Introduction

The nonlinear, multiphoton process of two-photon absorption (2PA) has been gaining greater interest among a number of multidisciplinary areas, particularly in the rapidly developing fields of multiphoton fluorescence imaging, optical data storage and switching, optical sensor protection, telecommunications, laser dyes, 3-D microfabrication, and photodynamic therapy (PDT).\textsuperscript{1-5} The demands of such applications exceed properties and reliabilities delivered by current organic materials, underscoring the need for increasingly sophisticated nonlinear optical organic materials. In particular, compounds that undergo strong nonlinear, multiphoton absorption are being investigated as materials for a wide variety of potential applications in areas ranging from optical information storage, 3-D optical memories, biophotonics, materials science, and photochemistry. For example, it is projected that a multiphoton-based 3-D optical volumetric memory will provide up to three orders of magnitude more information in the same size enclosure relative to a 2-D optical disk memory.\textsuperscript{1}

Two-Photon Organic Photochemistry

Although a wealth of information regarding the understanding and applications of photochemical transformations has been obtained over the last half century, comparatively few studies of multiphoton induced organic photochemistry have been reported. In fact, in most books of organic photochemistry, there is scarcely a mention of simultaneous two-photon induced photochemistry. A brief description can be found in \textit{Excited States in Organic Chemistry}\textsuperscript{6} and \textit{Principles and Applications of Photochemistry}.\textsuperscript{7} This said, the underlying principles of multi- or two-photon absorption are particularly meritorious, and warrant much further investigation (particularly with the advent of commercially available ultrafast pulsed lasers). In fact, the field of two-photon organic photochemistry is in its infancy, not unlike the field of single photon organic photochemistry 50 years ago before the pioneering work of George Hammond, Michael Kasha, Howard Zimmerman, and others.

Multiphoton absorption can be defined as simultaneous absorption of two or more photons via virtual states in a medium. The theory of simultaneous absorption of two-photons was developed by Goeppert-Mayer in 1931,\textsuperscript{8} but remained mainly a conceptual curiosity because light sources were not available of suitably high intensity. It was not until the early 1960s that the two-photon absorption process was experimentally verified by Kaiser and Garrett\textsuperscript{9} who used pulsed lasers that provided very high intensity. Normally, molecules are promoted from the ground state to an excited state through resonant single photon absorption. However, under appropriate conditions, this excitation can be accomplished by two-photon absorption (2PA). In 2PA, molecules exposed to high intensity light can undergo near simultaneous absorption of two longer wavelength photons mediated by a so-called “virtual state,” a state with no classical analog. The combined energy of the two photons stimulates the molecule to a stable excited state. If the two photons are of the same energy (wavelength), the process is referred to as degenerate 2PA. On the other hand, if the two photons are of different energy (wavelength), the process is nondegenerate 2PA. When light passes through molecules, a virtual state may form, persisting for a very short duration (on the order of a few
From the Executive Director

D. C. Neckers, Executive Director, Center for Photochemical Sciences, Bowling Green State University

Recently my graduate students have really caught me off guard. They’ve presented me with papers and communications from journals I’ve been subscribing to for almost as long as I’ve been in chemistry. That is no big deal. What is a big deal is that they’ve given me a photocopy of an interesting article a day or week before my hard copy arrives. That didn’t and couldn’t happen before because my hard copy of JACS or J. Org. Chem. always arrived before the library’s copy. Now the on-line version is sometimes weeks in front of the arrival of the hard copies. My students, and I too, have much broader and more rapid literature access right at our fingertips.

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I guess one must ask what is the future of hard copy anything?

This is an important question we can, and continually do, ask about The Spectrum. Printing hard copy is expensive and slow. Mailing hard copies is increasingly costly. When it comes to reference books, the old-fashioned setting of copy in type still happens but it’s on the way out. One wonders what is the future of series that take 9 to 12 months from the time of author submission to the date of publication. By the time review articles appear, several more papers in the field may have already been published.

I’d like to seek the input of our readers. The Internet has revolutionized the passing of information.

• How will this impact the way scientists obtain, seek and retrieve critical technical data and information?
• Will paper and hard copy soon be obsolete?
• Will literature searches be dated as a result? Or will they be more complete because more information is easier to obtain?
• Just how do you feel about Internet journal subscriptions?
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My questions are asked in all seriousness and I really want your input. Please use the following email address (dcnecke@bgnet.bgsu.edu) and let me know what you think. If we get a good sampling of responses from our readers we’ll publish your thoughts in a future Spectrum. Thanks in advance.
The 2PA can result only if a second photon arrives before the decay of the virtual state. Thus, 2PA involves the concerted interaction of both photons that combine their energies to produce an electronic excitation analogous to that conventionally caused by a single photon of a correspondingly shorter wavelength.

Two-photon transitions can be described by two different mechanistic processes. For nonpolar molecules with a low energy, strongly absorbing state near the virtual level, only excited states that are forbidden by single photon selection dipole rules can be populated via two-photon absorption. The probability that this low energy state can contribute to the virtual state is predicted by Heisenberg’s uncertainty principle with a virtual state lifetime approximated as $h/(4\pi\Delta E)$, where $h$ is Planck’s constant and $\Delta E$ is the energy difference between the virtual and actual states. Using this equation, it is predicted that an allowed state can contribute to the formation of the virtual state for time $t_{\text{virtual}}$, which is equal to about $h/(4\pi\Delta E)$ with the transition probability proportional to $\Delta \mu^2$. In polar molecules, strong 2PA can also occur by a different mechanism in which a large change in dipole moment ($\Delta \mu > 10 \text{ D}$) occurs upon excitation of the ground state. Single photon allowed states can then be accessed via 2PA, and the virtual state lifetime is proportional to $\Delta \mu^2$, while the transition probability also scales with $\Delta \mu^2$. In this case, both the ground and excited states can participate in the formation of the virtual state, which enhances 2PA.

One of the major features that distinguishes 2PA from single photon absorption, apart from selection rules, arises by considering the rate of energy (light) absorption as a function of the incident intensity. In the single photon process, the rate of light absorption is directly proportional to the incident light intensity ($\text{d}q/\text{d}t \propto I$), and is independent of the rate of photons passing through the molecule. By contrast, the 2PA process depends on both spatial and temporal overlap of the incident photons, therefore the rate of light absorption is proportional to the square of incident light intensity ($\text{d}q/\text{d}t \propto I^2$).

Applications of Two-Photon Photochemistry

The quadratic, or nonlinear, dependence of two-photon absorption on the intensity of the incident light has substantial implications. For instance, in a medium containing one-photon absorbing chromophores, significant absorption occurs along the path of a focused beam of suitable wavelength light, which can lead to out-of-focus excitation. In a two-photon process, negligible absorption occurs except in the immediate vicinity of the focal volume of a light beam of appropriate energy. This allows spatial and radial resolution about the beam axis, which circumvents out-of-focus absorption and is the principle reason for two-photon fluorescence imaging. Using near-IR radiation to induce fluorescence in biological samples which contain fluorophores, dynamic processes in living cells can also be imaged. The 2PA processes greatly reduce photobleaching of the fluorophore and photodamage of cells, which would occur using conventional UV excitation sources. Two-photon scanning laser microscopy can be used as a nondestructive diagnostic and evaluation tool to probe surfaces, interfaces, and fractures in materials. Recently, we have shown defects in substrates coated with fluorophore-labeled poly(methyl methacrylate) (PMMA) can be detected through nondestructive 3-D optical sectioning using two-photon fluorescence microscopy. Two-photon fluorescent imaging provides a well-defined three-dimensional image of the subsurface morphology and demonstrates the possibility of using this method as a tool in failure analysis of materials.

Two-Photon Microfabrication

In contrast to the linear dependence of single photon absorption on incident light intensity in conventional photopolymerization, the quadratic dependence of photoexcitation on light intensity in 2PA can be exploited to confine polymerization to the focal volume and achieve fabrication of microstructures via 3-D spatially resolved polymerization. Near-IR two-photon induced polymerization was demonstrated using free-radical and cationic photoinitiator systems. Recently, we reported the near-IR two-photon induced polymerization of acrylate monomers using a commercially available photoinitiator system based on a visible light absorbing dye. Two-photon initiated polymerization was conducted at 775 nm via direct excitation of a commercially available dye (5,7-diiodo-3-butoxy-6-fluorone, H-Nu 470) in the presence of an arylamine, and (meth)acrylate monomer, resulting in an electron transfer-free radical initiation process. The excitation wavelength was well beyond the linear absorption spectrum for
5,7-diiodo-3-butoxy-6-fluorone (strong and weak absorption maxima at 330 and 470 nm, respectively). The formation of polymeric microstructures with a variety of dimensions was accomplished with the H-NU 470 initiator/acrylate monomer and other initiator systems (Figure 1a). In particular, arylketone photoinitiators such as isopropylthioxanthone (ITX), benzoin methyl ether (BME), and an acylphosphine oxide (Irgacure 819, CIBA) were found to be effective initiators (all have $\lambda_{\text{max}} < 400$ nm), resulting in well-defined microstructures.

Cationic photoinitiated polymerization of epoxides, vinyl ethers, and methylenedioxolanes have received increasing attention, due in large part to the oxygen insensitivity of the cationic process. Commercially available diaryliodonium (CD-1012, Sartomer) and triarylsulfonium (CD-1010, Sartomer) salts were found to initiate polymerization of multifunctional epoxide and vinyl ether monomers, affording well-defined microstructures (Figure 1b).

Typical multifunctional epoxide monomers investigated were a mixture of poly(bisphenol A-co-epichlorohydrin), glycidyl end-capped and 3,4-epoxycyclohexylmethyl 3,4-epoxy-cyclohexanecarboxylate (K126, Sartomer) using CD-1012 (18 $\mu$m line width, progressively increasing line spacing beginning with 72 $\mu$m, from bottom to top).

Figure 1. Optical micrographs of microstructures created via two-photon polymerization of (a) an acrylate monomer (SR349) using the H-NU 470 initiating system (9 $\mu$m line width, 100 $\mu$m line spacing) and (b) a mixture of poly(bisphenol A-co-epichlorohydrin), glycidyl end-capped and 3,4-epoxycyclohexylmethyl 3,4-epoxy-cyclohexanecarboxylate (K126, Sartomer) using CD-1012 (18 $\mu$m line width, progressively increasing line spacing beginning with 72 $\mu$m, from bottom to top).

Two-Photon Photocycloaddition

The photochemical [$\pi 2s + \pi 2s$] cycloaddition reaction has proven useful in organic synthesis, resulting in formation of two new carbon-carbon bonds and a maximum of four new stereogenic centers. The first [2+2] photocycloaddition reaction reported was the formation of carvone camphor on exposure of carvone to sunlight by Ciamician in 1908. Both intermolecular and intramolecular photocycloaddition reactions have received considerable attention over the past several decades, emphasizing the importance of this synthetic transformation. The [2+2] photodimerization of cinnamate-derivatized polymers facilitated the development of early photoresists that helped launch photolithography and the printed circuit board industry.

Under irradiation with UV light, psoralens, naturally occurring coumarin derivatives, are known to react with pyrimidine bases in DNA. Various physiological actions such as skin erythema, inactivation of DNA virus, and disorders in the development of sea urchin eggs fertilized with sperm have been attributed to this photoreaction. The formation of interstrand crosslinking through C4-cycloaddition of 3,4- and 4',5'-double bond with the 5,6-double bond of the pyrimidine bases, especially thymine, in DNA has been correlated with biological effects of the photoexcited furocoumarin. The crosslinked DNA inhibits replication, causing cell death. This is the principle behind the use of psoralens in photodynamic cancer therapy.

The 2+2 photocycloaddition of 5,7-dimethoxycoumarin (DMC) was investigated as a model system for the study of the photoinduced reaction of psoralsens and pyrimidine bases in DNA. Unlike other coumarins, DMC only has the reactive pyrone 3,4-double bond which causes the photoreactivity. Thus, fewer photoproducts of DMC with thymine are formed due to this lack of bifunctionality. As a result, this led to easy isolation and analysis of photoproducts. The study of photocycloaddition of DMC with thymine provided a good model for the photoreaction of psoralsens with pyrimidines, while the study of the photodimerization of DMC provided the fundamental understanding for this model reaction. Single photon induced photodimerization of 5,7-dimethoxycoumarin was reported in the early 1990s. It was reported that photodimerization of DMC generated three different isomers: syn head-to-head, syn head-tail, and anti head-to-tail.

In order to compare two-photon induced photodimerization with the corresponding single-photon induced reaction, we chose the photodimerization of DMC. In anisole under long wavelength UV irradiation (350 nm broadband) two photodimers were observed (Scheme 1). Since the $\lambda_{\text{max}}$ of DMC is 320 nm, experiments were conducted by...
irradiating DMC in anisole at 660 nm (110 fs pulse width). GC/MS analysis confirmed dimer formation, while HPLC analysis allowed identification of the specific photodimers through comparison with authentic samples prepared via single-photon photolysis. It was found that the same two photodimers observed upon UV exposure of DMC were formed after two-photon excitation, and the dimers were formed in nearly the same ratios. Further confirmation of two-photon absorption of DMC at 660 nm was secured by recording its two-photon upconverted fluorescence spectrum and observing the characteristic quadratic dependence of emission intensity as a function of incident (excitation) intensity.

Two-Photon Photochromism

Photochemical transformations induced by 2PA have potential applications. Rentzepis et al. reported two-photon induced photochromism of spiropyran derivatives using 1064 nm radiation.20,21 Analogous to single photon absorption facilitated isomerization, the spiropyran underwent ring-opening isomerization to the zwitterionic colored merocyanine isomer. Like many spiropyrans, spirooxazine and fulgide-type compounds are known to undergo photoisomerization from a colorless to highly colored isomer.22 Unlike the spiropyrans, the thermally and photochemically stable spirooxazine and fulgide-type compounds have been reported which underwent numerous single-photon photochemical isomerization (color) and reversion cycles without significant degradation.23 Optical data recording potentials as high as 100 million bits/cm² have been reported for fulgide-type materials. In an effort to develop a more photostable material for two-photon holographic imaging and information storage, fulgide 1 (λmax = 385 nm) was chosen for study, particularly since its single-photon photochromic behavior is well established (Figure 2).24

Two-photon induced photochromism of 1 was demonstrated through determination of the kinetics of fs near-IR (775 nm) photoisomerization performed using a pump-probe experimental setup.11,25 As seen in Figure 3, formation of the fulgide photoisomer (2) was monitored as a function of time. Plots of absorbance at 585 nm (log \( I_0/I \)) versus

![Scheme 1](image1)

![Figure 2](image2)

![Figure 3](image3)
time (s) were linear for the formation of the longer wavelength of the ring-closed photoisomer. The photoisomerization rate constants thus obtained were $2.53 \times 10^{-3} \text{s}^{-1} \pm 0.3 \times 10^{-3} \text{s}^{-1}$ and $6.99 \times 10^{-3} \text{s}^{-1} \pm 0.5 \times 10^{-3} \text{s}^{-1}$ at irradiation intensities of 3.5 and 7.0 mW, respectively. As can be seen from the rate constants as a function of irradiant intensity, a near-quadratic dependency was observed for the photoisomerization of 1 as a function of intensity of the 775 nm fs pump beam, supportive of a two-photon induced process.

Preliminary 2-D interferometric recording was performed using a Mach-Zehnder interferometry setup using a Clark CPA2001 775 nm fs laser as the irradiation source (Figure 4). Photoinduced changes were observed in the regions of high light intensity (bright interference fringes) in a thin film of poly(styrene)/fulgide 1 composite, demonstrating a proof of principle for effecting photochromic transformations in localized regions (Figure 4) as a model for holographic information storage.11,25 The dark lines in the image of Figure 4 result from high intensity bright fringe-induced photoisomerization of fulgide 1 (13 µm line width and 155 µm line spacing). Current efforts involve two-photon holographic volumetric recording in this material.

**Conclusion**

The vast number of advantages associated with nonlinear absorption-induced processes is fast propelling two-photon absorbing materials and photochemical transformations to the forefront of several important fields. Such materials and processes can be expected to be employed in 3-D volumetric optical recording, nondestructive 3-D imaging, optical sensor protection, photodynamic therapy, and 3-D microfabrication. We can expect to witness breakthroughs in years to come, due to the harnessing of spatially-resolved two-photon induced photochemical reactions in organic materials.

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**References**

13. The fluorone dye, 5,7-diodo-3-butoxy-6-fluorone (H-NU 470) and N,N-dimethyl-2,6-diisopropylaniline, were obtained from Spectra Group Limited, Inc.

**About the Author**

Kevin D. Belfield received a Ph.D. in chemistry from Syracuse University in 1988, where he studied the kinetics and mechanism of thermal rearrangements of organic molecules with John E. Baldwin. As a postdoctoral researcher at SUNY College of Environmental Science and Forestry with Israel Cabasso, he worked in the area of synthesis and characterization of functionalized polymers and synthetic polymer membranes. He then pursued postdoctoral research in mechanistic organic chemistry with William von E. Doering at Harvard University, utilizing photochemical reactions and determining radical stabilization energies of polyenyl radicals through spectroscopically-aided kinetic studies. He joined the faculty of the Chemistry Department at the University of Detroit Mercy in 1992. In 1998, he moved to the University of Central Florida in 1998 to establish a program in the synthesis and characterization of nonlinear optical organic materials and polymers. He is an Associate Professor in the Department of Chemistry, School of Optics/Center for Research and Education in Optics and Lasers (CREOL), and the Department of Mechanical, Materials, and Aerospace Engineering. His address is Department of Chemistry and CREOL/School of Optics, University of Central Florida, P.O. Box 162366, Orlando, FL 32816-2366; e-mail: kbelfiel@mail.ucf.edu.
Novel Exploration of the Ability of Singlet Oxygen to Photobleach P. taeda Pulps

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Introduction

Pulp and paper manufacture is a central part of a global, diverse, and dynamic industry that contributes to products that in general economists equate with increased quality of life, that is, tissue, boxes and packaging, board products, paper and writing materials, lumber/wood, veneer, resins, and even surplus energy. The commercial nature of the industry typically garners a high ranking in productivity and contribution to the US national GNP with an output of 87 million metric tons (30% world total) of pulp and paper products (excluding lumber products) at the end of the last century. Despite its production efficiency and contribution to state economies, the industry relies heavily on capital expenditures that unfortunately mitigate overall annual growth to several percent. New, fundamental breakthroughs in its technological operations are therefore required to provide an avenue for future improved growth. The partnership of the Institute of Paper Science and Technology (IPST) with industry addresses the need for basic chemistry knowledge in the industry. The partnership is an academic/industrial relationship 60 years in the making before the creation of the NSF Science and Technology Centers (STCs) established in 1989 to “help maintain US preeminence in science and technology and ensure the requisite pool of scientists with the quality and breadth of experience required to meet the changing needs of science and society—ingredients essential to successful economic competitiveness”.

IPST is a private graduate school that is academically allied with the Georgia Institute of Technology and remains the principal research arm of the US and currently the global pulp and paper industry. Since many of the capital expenses incurred by the industry are associated with increasing the efficiency of the pulping and bleaching chemical reactions (the core of the industry processes), the work at IPST described in this paper will focus on chemical bleaching reactions. The generation of high-value products in the industry begins with the incipient pulping and bleaching (a fine tuning of pulping) reactions. Pulping is a process practiced globally to remove the “lignin” in woody tissue from the valuable carbohydrates (cellulose and hemicelluloses) and is dominated by the well-known kraft process and its sundry modifications. Figure 1 displays the three essential building blocks or propanoid phenol units, C_{
u} “monomer” units of lignin that are assembled via an enzyme-mediated radical coupling process (lignification) that generates “lignin.” Lignin (from Latin, lignum, wood) is a three-dimensional matrix of these units condensed at different C-C bond sites as shown in Figure 2.

In general, the bulk (greater than 50%) of lignin linkages in softwoods (such as pine and spruce) are β-O-4 (the β carbon of one propyl monomer is connected to the p-hydroxy oxygen of another monomer). The kraft pulping process consists of hydrolyzing the linkages between monomers by the introduction of a highly basic hydrosulfide solution at high temperatures and pressures as shown in Figure 3. The basic conditions deprotonate the phenol hydroxy groups and resonate the phenoxy anion to form quinone methides (QM) that are susceptible to nucleophilic attack by hydrosulfide anion. The hydrosulfide anion attacks the α-carbon of a QM to reform the phenolate anion and subsequently attacks the β-carbon to form a thirane structure with the concomitant expulsion of the β group (pulping concept).

Bleaching is an extension of pulping and is designed to remove the residual lignin that cannot be effectively removed in pulping without complete degradation of the
carbohydrates. A breakthrough in bleaching that we have made recently was determining that singlet oxygen could effectively be employed to oxidatively attack the last vestiges of lignin remaining in kraft softwoods, specifically Southern pine, *Pinus taeda*, having lignin contents of 4%.

The Applicability of Singlet Oxygen to Selectively Photobleach the Lignin in *P. taeda*

Singlet oxygen can be generated via a step-wise photochemical process. In general, the photochemical process is very facile and requires a photosensitizing agent to transfer the excitation energy to the triplet ground state of oxygen ($^{3}$O$_{2}$). The photochemical reaction scheme is shown in the following equation:

$$\text{Sensitizer} + h\nu \rightarrow \text{Sensitizer}^* + ^{3}\text{O}_2 \rightarrow \text{Sensitizer} + ^{1}\text{O}_2$$

The focus of this work was to generate singlet oxygen *in situ* and allow it to react with residual lignin in pulp samples without deleteriously affecting the native carbohydrate component. Past work in the realm of “photobleaching” has dealt with model compounds and methylene blue as the photosensitizing compound. These schemes, however, were not predictive of the behavior of singlet oxygen in more complex *P. taeda* matrices (that include carbohydrates and organic processes.

Figure 2. Shown at the left is a modern day depiction of the currently accepted macrostructure of lignin in wood as constructed from various spectroscopic and chemical analyses of various lignin functionalities of wood. Unfortunately, a true depiction of native lignin is well nigh impossible given the difficulty of extracting a “pure” sample and its noncrystalline, highly amorphous nature.

Figure 3. The sulfidolytic cleavage of β-aryl ether bonds in propyl phenol units as part of the overall chemical process of kraft pulping.
fatty acids) and did not employ high quantum yield photosensitizing agents. We employed Rose Bengal (RB), whose structure is shown in Figure 4, that has a quantum yield of 0.76 for singlet oxygen production in water.\(^7\) Interestingly, RB aggregated easily with the lignin functionalities in the pulp as evidenced by its partitioning between the pulp and aqueous phase as a function of lignin content (the pulp uptake of RB was greatly enhanced with increasing amounts of lignin in the pulp). Since singlet oxygen is highly electrophilic, electron transfer reactions with lignin were highly probable, given free energies of several hundred millivolts. Presumably, \(\pi-\pi\) stacking interactions between the polyaromatic RB sensitizer and the aryl lignin units enhanced the tendency of RB to associate with the lignin.

The oxidative ability of \(1^O_2\) is also known to be affected by a variety of factors, including pH and concentration of hydroxyl anions. Work in the bleaching of textiles by \(1^O_2\) demonstrated that the optimal pH for \(1^O_2\) production is approximately 9-10.\(^8\) We determined that an untreated pulp sample that is allowed to stand in deionized water (pH = 6.0) for long periods of time (> 200 hours) would achieve a steady state pH of 9.2, roughly what is required for optimal \(1^O_2\) activity. Unfortunately, water tends to quench \(1^O_2\) via coupled vibrational interactions causing the lifetime window to be on the order of several microseconds. Nonetheless, the close proximity of the sensitizer to the lignin was sufficient to provide ample opportunity for the photochemistry. Although singlet oxygen reactions with lignin species are favorable as substantiated by past literature reports of lignin model compounds,\(^9,10\) lignin requires an alkaline environment to encourage its dissolution. We found that the best pH for removal of lignin was approximately 10. We found that we could photochemically remove approximately 85% of the lignin in a sample having a starting lignin content of about 3%. Figure 6 illustrates a generic reaction model for the manner in which singlet oxygen causes photobleaching.

**Reaction Conditions for Photochemistry**

All work was done in deionized water that had a concentration of 1-2% mass/mass of *P. taeda* pulp/water while the homogeneity of the suspension was maintained by thorough mixing. Figure 5 provides an illustration of the setup that was employed. The system was designed to capture the light emitted from the lamp, while the suspension was maintained at 45 °C for the duration of the experiment. We used a 0.5% mass/mass concentration of RB relative to pulp, but 17% relative to the amount of lignin in the starting sample (0.7 mmoles per 100 g of pulp sample).

Based on an average \(M_w\) of 50 kDa for lignin, the level of RB added corresponds to approximately 10 molecules of RB/\(C_9\) lignin monomer unit. Although this

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**Figure 4.** The chemical structure of Rose Bengal, the photosensitizer that was employed in our photobleaching schemes.

**Figure 5.** The photochemical apparatus that was employed for photobleaching of the softwood pulp samples. A 400 W Hanovia lamp was employed, while the sample was mixed and the internal temperature of the 1% pulp sample suspension was maintained at 45 °C with an ice bath.
appears high, two factors competing against its photobleaching activity are the overwhelming excess of carbohydrates in the *P. taeda* sample (97% of sample) and the depletion of RB by singlet oxygen.

**Photobleaching Proves to be a Promising Bleaching Process**

Several fundamental physical and chemical features were monitored to assess the ability of singlet oxygen to successfully bleach *P. taeda* samples: (1) reduction in total lignin content (photobleaching), (2) reduction in the overall chromophoric content of *P. taeda* (the “colored” lignin forms) and (3) impact on carbohydrates.

The photobleaching results are displayed in Figure 7. The effects of lignin leaching as a function of base content and heat are investigated separately with 0.1 N NaOH concentration and 45 °C (temperature achieved during the irradiation). Past work in lignin leaching (autodelignification based on residual NaOH content and temperature) suggests that 20% leaching at this temperature is not unusual, especially at protracted times after almost complete residual NaOH consumption. Interestingly, we observed 36% lignin removal even in the absence of the photosensitizer, which realistically is approximately 16% after accounting for lignin leaching. Delignification in the absence of RB is not unexpected since carbonyl groups in lignin (very small native mole-percentage, perhaps less than 1 percent) can photosensitize the formation of singlet oxygen.

RB provided significant delignification that was enhanced in an alkaline environment. Lignin removal up to 90% was surprising, but not unexpected given the reactive nature of singlet oxygen and the complete absence of RB after the irradiation from oxidation reactions.

A more fundamental investigation into the chemical basis for the effect of singlet oxygen on lignin was obtained by systematically extracting the lignin from pulp using published methods and reacting it with singlet oxygen generated chemically (hypochlorite/hydrogen peroxide) at 10% yield. We did not employ the lamp to avoid the direct photolysis of the lignin samples from the high intensity irradiation. We discovered that the carboxylic acid content of lignin increased by almost 40% (from an original value of 0.28 mmol COOH groups/g lignin), while the 5,5-aromatic lignin forms (condensed monomer structures) decreased by about 5%. The increase in acid groups helps to account for increased dissolution in alkaline environments, while the condensed lignin concentration decrease is probably a likely result of more favorable electron transfer reactions. In fact, pulps that had a significantly higher condensed lignin content were significantly more reactive toward singlet oxygen. This latter finding was supported during our studies of the decrease
The Spectrum

The spectrum in chromophoric content (lignin absorbance) of the pulp samples.

For example, we took *P. taeda* samples that had approximately 30-50% higher condensed 5,5-aromatic lignin contents (pulps that had been treated with high oxygen pressures to encourage radical coupling reactions), and found significantly increased delignification kinetics in these samples. Figure 8 clearly demonstrates the accelerated kinetics for the enriched condensed lignin sample over the sample containing “normal” lignin. Clearly, the enrichment in the condensed lignin structures exerts a profound influence on the manner in which the singlet oxygen behaves. This may be a function of the more favorable electrochemistry as indicated earlier in which the condensed structures have lower oxidation potentials from the resultant conjugation (coupling of aromatic units).

In general, singlet oxygen degrades native pulp lignin. Figure 9 provides a UV/VIS spectrum of the absorbance difference in a lignin sample from a standard kraft pulp degraded by singlet oxygen that is dissolved in 1/1/0.1 dioxane/water/0.1 N HCl (0.09 mg/L). The difference of approximately 0.01 absorbance units although not apparently significant is dramatically reflected in the color of the lignin samples. The singlet oxygen treated sample is light in color in comparison to the nontreated dark brown colored sample.

Finally, the issue of the carbohydrate chemistry is most important to the ultimate success of singlet oxygen as a viable bleaching chemical. The damage sustained by the carbohydrates in pulp samples is reflected by the intrinsic viscosity, a measure of the integrity of the cellulose polymer in the sample. Normally, cellulose chain lengths in kraft pulps are approximately 10,000 repeat glucose units that correspond roughly to a CED (cupriethylene diamine) viscosity of about 25-35 centipoise. The object of any pulping or bleaching technology is to maintain the natural viscosity to preserve the physical characteristics of the sample. Not surprisingly, although singlet oxygen attacks electrophilic structures with a high specificity, the byproducts of the reaction are not entirely discriminatory in their reactivity. For example, as shown in Figure 6, superoxide is a direct byproduct of the electrophilic aromatic attack and has been shown to disproportionate to various oxygen species such as hydroperoxyl radical and hydroxyl radical in the presence of metals. The latter oxygen species are responsible for carbohydrate degradation in *P. taeda* and have been controlled to some extent in this lab by the use of MgSO$_4$ that is generally thought to help complex various active metals such as Mn$^{2+}$ and Fe$^{2+}$ in a colloidal “solution” to prevent Fenton chemistry from occurring. Figure 10 displays the initial high selectivity displayed by the chemical bleaching system, not unlike what would be expected; yet, at 60% delignification, a steep drop in the integrity of the sample is observed. This peculiar finding may reflect the increased reactivity competition between the lignin and the carbohydrates for the local oxygen speciation.

We tested the ability of MgSO$_4$ to reduce the level of hydroxy radicals from hydrogen peroxide breakdown and thereby maintain the system viscosity. Figure 11 shows a typical profile of the sample viscosity as a function of time. We noticed, for example, that the use of bubbled oxygen in the reactor cavity increased the rate of carbohydrate degradation, presumable because of increased reactive specie concentration. On the other hand, including the Mg$^{2+}$ salt reduces this likelihood dramatically, but does

![Figure 8](image1.png)  
**Figure 8.** A plot of the changes in k/s, the absorption/scattering coefficient ratio, versus irradiation time for a normal lignin sample versus the same sample that is artificially enriched in condensed lignin units.

![Figure 9](image2.png)  
**Figure 9.** UV/VIS spectra of lignin dissolved in dioxane before and after singlet oxygen reaction.
not staunch it altogether, presumably because of local species competition at the lower lignin concentrations. The 60% delignification mark is reached at about 10 hours at which point the viscosity is well preserved; however, even with Mg$^{2+}$, the selectivity is not absolutely preserved. Further studies with more condensed lignin structures at lower concentrations (< 2%) suggest that despite the preferential reactivity of singlet oxygen with lignin, the ensuing radicals from the superoxide speciation are even more indiscriminatory in their reactivity and have greater opportunity to react with cellulose since the lignin levels are so much lower. Strangely enough, this indiscriminatory reactivity is beneficial for reducing the chromophoric content significantly (by approximately 50%) since the activity of the radicals is not mitigated.

**Concluding Remarks**

The studies described above have been part of a new study to explain the ability of reactive oxygen species such as singlet oxygen to selectively degrade the lignin in wood pulp. Our work above, for example, has demonstrated that despite the initial kinetics of the singlet oxygen-lignin reaction, reaction selectivity is a function of the oxygen species in the environment and reaction thermodynamics. Although cellulose is five to six times slower to react with hydroxy radicals than lignin,$^{15}$ its proclivity to react is exponentially increased when the lignin concentration in the environment decreases by 60%. Nonetheless, these studies represent the first known study to examine the photochemical ability of singlet oxygen to selectively degrade the lignin in *P. taeda*.

**References**


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Saint John’s wort has been in the news quite a lot in recent years. In the late 1980s, several studies showed that red pigments derived from this herb—hypericin and pseudo-hypericin—inactivated or blocked the reproduction of certain retroviruses in mice. This led to speculation that these compounds might be effective against human retroviruses, including the human immunodeficiency virus (HIV), the virus associated with AIDS. Indeed, subsequent preclinical studies showed that hypericin could destroy the equine infectious anemia virus and the related human immunodeficiency virus. By the start of 2001, no clinical studies have demonstrated that hypericin or Saint John’s wort is an effective treatment for AIDS, although research in this area continues.

Another line of research has shown that Saint John’s wort may effectively treat mild to moderate depression and seasonal affective disorder, while causing fewer side effects than certain standard anti-depressant drugs. Many studies have examined the biochemical basis of this anti-depressant effect. Surprisingly, crude extracts of the plant only exhibit weak activity in biochemical assays related to the neurochemical mechanisms of widely used antidepressants, such as inhibitors of monoamine oxidase (nardil and parnate) and serotonin reuptake (prozac, zoloft, and paxil). Crude extracts of Saint John’s wort have shown affinity for nerve cell receptors that normally bind to gamma-aminobutyric acid (GABA), an important neurotransmitter in the brain. Barbiturates, benzodiazepines (Valium and Xanax), and alcohol all exert their profound effects on the central nervous system by binding to GABA receptors. At present, it must be concluded that the mechanism by which Saint John’s wort alleviates depression is unknown. It is possible that the combined action of numerous mechanisms accounts for the overall effect. Future laboratory studies must be directed toward determination of the active ingredient(s) and its mechanism of action, and clinical studies should employ more standardized doses, longer lasting trials, and comparisons with more modern anti-depressants.

Study of the myriad of possible therapeutic effects of Saint John’s wort and hypericin continues, as it has at least since the time of John Gerard, a sixteenth century English herbalist. This gives many researchers high hopes for Saint John’s wort. At the same time, photobiologists have been studying the function of naturally-occurring hypericin-like pigments in two free-living microorganisms, *Stentor coerulus* (see Figure 1) and *Blepharisma japonicum* (see Figure 2). The hypericin-like pigments in these microorganisms, known as “stentorin” and “blepharismin”, are photoreceptive pigments that control cellular locomotion (see Figure 3). Understanding the mechanism by which these pigments control cellular movements may clarify the mechanisms by which hypericins act as anti-viral agents and Saint John’s wort as an antidepressant. At a more fundamental level, study of the light-induced movement responses of *Stentor* and *Blepharisma* may provide a more basic understanding of the chemistry of hypericins, a truly unique class of biological pigments.

**Biology of the Ciliates**

*Stentor coerulus* and *Blepharisma japonicum* are classified as Ciliates, a taxonomic group of single-celled, non-photosynthetic organisms that are covered with cilia, short whip-like appendages that beat back and forth to propel
them through the water or sweep food into their mouthlike openings. The cilia are typically arranged in precise and species-specific patterns on the surface of the cell and are firmly embedded in a one-micrometer thick protein-rich layer that coats the outside of the cell membrane. There are about eight thousand known species of ciliates, but probably many more unidentified species. The ciliates have little economic significance with the possible exception of *Balantidium coli*, a species that inhabits the human gut and causes a rare form of dysentery. *Stentor* and *Blepharisma* belong to a group of ciliates called the polyhymenophorans, all of which have complex ciliary structures. The polyhymenophoran ciliates are an ancient group, as fossils of these ciliates have been dated to 100 million years ago.

Biologists have studied light-induced responses of the ciliates for over 100 years. Sixteen species of ciliates exhibit some form of phototaxis, moving toward the light (positive phototaxis), away from the light (negative phototaxis), or perpendicular to the light direction (transverse phototaxis). Of these sixteen species, five—including *Stentor* and *Blepharisma*—are brightly colored due to the presence of vesicles that contain pigment granules. These vesicles lie between the rows of cilia, directly beneath the cell membrane. In all five brightly colored species, the pigment granules presumably contain the photoreceptive pigment that controls phototaxis.

The *Stentor* cell is typically about 0.35 millimeters long, although some strains can grow up to 2 millimeters in length. It assumes a pear-like shape when swimming, but is otherwise shaped like a trumpet. This species is named after the ancient Greek warrior Stentor, who, according to Homer, had a trumpet-like voice and “could cry out in as great a voice as fifty other men.” *Stentor* cells have longitudinal rows of granules (0.3 to 0.7 micrometers in diameter) that are blue-green in color because they contain the pigment stentorin, which strongly absorbs red and UV radiation.

*Blepharisma* is about the same size as *Stentor*, but is spindle-shaped. *Blepharisma* is named after the Greek word for eyelid (blepharon), because its long cilia resemble eyelashes. These cells have pigment granules (0.35 micrometers in diameter) that make them appear red under dim light because of the fluorescence emitted by the pigment blepharismin. *Blepharisma* turns blue-green under strong light, however, because blepharismin is transformed to “blue-blepharismin”, which is only weakly fluorescent.

**Behavioral Responses to Light**

*Stentor coeruleus* was one of the first ciliates used to study light-induced movement responses. A ring of hair-like cilia surrounds its large open end, which serves as a
mouth and anus and is most sensitive to light and other stimuli. More sparsely distributed cilia cover the rest of its surface. At the narrow posterior end lies the “foot”, which often attaches to stationary objects. Stentor avoids the light and tends to collect in shaded regions by using a trial and error method—it constantly rotates about its long axis to sample the light environment, then aims toward the darkness.

*Stentor* and *Blepharisma* exhibit step-up photophobic responses: an increase in the light level causes the cilia to reverse the direction of their beating, so that the cell stops, reverses direction, and moves out of the light. These two species also exhibit negative phototaxis, in that they move away from a directional light source. When cells are irradiated with a light beam that converges to a focal point and then diverges, they swim through the bright focal point and away from the light source. This indicates that they truly sense the light direction and not merely the fluence rate (“intensity”) of the light. In nature, the step-up photophobic response and negative phototaxis presumably allow *Stentor* and *Blepharisma* cells to move toward the bottom of a pond, where they can avoid zooplankton predators or develop into cysts—dormant and resistant capsules.

*Stentor* and *Blepharisma* are killed when exposed to very bright light. Thus, while a one second pulse of light at 0.1 W m$^{-2}$, roughly equivalent to the light level during late twilight, elicits a stop response in *Stentor*; light of 5000 W m$^{-2}$, roughly equivalent to the light level during a clear midday, kills the cells. Under high levels of light, stentorin generates reactive oxygen species that react with proteins, lipids, and other molecules and this damage eventually kills the cells. Light-induced generation of reactive oxygen species is also responsible for the phototoxic effects of hypericin given to human patients, many of the symptoms of the metabolic disease porphyria, and the harmful effects of bright sunlight on plants.

The effect of very bright light on *Blepharisma* is more complicated. Cells grown under dim light (which appear red due to fluorescence from the pigment blepharismin) are killed by light of 30 W m$^{-2}$ because reactive oxygen species are generated. If the cells are first exposed to light of about 3 W m$^{-2}$, however, they are transformed into blue or colorless cells that can survive treatment with light of 1500 W m$^{-2}$ for 20 minutes or more.

Since stentorin and blepharismin, the hypericin-like pigments of *Stentor* and *Blepharisma*, are responsible for the toxic effects of bright light, one may reasonably ask: Why synthesize these pigments in the first place? A possible answer is that in nature, *Stentor* and *Blepharisma* use these hypericin-like pigments to control movement of the cells out of dim light so they can avoid the phototoxic effect of very bright light.

### Chemistry of the Response to Light

The action spectrum for the photophobic response in *Stentor coeruleus* has a major peak near 610 nm, with minor peaks near 550 nm and 480 nm. This is similar to the absorption spectrum of a stentorin-binding protein that has been extracted from *Stentor* cells. In fact, the stentorin-binding protein is part of a large assembly of proteins that includes other proteins that do not bind stentorin. Blepharismin has been less intensively studied than stentorin. Research has shown, however, that the action spectrum for the photophobic response in *Blepharisma*, which has a major peak near 590 nm and minor peaks near 540 nm and 480 nm, is similar to the absorption spectrum of a blepharismin-binding protein.

As with all other photoreceptive pigments, the absorption of light by stentorin and blepharismin must generate chemical signals that are eventually converted into a physiological response—in this case, cell movement. While the generation of reactive forms of oxygen is clearly responsible for cell death under high levels of light, this reaction does not appear to play a role in the movement responses. Instead, an increase in acidity following absorption of light by these pigments appears to be a crucial chemical reaction needed for cell movement. Drugs that alter the internal acidity of the cells also reduce their responsiveness to light, but have little effect on the cells’ overall viability or responsiveness to other stimuli. The light-generated increase in acidity in *Stentor* apparently arises from the very rapid (~$10^{-12}$ second) transfer of a proton from the pigment stentorin to the associated stentorin-binding protein, and the subsequent release of this proton to the cytoplasm.

Recent studies suggest that the increase in acidity that follows light absorption also alters the electrical potential across the cell membranes of *Stentor* and *Blepharisma*. In particular, when microelectrodes are inserted into these cells, measurements show that the membranes have a resting potential of about -45 to -60 millivolts, meaning that the inside is more negative than the outside. About two tenths of a second after a pulse of light, the membrane undergoes a transient depolarization by about 20 millivolts, so that the difference between the inside and outside is smaller.

Such electrochemical responses are ubiquitous among organisms. Light depolarizes the photoreceptor cells of many invertebrates, even though they are only distantly related to ciliates and use a rhodopsin as the photoreceptive pigment.
pigment.\textsuperscript{36} In contrast, the rods and cones in the eyes of humans and other vertebrates hyperpolarize (become more negative) upon illumination.

A recent hypothesis links the acidifying effect of stentorin and blepharismin, the depolarization of the membrane, and cell movement.\textsuperscript{37} According to this hypothesis, an increase in cellular acidity alters certain membrane-bound proteins that regulate the flow of ions into and out of the cell and this depolarizes the cell membrane. Membrane depolarization, in turn, alters the properties of proteins responsible for transport of calcium ions into the cell, so that the cells take up more calcium. The increased concentration of intracellular calcium then alters certain biochemical reactions that control movement of the cilia. Experiments with specific inhibitors suggest that cGMP (cyclic guanosine monophosphate) plays an important role in transforming the light signal into a biochemical signal that controls cilia movement.\textsuperscript{38} This same compound is also important in controlling the vision of vertebrates and invertebrates.\textsuperscript{39}

Some people may think that study of the light-induced movement responses of insignificant microorganisms like \textit{Stentor} and \textit{Blepharisma} is an arcane pursuit with little significance to problems in the real world. However, the photo-receptive pigments in these organisms resemble hypericin, which has shown promise as an anti-viral agent. Moreover, hypericin is also present in Saint John’s wort, which appears to be an effective anti-depressant. In addition, the chemical pathways that \textit{Stentor} and \textit{Blepharisma} use to convert the light signals received by their pigments into physiological movement responses share many features with the physiological pathways used for vision, hormone signaling, and other responses in humans.

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**About the Author**

Peter A. Ensminger is a biomedical consultant and writer. This article is adapted from a chapter in his recent book, *Life Under the Sun* (2001, Yale University Press), a collection of photobiology essays. Ensminger graduated with honors from Rutgers University and earned a doctorate in biology from the University of Michigan. He was a post-doctoral fellow at Syracuse University and Cornell University and an Alexander von Humboldt Fellow at Freiburg University in Germany. More information about *Life Under the Sun* is available from the Yale University Press web site, <www.yale.edu/yup/lifesun> or the author’s own web site, <home.twcny.rr.com/geomanagement/ensmingr/lifesun.html>. Ensminger can be reached at <ensmingr@twcny.rr.com>.
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