPF-500 corrected spectra spectrofluorometer or the following laser fluorescence system. The excitation source for the two-photon studies was an amplified, Q-switched Nd-YAG laser. Its output characteristics at 1064 were full width half maximum pulse length of 20 ns and energies up to 500 mjoule. This output could be frequency doubled to produce 532 nm pulses of 15 ns duration and up to 250 mjoule energy. The laser output was loosely focused into the 1 cm long cuvette to produce a beam of approximately constant cross section over a path length of 2 cm with a mean radius of 2.5×10^{-2} cm. The laser energy was controlled both by adjusting the relative timing between the firing of laser oscillator and amplifier (affecting the net gain), and through the use of volume absorbing glass filters. The laser-excited fluorescence was viewed at right angles to the direction of laser propagation, dispersed in a Spectrolab f/1.6 monochromator and detected with a GaAs photomultiplier tube. The signal was processed via a gated integrator and recorded using an LSI-11 based computer.

RESULTS

The absorption spectrum of HPD is presented in Fig. 1. Absorption features at approximately 370, 504, 538, 566 and 612 nm are readily apparent. No significant HPD absorbance, referenced to buffer only, can be discerned in the spectral region 780 to 1100 nm. The single photon HPD fluorescence emission spectrum obtained with a spectrofluorometer utilizing 532 nm excitation is presented in Fig. 2A and demonstrates the fluorescence spectrum characteristic of HPD. Also shown in this figure is the excitation spectrum obtained while monitoring emission at 615 nm (Fig. 2B). A fluorescence spectrum qualitatively identical to this was obtained following 395 nm

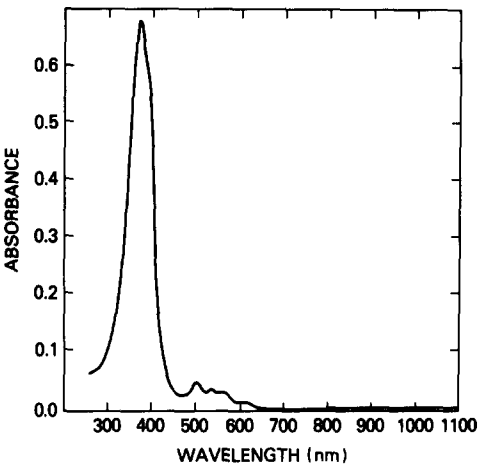

Figure 1. Absorption spectrum of hematoporphyrin derivative. Conditions as described under Materials and Methods. The slight discontinuity at 800 nm is a result of the change in the detector within the spectrophotometer, which occurs in passing from the infrared to the visible.

excitation into the Soret band. Figure 3A contains the two-photon fluorescence spectrum obtained following 1064 nm excitation with 50 μjoule laser pulses, corresponding to a peak power level of 2 GW/cm^2. This spectrum is seen to be virtually identical to the one-photon fluorescence spectrum obtained following 532 nm excitation by 6 μjoule of frequency doubled YAG

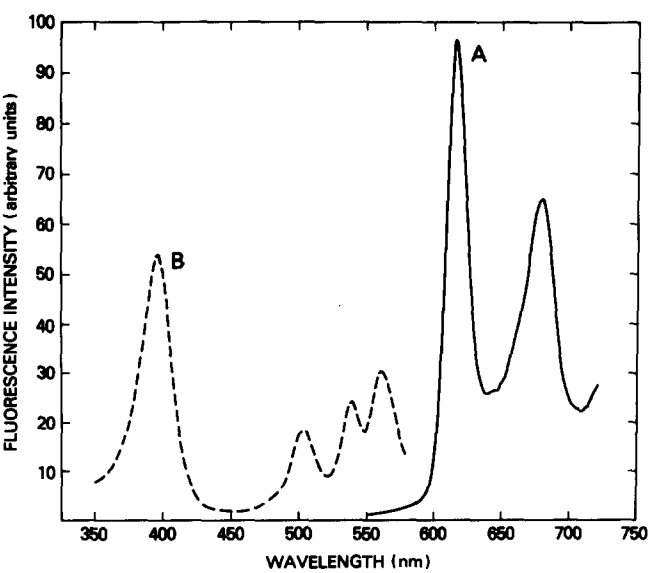

Figure 2. Corrected fluorescence spectra for hematoporphyrin derivative. Conditions as described under Materials and Methods. Curve A is the emission spectrum (for excitation at 532 nm). Curve B is the excitation spectrum (monitoring the emission at 615 nm).

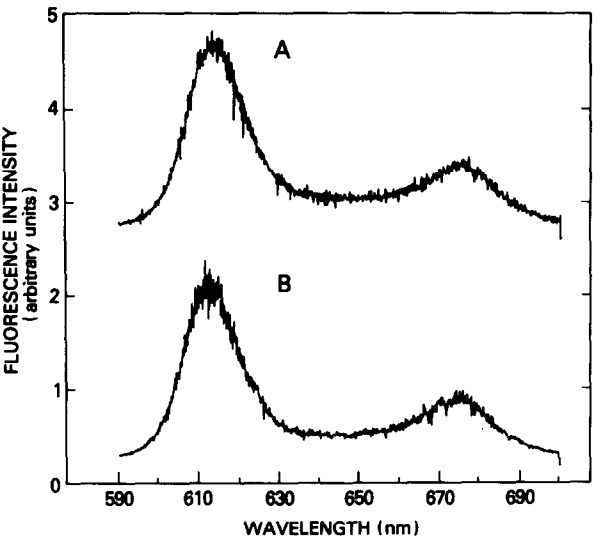

Figure 3. Fluorescence emission spectra of hematoporphyrin derivative resulting fro Nd-YAG laser excitation. Conditions as described under Materials and Methods. Curve A is the emission spectrum resulting from 1064 nm excitation at a laser energy of 50 mJ at the cuvette. Curve B is the emission spectrum resulting from 532 nm excitation (second harmonic of the Nd-YAG laser) at a laser energy of 6μJ at the cuvette.

output (Fig. 3B). The relatively minor spectral differences between Fig. 2 and Fig. 3 result from the different spectral responses of the two systems.

The laser-excited fluorescence intensity monitored through the monochromator at 615 nm (7 nm bandpass) is plotted logarithmically against laser intensity in Fig. 4A for HPD, and Fig. 4B for Photofrin II. The fitted lines are seen to have a slope of two in each case, thus corresponding to a quadratic intensity dependence and indicating a two-photon process.

DISCUSSION

The evidence of Figs. 3 and 4 clearly demonstrates that we are observing HPD emission following a direct two-photon excitation. A number of potential implications may follow from this observation. From a clinical viewpoint, it has been reported that human colonic adenocarcinoma-type tumors (Cx1) grafted on nude mice exhibit an increased susceptibility to acidophilic necrosis when exposed to 1064 nm laser radiation in the presence of HPD, as compared to 1064 nm laser irradiation alone (which resulted in coagulation necrosis) (6). The results demonstrated herein suggest the possibility that the reported HPD

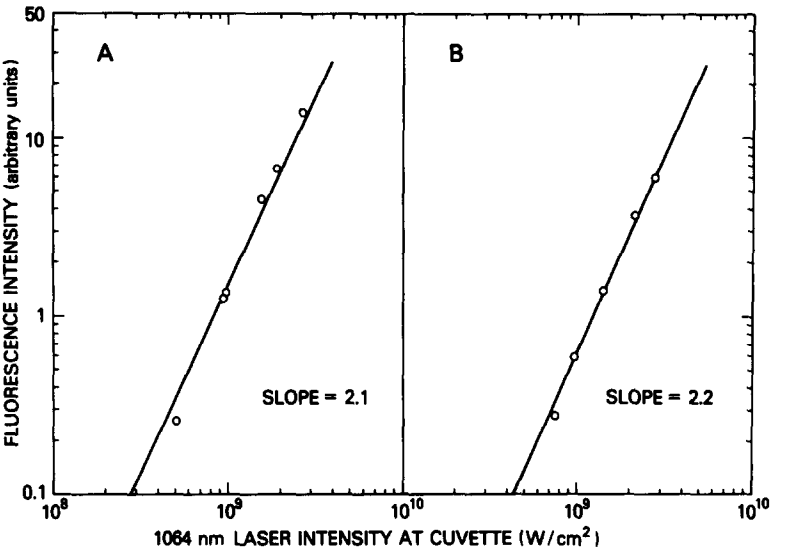

Figure 4. Logarithmic plot of relationship between fluorescence intensity (normalized signal at 6150 ±7 angstroms) and laser power at 1064 nm. Conditions as described under Materials and Methods. Curve A is for hematoporphyrin derivative. Curve B is for Photofrin II. The two-photon excitation probability for HPD can be obtained by multiplying the ordinate value by $5 \pm 3 \times 10^{-4}$.

enhanced 1064 nm acidophilic necrosis may be a two-photon process. More fundamentally, these results suggest the possibility that photosensitized oxidations may be driven by photons with energies lower than those required for single photon excitation, thus potentially presenting the opportunity to obtain greater excitation selectivity and to drive photooxidation reactions in the absence of additional complicating photo-activated processes allowed by the higher energy photons. We are observing the same fluorescence emissions from two-photon excitation that result from direct single-photon HPD excitation. It is therefore reasonable to predict that subsequent to attainment of S_1 the sensitizer triplet will be formed by intersystem crossing as occurs following direct single photon excitation at 532 nm. Oxidation could then be achieved by either direct interaction of the sensitizer triplet with the substrate, or via singlet molecular oxygen generated by energy transfer from the sensitizer triplet to $^3O_2$.

The experiments reported herein differ formally and fundamentally from those of Andreoni et al. (7), who reported a two-step activation of HPD

utilizing 337.1 nm radiation from a N_2 laser. This excitation wavelength lies within the strong Soret absorption band and involves direct excitation of HPD followed by the sequential absorption of the second photon into some higher lying S_n state(s). In marked contrast, the experiments reported herein utilized photons non-resonant with any dipole allowed HPD transition, and therefore were outside of the one-photon HPD absorption spectrum. The demonstration of fluorescence indicated the attainment of S_1. It is not likely that higher lying S_n states above S_1 would be reached via additional 1064 nm two-photon absorptions, although in principle the possibility for this exists.

For the reasons outlined above, these experiments may possibly suggest some potential mechanistic and clinical implications for HPD cancer photochemotherapy. More generally, however, they also suggest the potential for driving photosensitized oxidation reactions utilizing relatively low energy photons, outside of the one-photon absorption spectrum of the photosensitizer.

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REFERENCES

1. Forbes, I.J. (1984) Med. J. Australia 140: 94-96.

2. Benson, Jr., R.C., Kinsey, J.H., Cortese, D.A., Farrow, G.M., and Utz, D.C. (1983) J. Urology 130: 1090-1095.

3. Tsuchiya, A., Obara, N., Miwa, M., Ohi, T., Kato, H., and Hayata, Y. (1983) J. Urology 130: 79-82.

4. Hayata, Y., Kato, H., Konaka, C., Ono, J., and Tikizawa, N. (1982) Chest 81: 269-277.

5. Certain commercial equipment, instruments, or materials are identified in this paper in order to adequately specify the experimental procedure. In no case does such identification imply recommendation or endorsement by the National Bureau of Standards or the National Institutes of Health, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

6. Patrice, T., Le Bodic, M-F., Le Bodic, L., Spreux, T., Dabouis, G., and Hervouet, L. (1983) Cancer Research 43: 2876-2879.

7. Andreoni, A., Cubeddu, R., DeSilvestri, S., Laporta, P., and Svelto, O. (1982) Chem. Phys. Lett. 88: 37-39.