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A Two-Photon Antenna for Photochemical Delivery of Nitric Oxide from a Water-Soluble, Dye-Derivatized Iron Nitrosyl Complex Using NIR Light

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Abstract: The experiments described here demonstrate the use of two-photon excitation (TPE) to sensitize nitric oxide (NO) release from a dye-derivatized iron/sulfur/nitrosyl cluster $Fe_2(\mu\text{-}RS)_2(NO)_4$ (**Fluor-RSE**, RS = 2-thioethyl ester of fluorescein) with near-infrared (NIR) light in the form of femtosecond pulses from a Ti:sapphire laser. TPE at 800 nm leads both to weak fluorescence from the organic chromophore at λ_{max} = 532 nm and to NO labilization from the cluster. Since the emission from the reference compound **Fluor-Et** (the ethyl ester of fluorescein) under identical conditions (50/50 CH_3CN/phosphate buffer (1 mM) at pH 7.4) is considerably more intense, the weaker emission from **Fluor-RSE** and the NO generation indicate that the fluorescein excited states initially formed by TPE are largely quenched by energy transfer to the cluster core. The two-photon absorption (TPA) cross section of **Fluor-RSE** at 800 nm was determined to be $\delta = 63 \pm 7$ GM via the TPA photoluminescence technique. This can be compared to the TPA cross section of 36 GM reported for fluorescein dye in pH 11 aqueous solution and of 32 ± 3 GM for **Fluor-Et** measured under conditions comparable to those used for **Fluor-RSE**. Pulse intensity dependence studies showed that the quantity of NO released from the latter as the result of NIR photoexcitation follows a quadratic relationship to excitation intensity, consistent with the expectation for a TPE process. These studies demonstrate the potential utility of a two-photon antenna for sensitization of the photochemical release of an active agent (in this case, NO) from a photoactive pro-drug.

Introduction

The biological messenger nitric oxide (nitrogen monoxide) is a key player in numerous mammalian functions, including vasodilation, immune response, and neurotransmission.[1] NO has also been demonstrated to be a γ-radiation sensitizer, and this property may prove useful in enhancing the therapeutic effects of radiation treatment of hypoxic tumors.[2] As a result, there is an interest in developing materials for the controlled delivery of NO to specific biological targets. The key words are "controlled" and "specific" since systemic release could have serious physiological effects, most importantly, markedly reduced blood pressure inducing shock. One strategy to circumvent such an undesired side effect would be to develop thermally stable NO precursors that can be photochemically triggered upon demand to release nitric oxide.[3] In this regard, studies in this laboratory have been concerned with characterizing the quantitative photochemistry of transition metal compounds that may serve this role, such as metal nitrosyl and nitrito complexes[4,6,7] and the iron/sulfur/nitrosyl clusters known as the Roussin's salts and esters.[5,8]

A desirable characteristic of photochemical drugs would be activation by excitation wavelengths effective for tissue trans-

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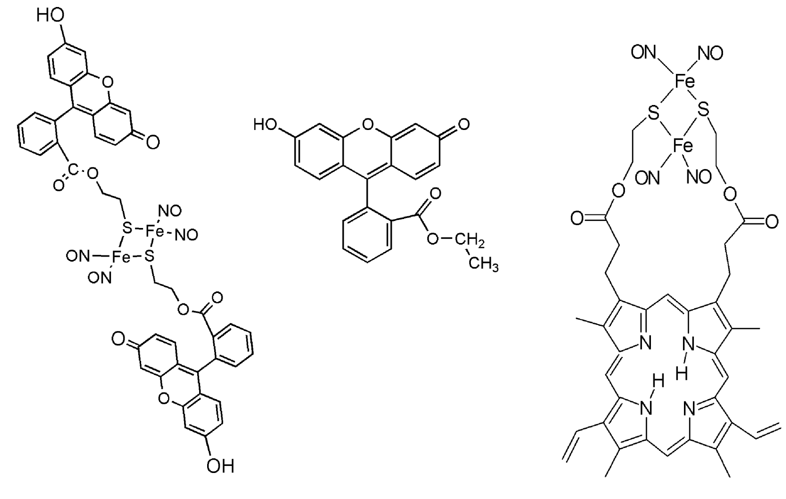

Fluor-RSE Fluor-Et PPIX-RSE

Figure 1. Formulas for **Fluor-RSE**, **Fluor-Et**, and **PPIX-RSE**.

mission. For this reason, our current focus is on designing compounds with antenna chromophores that have high absorption cross sections at longer wavelengths and that sensitize NO photochemical labilization from the linked metal center. We have demonstrated this for several supramolecular complexes whereby pendant chromophores led to markedly improved light gathering efficiency in the near-ultraviolet and visible wavelength ranges and consequently more effective photochemistry at the metal center serving as the nitric oxide precursor.[6d,8b] However, for mammalian tissue, light penetration is most favorable at a 800−1100 nm window in the near-infrared (NIR).[9] Since the excited states responsible for the desired NO release largely lie at higher energy, we have adopted a modified strategy to address this issue, namely, the use of an antenna with a sufficiently large cross section for two-photon excitation (TPE) at NIR wavelengths to serve as the sensitizer of the desired photochemistry. A second and perhaps equally important advantage of such multiple photon excitation is the much greater spatial resolution of TPE techniques, which is already a feature utilized with imaging via confocal microscopy and related methods.[10,11]

In this context, we have recently reported[12] the qualitative observation that TPE of the bichromophoric complex **PPIX-RSE** (Figure 1) with femtosecond pulses of 810 nm light leads both to weak emission from the protoporphyrin IX center with a $\lambda_{\max}$ of 632 nm and to photochemical generation of NO. This observation was interpreted in terms of the porphyrin chromophore acting as a two-photon antenna to sensitize the photoreactivity of the iron/sulfur/nitrosyl core. However, the solubility properties of **PPIX-RSE** and recognition that porphyrins are generally poor two-photon absorption (TPA) chromophores (for PPIX, $\delta = 2$ GM at 790 nm, where 1 GM = 10^{-50} cm^4 s photon^{-1})[13] led us to examine antennae with larger TPA cross sections. Described in the present article is an investigation of the two-photon photochemistry and photophysics of the supramolecular complex **Fluor-RSE**, which has two fluorescein moieties with much larger TPA cross sections ($\delta = 38$ GM at 782 for fluorescein in pH 11 aqueous solution)[14,15] as pendant groups on the **RRS** cluster (Figure 1).

Like **PPIX-RSE**, **Fluor-RSE** is a derivative of the iron/sulfur/nitrosyl compounds known as the Roussin's salts.[16] The Roussin's red salt anion (**RRS**, $Fe_2(\mu\text{-}S)_2(NO)_4{}^{2-}$) has been shown to be photoactive toward NO release with a moderately high quantum yield.[5] Furthermore, cell culture experiments (with V79 Chinese hamster lung cells) demonstrated that white light photolysis of **RRS**-treated cells simultaneous with γ-radiation exposure led to marked decreases in cell survival relative to controls also submitted to γ-radiation.[5a] This effect was attributed to sensitization of the radiation damage by concurrent photochemical NO release in the cells.[2] However, a limitation

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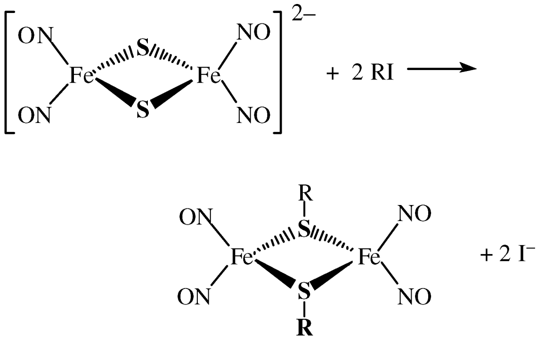
for Roussin's red salt in this regard is that the **RRS** chromophore does not absorb light appreciably at longer wavelengths. For this reason, related compounds known as the Roussin's red salt esters[17] (**RSE**, $Fe_2(\mu\text{-}SR)_2(NO)_4$) were prepared via reactions such as eq 1, and their photochemistry was investigated.[8] The premise of these studies is that the choice of the R substituent may allow control of optical properties and biological specificity of such materials.



(1)

Photochemical measurements involving direct excitation of the absorption bands characteristic of the iron/sulfur/nitrosyl clusters of the "simpler" RSE complexes (where R is an alkyl or aryl functionality) showed that these decomposed with moderate quantum yields ($\Phi_{RSE} = 0.02-0.13$) when irradiated in the near UV (366 nm).[8a] Furthermore, nearly 4 equiv of NO is released per mole of cluster undergoing photodecomposition. However, the spectra of these simple **RSE** are similar to that of **RRS** and accordingly display little absorptivity at longer visible wavelengths. To address the latter issue, **RSE** complexes were prepared with dye chromophores incorporated into the pendant R group, including protoporphyrin IX in the case of **PPIX-RSE** and fluorescein in the case of **Fluor-RSE** (Figure 1). Elsewhere, we have described the photochemical/physical properties of **PPIX-RSE**[8b] and **Fluor-RSE**[18] resulting from continuous photolysis with visible range light. The low intensity of the continuous photolysis excitation source defines these experiments as involving single-photon excitation (SPE). For both compounds, such excitation into the dye chromophore is followed by energy transfer to the iron/sulfur/nitrosyl core and sensitized reaction at that center, including NO release (see below). For the TPE experiments described here, the larger TPA cross section and the greater solubility in protic solvents make fluorescein a good candidate as a light-harvesting TPA antennae for the Roussin's clusters, and these properties are reported here for **Fluor-RSE**. The ethyl ester of fluorescein (**Fluor-Et**) was used as a metal-free model compound for comparisons.

Experimental Section

Materials. The synthesis and characterization of **Fluor-RSE** and **Fluor-Et** are described elsewhere.[18] Nanopure water was obtained for spectroscopic studies via a Millipore water purification system. Acetonitrile was distilled under dinitrogen from calcium hydride (CaH_2). Phosphate buffer solutions (1−10 mM) were prepared in Nanopure water, and their pH was adjusted to 7.4 using an Orion Research (Model 701 A) digital ionalyzer. Acetonitrile/phosphate buffer mixtures (50/50 v/v) used for spectroscopic studies were prepared by slowly diluting the buffered solutions with distilled acetonitrile.

All gases were purchased from Praxair Inc. and used as received unless further specified. Argon used for inert atmosphere preparation was passed through a Drierite/molecular sieve (4 Å) column to remove any remaining water. Solutions were deaerated by sparging with N_2 or Ar for approximately 1 min/mL or by three freeze−pump−thaw (f−p−t) cycles on a vacuum line. Inert atmosphere work was performed on a Schlenk line or in an argon-filled glovebox (VAC atmospheric company, Nexus model).

Instruments. UV−vis absorption spectra were measured using a HP8572 diode array spectrophotometer or Shimadzu dual beam UV-2401 PC spectrophotometer. All spectra were recorded in quartz UV−vis cells with 1.00 cm path lengths. Real time detection of nitric oxide in solution was accomplished using an amiNO-700 nitric oxide electrode from Innovative Instruments, Inc. The amiNO-700 is capable of quantitatively detecting nanomolar concentrations of NO in aqueous solution.

Two-Photon Excitation Fluorescent Measurements. TPE fluorescent measurements were carried out with the UCSB Optical Characterization Facility. Samples were excited with a tightly collimated (diameter ∼120 μm) high-intensity laser beam, and the corresponding up-converted fluorescence was detected at 90°. TPE was achieved using a mode-locked Ti:sapphire laser (Spectra Physics Tsunami) with excitation pulses of ∼100 fs and energy of ∼6 nJ operating with a repetition rate of 80 MHz. The emitted light from the sample was collected unto a high numerical aperture lens and focused onto the spectrometer. The radiation dispersed from the spectrometer was then detected by a thermoelectrically cooled CCD camera (Roper Scientific Spec 10:100B/TE). Solutions were irradiated for approximately 30 s with a central excitation wavelength (λ_{ex}) of 800 nm, and the corresponding TPE-induced fluorescence was monitored over the λ_{mon} range of 400−750 nm.

Two-Photon Absorption Cross Section Measurements. Although there are several experimental methods for determining molecular two-photon absorption (TPA) cross sections (δ),[19] all the measurements described in this work utilized the TPA photoluminescence technique described by Xu and Webb.[14] With this method, a sample was excited with a tightly collimated high-intensity laser beam ($d_0 \sim 120$ μm), and the emission was collected at 90° and detected (400−750 nm) using the spectrometer/CCD combination described in the previous paragraph. The integrated fluorescence intensity (480−700 nm) was utilized to determine the TPA cross section according to the equation

$$\delta = \frac{\Phi_{ref}\delta_{ref}c_{ref}P_{ref}^2 I}{\Phi c P^2 I_{ref}} K \tag{2}$$

where the subscript ref refers to the experimental measurements for the reference material under the same conditions, Φ is the photoluminescence quantum yield, c is the concentration, P is the average excitation power (P and P_{ref} were intentionally as similar as possible), K is a correction factor accounting for the difference in refractive indices for the respective reference and sample solutions, and I is the integrated fluorescence intensity. The derivation of this equation is described in greater detail in the Supporting Information. The reference utilized was fluorescein dye (10 μM in aqueous solution at pH 11), which has a $\delta = 38 \pm 9.7$ GM at 782 nm under these conditions.[14]

TPE with the NO Electrode. Nitric oxide concentrations photochemically generated were determined with a NO specific electrode (amiNO-700 from Innovative Instruments) that had been calibrated from 0 to 400 nM using injections of nitrite standards into acidified, aerated

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solutions containing the reducing agent sodium iodide. In these experiments, samples were prepared in the dark in aerated[22] solutions of 50/50 CH_3CN/phosphate buffer (10 mM) at pH 7.4, and then were irradiated at 800 nm with the Ti:sapphire laser for 30 s intervals under conditions analogous to those of the TPE photoluminescence measurements. The mixed solvent system was used owing to limited solubility of **Flour-RSE** in water and is the same medium used for previous SPE experiments.[18] Samples were placed into the optical train in a high-quality quartz UV−vis cell equipped with a mini stir flea. The nitric oxide electrode was then immersed into the solution and carefully positioned in order to avoid direct illumination by the excitation source. The solution was stirred using a micro stirrer. The quantity of NO photochemically produced via TPE was calculated by taking the difference between the minimum and maximum peak amplitudes before and after a photolysis interval and comparing this to the calibration curves. A neutral density filter wheel was utilized to attenuate the energy of the laser pulses to the desired level, and the energy of the pulses was determined using a thermopile optical power meter (Newport-815C).

Results and Discussion

TPE Fluorescence Measurements. Luminescence spectra of **Fluor-RSE** and the reference compound **Fluor-Et** have been recorded in this laboratory[18] using a continuous xenon lamp excitation source and monochromator set to 480 nm, under which conditions the excitation should be entirely in the SPE mode. Deoxygenated solutions of **Fluor-RSE** (1.88 μM) and **Fluor-Et** (1.80 μM) in 50/50 CH_3CN/phosphate buffer (10 mM) at pH 7.4 with matched absorbances (∼0.1) at λ_{exc} were used, and the emission spectra were integrated over the range of 485−700 nm. Although both samples displayed fluorescence (Figure 2), the integrated emission intensities for **Fluor-RSE** proved to be 15% that for **Fluor-Et**. Concomitant photochemistry of the iron/sulfur/nitrosyl cluster core (NO release) was also observed, thus in the former case, the principal decay pathway of the fluorescent state is apparently energy transfer from the light-gathering antennae to the iron/sulfur/nitrosyl cluster.

To test their potential TPE properties, similar deaerated solutions of **Fluor-RSE** and **Fluor-Et** were subjected to excitation using 800 nm light using the output from the Ti:sapphire laser (6 nJ pulses with half-widths of ∼100 fs at 80 MHz pulse frequency). The respective solution concentrations (3.81×10^{-6} and 1.92×10^{-5} M) were chosen to give similar absorbances (∼0.06) at the wavelength (400 nm) corresponding to twice the frequency of the excitation source. Notably, the recorded spectrum of neither solution displays any absorption at 800 nm; however, both displayed measurable fluorescence at energy greater than the excitation energy, indicating the operation of a TPE mechanism. The data shown in Figure 3 are the emission (450−750 nm) resulting from such 800 nm excitation of these solutions. The up-converted fluorescence was recorded for 30 s at a 90° angle from the excitation source. Notably, while both samples displayed emission as the result of two-photon excitation under these conditions, the integrated photoluminescence intensity for **Fluor-RSE** was 6.7% that of **Fluor-Et**. Since the two measurements were made under identical conditions, the ratio of the integrated TPE photoluminescence intensities can be calculated from eq 3.

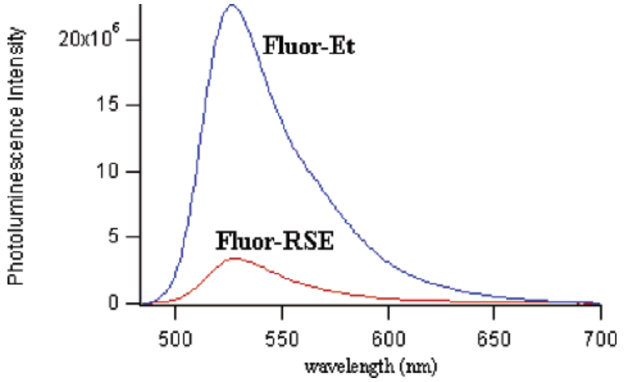

Figure 2. Steady-state luminescence spectra of **Fluor-Et** (1.80 μM) and **Fluor-RSE** (1.88 μM) in deoxygenated 50/50 CH_3CN/phosphate buffer (10 mM) solutions at pH 7.4 under argon, prepared with matched absorbances (∼0.1) at the excitation wavelength (480 nm).

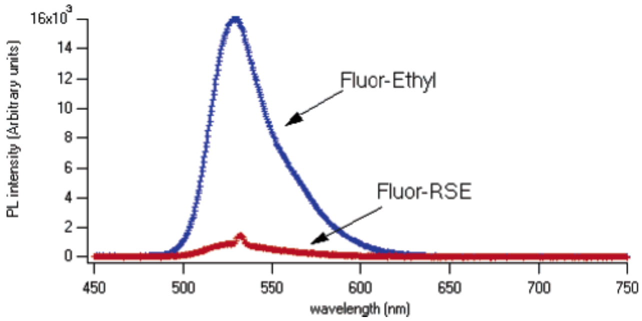

Figure 3. TPE PL spectra of **Fluor-RSE** (3.81×10^{-6} M) and **Fluor-Et** (1.92×10^{-5} M) in 50/50 CH_3CN/phosphate buffer (1 mM) at pH 7.4.

$$I_{\text{Fluor-RSE}}/I_{\text{Fluor-Et}} = \frac{\Phi_{\text{Fluor-RSE}}\delta_{\text{Fluor-RSE}}c_{\text{Fluor-RSE}}}{\Phi_{\text{Fluor-Et}}\delta_{\text{Fluor-Et}}c_{\text{Fluor-Et}}} \qquad (3)$$

Substituting the respective **Fluor-RSE** and **Fluor-Et** values for Φ (0.11 and 0.77),[18] δ (63 and 32 GM), and c (3.81×10^{-6} and 1.92×10^{-5} M) gives the predicted ratio of 5.6 ± 1.0%, in reasonable agreement with that determined experimentally.

TPA Cross Sections. The δ values were measured for **Fluor-RSE**, **Fluor-Et**, and fluorescein using the photoluminescence intensity ratio method described in the Experimental Section, with fluorescein in pH 11 aqueous solution ($\delta = 38$ GM at 782 nm)[14] used as the reference. For these measurements, solutions of **Fluor-RSE** and **Fluor-Et** were prepared in deaerated 50/50 CH_3CN/phosphate buffer (1 mM) at pH 7.4. The samples were

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(22) As we have earlier shown,[5,8] both Roussin's red salt and the red salt esters show much higher net photodecomposition when irradiated in the presence of dioxygen (usually air) than in deaerated solution. This effect can be attributed to oxygen trapping of the $Fe_2S_2(NO)_3$ clusters formed by NO photodissociation in competition to the fast back reaction with NO to regenerate the starting complex. Flash photolysis studies have confirmed this analysis. It is for this reason that luminescence measurements for the Roussin's salt esters with pendant chromophores are normally carried out in deaerated solutions in order to reduce the complications resulting from net photoreactions. However, when the goal is to evaluate the photoreaction chemistry itself, the photolyses are carried out in aerated solutions. Control experiments have demonstrated that the luminescence behavior of aerated and deaerated solutions of **Fluor-RSE** are initially equivalent; that is, there is no evidence of the luminescence being directly quenched or enhanced by the presence of dioxygen. The only effects in these cases are the results of different long-term stabilities of these solutions.

(23) Baker, E. S.; Bushnell, J. E.; Wecksler, S. R.; Lim, M. D.; Manard, M. E.; Dupuis, N. F.; Ford, P. C.; Bowers, M. T. *J. Am. Chem. Soc.* **2005**, *127*, 18222−18228.

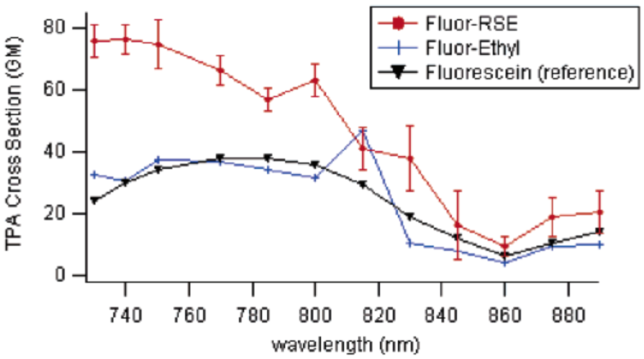

Figure 4. TPA cross section of **Fluor-RSE**, **Fluor-Et**, and fluorescein.

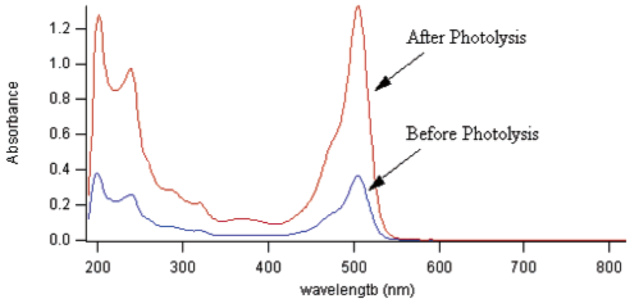

Figure 5. Optical absorption changes of **Fluor-RSE** (9.1 μM) in 50/50 CH_3CN/phosphate buffer (10 mM) at pH 7.4 before and after TPE at 800 nm for ~10 min of irradiation.

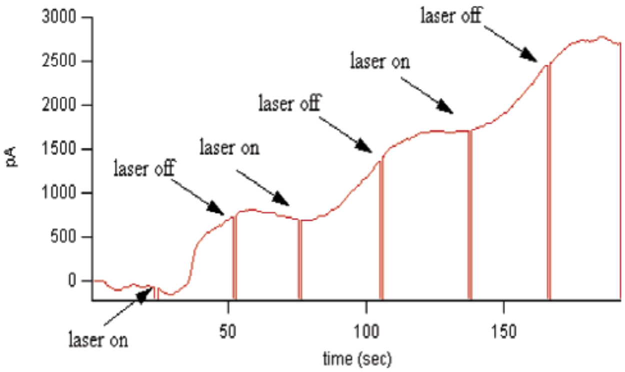

Figure 6. NO electrode response due to TPE of **Fluor-RSE** (9.1 μM) in 50/50 CH_3CN/phosphate buffer (10 mM) at pH 7.4 with ~100 fs pulses operating at a repetition rate of 80 MHz, a central wavelength at 800 nm, and an average power of 450 mW.

prepared in the dark, blanketed with argon, and kept from exposure to extraneous light before and during the measurement. Previous photochemical experiments of **Fluor-RSE** demonstrated that the complex undergoes photodecomposition to a more luminactive fluorophore.[18] Therefore, to avoid sample degradation, data collection times were limited, and each sample was only used for two data points in the δ versus λ_{ex} spectrum. Control experiments were performed under identical conditions to ensure the integrity of the samples during collection times, and in these experiments, the fluorescence intensity of **Fluor-RSE** changed less than 5% after TPE at 800 nm for a period of approximately 5 min.

Figure 4 shows the results of these TPA cross section measurements over the wavelength range of 730−890 nm. The δ values for fluorescein and **Fluor-Et** are similar, with $\delta = 38$ and 32 ± 3 GM, respectively, at 800 nm. Thus, the synthetic modification of fluorescein by forming the ethyl ester of the carboxylic acid functionality resulted in little change to the two-photon photophysics of this complex. Although the TPA cross section measurement for the **Fluor-RSE** showed a similar TPA spectral profile, the δ value proved to be somewhat higher at 800 nm and at shorter λ_{ex} than for the free chromophores. The reason for the enhanced δ observed for **Fluor-RSE** is unknown, but it may simply be the result of having two independent dye chromophores. On the other hand, δ is believed to correlate with the quadrupole moment of the molecule,[19−21] and given the molecular formula illustrated (Figure 1), it seems likely that **Fluor-RSE** with two pendant chromophores about an iron/sulfur/nitrosyl core would have a larger quadrupole moment than does **Fluor-Et** or fluorescein.

Does TPA by Fluor-RSE Lead to Photoreaction? Figure 5 illustrates the changes in the absorption spectrum of an aerated[22] solution of **Fluor-RSE** before and after TPE with 800 nm light (~100 fs pulses operating at 80 MHz with an average power of 450 mW) for a total of ~10 min of irradiation. Under these conditions, the changes in the optical spectra (principally strong absorption increases across the spectrum) closely parallel those seen for SPE studies carried out using continuous photolysis of analogous solutions with 436 nm light.[18] Clearly TPE with 800 nm light leads to sensitized photochemical reactions. As discussed with regard to the SPE photochemistry of **Fluor-RSE**,[18] we have no ready explanation for the absorption increases observed upon photodecomposition, other than to suggest that these may be due to differences in the pK_a's of the fluorescein chromophores between **Fluor-RSE** and the fluorescein ethyl thiolate photoproduct. Ion mobility mass spectrometry and computation experiments[23] are underway with the goal of gaining insight into three-dimensional structural features that might influence photophysical properties.

Photochemical Production of NO from TPE of Fluor-RSE with NIR Irradiation. The goal of these experiments was to establish quantitatively the NO production via reaction of the RRS cluster resulting from photosensitization by TPE of the pendant chromophores. A nitric oxide specific electrode and the procedures described in the Experimental Section were used to evaluate the NO concentration generated by 800 nm excitation of an aerated **Fluor-RSE** solution (9.1 μM) in 50/50 CH_3CN/phosphate buffer (10 mM) at pH 7.4. Immediately upon exposing the solution to the laser, the electrode displayed substantial current increases, indicating the photochemical production of NO, which leveled off once excitation was terminated (Figure 6). There is a lag time in the electrode response that we attribute to diffusion of NO from the excitation point to the electrode. This was also seen with SPE experiments.[18] Control experiments in which solutions were irradiated without the **Fluor-RSE** present showed no change in the current due to exposure to the laser. The concentrations of nitric oxide photochemically generated from TPE were obtained by taking

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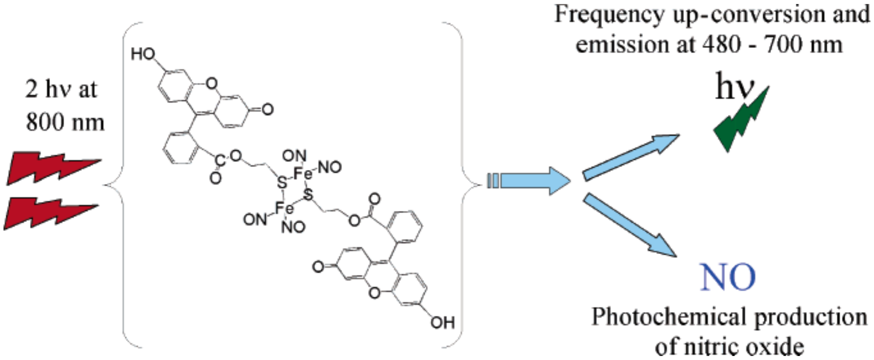
(24) Continuous photolysis investigations of Roussin's red salt and the simple esters demonstrated that net photodecomposition quantum yields (Φ_d) are dependent on the photon flux of excitation (I_a).[5,8] The Φ_d decreases somewhat upon increasing I_a in a manner consistent with reversible NO labilization to give cluster intermediates that are trapped by solution O_2 competitive with back reaction with NO to regenerate the starting complex. The extent of back reaction is dependent on the steady-state concentrations of NO and the cluster intermediates that are in turn dependent on I_a. Such considerations might have some modest impact upon Figure 5, thus explaining the slope slightly less than 2.0. However, the [NO] generated under TPE conditions is low, so that impact is likely to be minor.

Scheme 1. Schematic Illustrating the Consequences of TPA at 800 nm by **Fluor-RSE**



the difference between the minimum and maximum peak heights before and after an illumination period and calculated from the calibrations performed before and after photolysis.

Figure 6 illustrates such a NO electrode measurement for repeated TPE excitation for a single solution. In this experiment, the concentration of NO photochemically produced during the first 30 s of illumination was ∼4.7 nM, and if one assumes that the red salt esters photochemically generate ∼4 equiv of NO for each mole of RSE photodecomposition (as in SPE), then this would correspond to ∼0.015% depletion of the initial **Fluor-RSE** within this time period. Experiments of this type generally gave reproducible data within an experimental uncertainty of ±15%.

The observation of NO production via electrochemical detection and the changes in the optical absorption spectrum upon using 800 nm laser pulse to excite solutions of **Fluor-RSE** both point to the operation of a photochemical mechanism initiated by simultaneous two-photon absorption (Scheme 1). However, since it is possible that these observations might be the result of thermal excitation or some other linear process, it would be desirable to provide alternative evidence for a TPE process. According to the "formal intensity law",[19a] a photochemical or photophysical process can be described in terms of an intensity dependence. For example, the transition probability $W^{(n)}$ of an n photon absorption (nonresonant case) is a linear function of I^n, the specific case of TPA being proportional to I^2. Thus a plot of log $W^{(n)}$ versus log I should take the form log $W^{(n)} = n \log I + C$, where C is a constant independent of I. For a TPA process, the parameter being measured (photoluminescence intensity, NO release, etc.) must be a function of the intensity squared; that is, the slope of the log−log plot of the measured quantity versus intensity should equal 2.

In this context, experiments were undertaken in order to measure the quantitative NO release as a function of irradiation intensity. The experimental setup was that described previously for the TPE of **Fluor-RSE** in conjunction with the NO electrode, the only difference being that the irradiation intensity was varied by the use of neutral density filters. The irradiation intensities were measured using a thermopile optical power meter and were found to be 211, 375, and 450 mW for the lowest, medium, and highest intensities, respectively.[24] (Several measurements at lower irradiation intensity were attempted, but these resulted in minimal changes in the NO electrode current.) The samples were irradiated for 30 s while the [NO] was measured using the NO specific electrode. Three data points were collected for each sample, and the average [NO] for the photochemically released nitric oxide was determined using the calibration curves. The respective concentrations of NO detected in these measurements were 1.3 ± 0.2, 3.8 ± 0.5, and 4.7 ± 0.7 nM. The plot of log [NO]/s versus log P (Figure 7) displays a slope of 1.8 ± 0.15 consistent with the quadratic dependence of NO release on excitation intensity. In other words, this slope is consistent with a mechanism for nitric oxide generation resulting from two-photon excitation of the pendant chromophore(s) of **Fluor-RSE** with NIR light, followed by energy transfer to the cluster core resulting in sensitization of the photochemical production of NO.

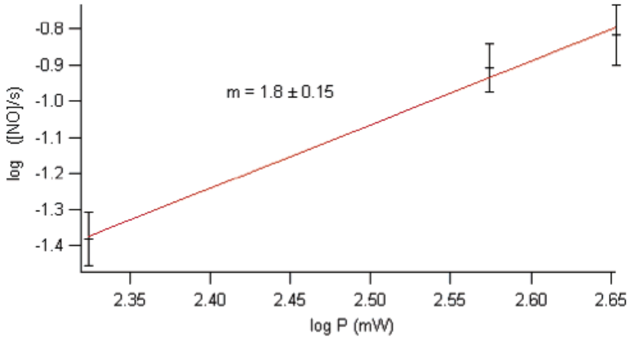


Figure 7. Log [NO]/s versus log P plot, where [NO]/s is the concentration of nitric oxide released in 30 s under 800 nm excitation, and P is the average power of the pulsed laser (100 fs pulses at 80 MHz) which is proportional to the pulse intensity I. Slope = 1.8 ± 0.15.

If it is assumed that the products and quantum yields of **Fluor-RSE** photoreactions are the same for TPE as for SPE, then one should be able to use the parameters of the experiments determined here to estimate the quantity of NO that should be photochemically generated by TPE under these conditions. From the rate equation for TPA by a molecule,[25] the intensity of light that is absorbed by this mechanism changes upon passing through a sample according to

$$\Delta I = \frac{\gamma \cdot l \cdot I_0^{\,2}}{\gamma \cdot l \cdot I_0 + 1} \tag{4}$$

where $\Delta I = I_0 - I$, I_0 is the input intensity for each laser pulse in W/cm^2, I is the transmitted light, and l is the path length (in cm). The term γ is defined as $\delta n/2h\omega$, where δ is the TPA cross section (in cm^4 s/photon), n is the concentration of the solution in molecules·cm^{-3}, h is Planck's constant (1.05 × 10^{-34} J·s), and ω is $2\pi c/\lambda$. I_0 is calculated from

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(25) Freidrich, D. M. *J. Chem. Educ.* **1982**, *59*, 472−481.

$$I_0 = \frac{4P}{\pi \cdot f \cdot \tau \cdot {d_0}^2} \tag{5}$$

where P is the (average) laser power (in W), f is the repetition rate, τ is the pulse duration, and d_0 is the diameter of the laser beam (assuming that the laser is collimated). If each pulse contains N_0 photons before entering the sample, and ΔN is the number of photons reduced by TPA, then the number of excited states generated by TPA from a single pulse will be $\Delta N/2$. Substituting into eq 4 gives

$$\frac{\Delta N}{N_0} = \frac{\Delta I}{I_0} = \frac{\gamma \cdot l \cdot I_0}{\gamma \cdot l \cdot I_0 + 1} \tag{6}$$

Therefore

$$\mathrm{ES}(\mathrm{pulse}^{-1}) = \frac{N_0}{2} \cdot \frac{\gamma \cdot l \cdot I_0}{\gamma \cdot l \cdot I_0 + 1} \tag{7}$$

where ES(pulse^{-1}) is the number of excited states formed by TPE in each pulse. Since $N_0 = P/(fh\omega)$, one can calculate the excited states formed per second from

$$\mathrm{ES}(\mathrm{s}^{-1}) = \frac{P}{2h\omega} \cdot \frac{B}{B+1} \tag{8}$$

where $B = \gamma\ l\ I_0$. Since $B \ll 1$, eq 7 simplifies to

$$\mathrm{ES}(\mathrm{s}^{-1}) = \frac{BP}{2h\omega} \tag{9}$$

For the average laser power (P) of 500 mW (0.5 W), a pulse duration (τ) of 100 fs, operating at a repetition rate (f) of 80 MHz, $\lambda_{\mathrm{exc}} = 800$ nm, and a beam diameter of 120 μ, I_0 is found to be 5.7×10^8 W/cm^2. The experimentally determined cross section (δ) for **Fluor-RSE** is 63 GM, or 6.3×10^{-49} cm^4 s/photon, at 800 nm. For these measurements, a quartz cuvette with a 1.0 cm path length, a sample volume of 3.5 mL, and a concentration of **Fluor-RSE** approximately 1.0×10^{-5} M were used. For these conditions, $\gamma = 7.56 \times 10^{-14}$ cm/W, entering these values into eq 5 estimates the rate of excited state formation under these experimental conditions to be ES = 1.51×10^{13} cm^{-3} s^{-1} or equivalently 21 nM/s.

We have determined previously from SPE experiments that the quantum yield for photodecomposition of **Fluor-RSE** at $\lambda_{\mathrm{irr}} = 436$ nm is $\Phi = 0.0036 \pm 0.005$, and that the compound releases 3.2 equiv of NO per mole of cluster decomposed.[18] Thus the quantum yield for NO release from this system is approximately 0.01 or 1.0% at 436 nm. According to this estimate, approximately 0.21 nM of NO per second upon TPE of **Fluor-RSE** at 800 nm would be generated, leading a concentration of NO of approximately 6.3 nM after 30 s. Experimentally, the amount of NO generated from TPA of **Fluor-RSE** for 30 s was found to be ~4.7 nM. Given a number of uncertainties inherent to the experiment and to the estimate (one being the assumption that the laser pulse is a uniform cylinder of light rather than having a Gaussian shape in time and in radial distribution), the estimated and experimental values are remarkably (more likely, fortuitously) close. Nonetheless, this calculation provides further support for the operation of a two-photon excitation mechanism.

Summary

These studies have successfully demonstrated the viability of utilizing two-photon excitation with near-infrared light for the photochemical activation of a model pro-drug, the fluorescein dye-derivatized iron/sulfur/nitrosyl complex **Fluor-RSE**. The potential advantages of using NIR TPE for photochemical drug delivery are 2-fold: deeper light penetration through tissue and enhanced spatial selectivity for drug activation. TPE photoluminescence measurements of **Fluor-RSE** show visible range emission from the fluorescein chromophore(s), indicating frequency up-conversion of the excitation light at 800 nm but at a marked decrease in PL intensity relative to that of the reference compound **Fluor-Et**. The decreased intensity is an indicator of efficient energy transfer from the fluorescein chromophores to the iron/sulfur cluster as seen previously with single-photon excitation with near UV wavelengths. The TPA cross section of **Fluor-RSE** was determined to be 63 ± 5 GM at 800 nm using the TPE PL technique, and this is a somewhat larger δ than that found for **Fluor-Et** or for fluorescein itself. Last, NIR TPE of **Fluor-RSE** leads to the release of NO into the solution as determined quantitatively using a NO specific electrode. The quadratic dependence of the NO generated on the excitation pulse intensities shows unequivocally that NO is being produced via a two-photon process.

Acknowledgment. These studies were supported by the National Science Foundation (CHE-0352650).

Supporting Information Available: Complete ref 21 and more detailed derivation of eq 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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