3-Dimensional Submicron Polymerization of Acrylamide by Multiphoton Excitation of Xanthene Dyes

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Introduction. The process of two-photon excitation (TPE) was first predicted in 1931 by Goppert-Mayer[1] and has been utilized among spectroscopists for some time.[2] With the advent of convenient femtosecond lasers the utility of multiphoton excitation processes has undergone a rebirth in the past few years. In particular, applications involving high-resolution fluorescence microscopy have greatly benefited. Since efficient TPE requires high peak laser power, the power can be precisely adjusted such that absorption only occurs at the point of focus, providing intrinsic three-dimensionality.[3] Recently, there has also been considerable interest in utilizing the inherent 3-D confinement in micron and submicron fabrication applications. Cheng and coworkers demonstrated that TPE could "write" three-dimensional patterns by completely photobleaching a fluorescent dye embedded in a polymer matrix.[4] Several groups have demonstrated TPE photolithography and photopolymerization in solvent-free systems.[5−9] Albota et al.[10] have developed photoactivators with very large TPE cross sections in order to improve the speed of the process.

In this report, we extend this concept to the more general solution chemistry situation of assembling building blocks within solvent to form 3-D structures. Our group's primary interest is in 3-D biological fabrication applications involving a variety of biomaterials, biosensors, and biomolecule spatial positioning, where the ability to fabricate from an aqueous environment is a fundamental requirement.[11] Polyacrylamide is a convenient model system due to both its biocompatibility and the fact that photopolymerization using dye/coinitiator systems has been well studied under one-photon excitation.[12−18] We compare the efficiency of TPE polymerization for several. Optical and scanning electron microscopy (SEM) were used to analyze resulting structures.

Experimental Section. a. Reagents. Photosensitizers were obtained from Sigma (rose bengal, eosin, erythrosin) or Exciton (rhodamine B, coumarin 519) and used without further purification. Acrylamide−bis(acrylamide) 29:1 (Fisher, electrophoresis grade) was prepared in PBS (pH 8). Triethanolamine (Aldrich) was used without further purification. Norlan #83H optical adhesive is a proprietary prepolymer urethane and photoiniator mixture that is UV photopolymerized.

b. Fabrication Instrument. The fabrication apparatus consists of a laser scanning confocal microscope (Biorad MRC600) modified for near-infrared excitation and a femtosecond titanium sapphire oscillator (Coherent 900-F). A schematic is shown in Figure 1a. The laser was operated at 800 nm with a repetition rate of 76 MHz at 100 fs pulse duration with an average power at the sample of approximately 100 mW.

The laser scanner is configured to perform line and rastor scans. Optical diagnostics are measured via either two-photon excited epi-illuminated fluorescence arising from the photoactivator or transmitted light. Line scans were repetitively performed until polymerization was observed via an appearance of a change in refractive index. While not rigorously quantitative, this method is suitable for comparison of changes of polymerization efficiency, and the results were self-consistent between sets of experiments.

c. TPE Cross Sections. To compare photosensitizer efficiency at a given wavelength, the TPE cross section values are required. These measurements were performed by an epi-illumination fluorescence setup as shown in Figure 1b. The fluorescence was separated with a long wave pass dichroic mirror, detected by a PMT (Hammamatsu R4632) and boxcar averaged (Stanford Research Systems SR250). The laser power was attenuated with a $\lambda/2$ plate and Glan-Laser polarizer, and power dependencies indicated that power levels used (50 mW) were not in saturation.

While absolute TPE cross sections are difficult to measure, a value relative to a known fluorophore can readily be determined. For example, a value for rose bengal was measured relative to the known fluorescein cross section[19] of 10^{-49} cm^4 s by using the following ratiometric expression:

$$\delta_{rb} = \frac{I_{rb}\delta_{fl}\phi_{fl}}{I_{fl}\phi_{rb}}$$

where I_{fl},δ_{fl}, ϕ_{fl}, and I_{rb}, δ_{rb}, ϕ_{rb} are the measured fluorescence intensities, TPE cross sections, and one-photon excited fluorescence quantum yields of fluorescein and rose bengal, respectively. Within the same family of chromophores it is reasonable to assume similar quantum yields for one- and two-photon excitation since the decay mechanism of a stationary state should be independent of the excitation pathway.

Results and Discussion. a. Photochemistry. The photosensitizers successful in TPE polymerization were the xanthene dyes rose bengal, erythrosin, and eosin Y. No polymerization was observed for coumarin 519 and rhodamine B. The photoexcitation scheme for rose bengal is shown in Figure 2. Simultaneous two-photon excitation at 800 nm occurs via a virtual state and accesses S_2 which undergoes rapid radiationless decay to S_1, interconverting to the nearby long-lived triplet state with near unit quantum efficiency. Note that for fluorescein-type chromophores the one-photon transition to S_2 is symmetry-forbidden but is the most intense transition in two-photon excitation, having a 820 nm

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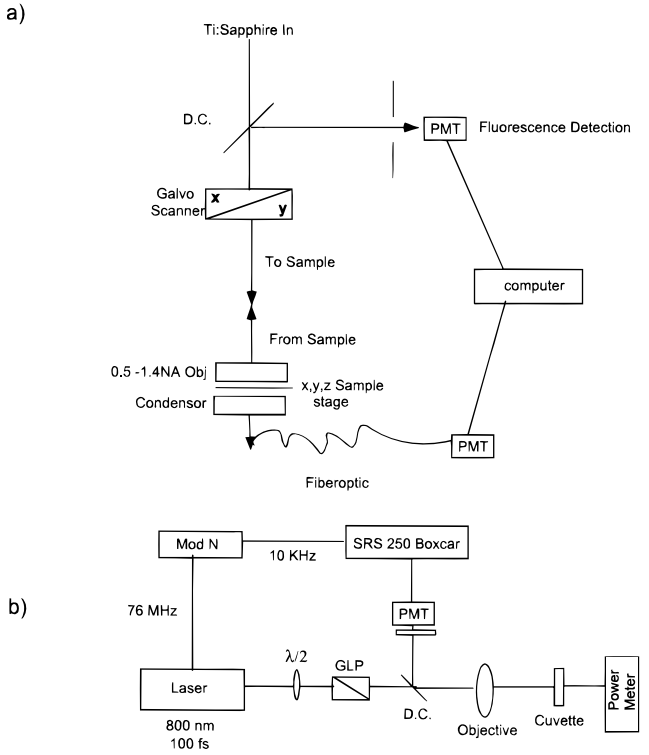

Figure 1. Schematics of the instruments used in these studies: (a) femtosecond titanium sapphire laser at 800 nm coupled to a modified Biorad MRC600 laser scanning confocal microscope; (b) epi-illumination setup for measuring relative TPE cross sections by fluorescence detection. D.C. = 800T/600R dichroic mirror and filter = BG-39 color glass IR blocking filter.

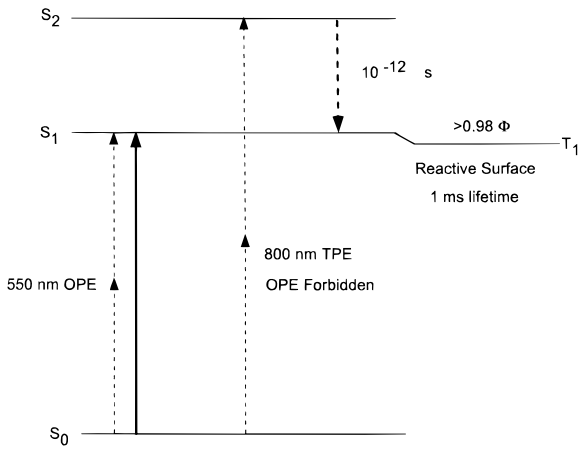

Figure 2. Joblanski diagram of the operative photophysics of TPE of rose bengal for preparation of the reactive triplet state. The dashed arrows indicate excitation is occurring through a virtual state. The other fluorescein derivatives have analogous photophysics.

maximum.[19] The subsequent free radical propagation steps involving the co-initiator (triethanolamine) have been well-studied and are not addressed here.[12−18]

b. Determination of Minimum Feature Sizes. In Figure 3a a transmitted light image of a stepped "pyramid" of polyacrylamide is shown, demonstrating the 3-dimensional nature of the process. This structure was fabricated using a mixture of 40 wt % acrylamide in PBS (pH 8), 0.1 M triethanolamine, and 2×10^{-4} M rose bengal. Optically, this structure was created using a 0.75 numerical aperture 20× objective by performing rastor scans of increasing optical zoom while concurrently changing the focal plane. This structure required a total fabrication time of approximately 1 min, corresponding to a fabrication rate on the order of 5 μm^3/ms. As a control to ensure the absorption occurred via two-photon process, the femtosecond laser was placed in continuous wave operation, and no photopolymerization was observed.

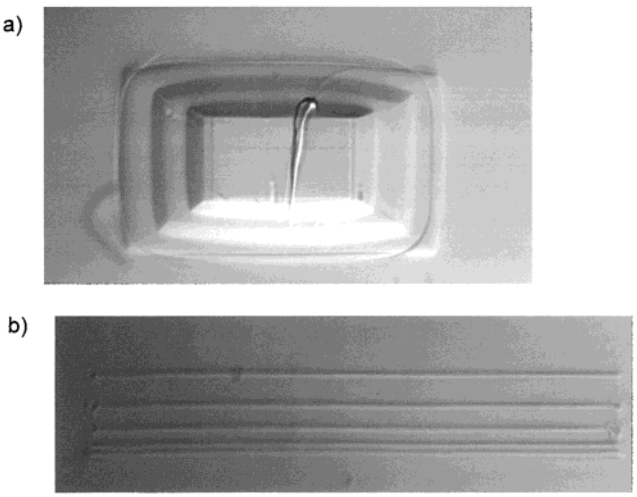

Figure 3. Transmitted light images of fabricated polyacrylamide objects using a 0.75 NA objective. (a) A three level "pyramid" with step height of 1 μm. The defect on the top level is due to an artifact in the laser scanner. (b) Five unevenly spaced rods with widths of approximately 500 nm.

A fundamental issue is the obtainable size limit achievable through this process. From the Abbe relation

$$d_{\min} = \frac{1.22\lambda}{2\mathrm{NA}}$$

where $d_{\min}$, λ, and NA are the minimum spot size, wavelength, and numerical aperture of the objective, respectively, lateral resolution of approximately 900 nm would be expected with a 0.75 NA objective at 800 nm excitation. The transmitted light image in Figure 3b shows five rods of polymerized acrylamide. We attempted to verify the resolution performance from these simple structures via SEM; however, due to the hydrogel nature of polyacrylamide, no samples survived the preparation. Similar rods were TPE fabricated (same optics) from a more durable (i.e., suitable for SEM) polyurethane optical adhesive (Norlan #83H). As determined by SEM, these structures resulted in lateral resolution of approximately 500 nm, indicating the resolution in TPE fabrication approaches that of the corresponding OPE wavelength.[20] Note that in all cases the final resolution may not be completely determined by the optical spot size since free-radical polymerization may extend somewhat beyond the focal volume before chain termination occurs. To estimate the axial resolution or minimum obtainable z thickness, SEM images of a fabricated polyurethane stepped "pyramid" were obtained at different projection angles. Analysis of the step function in each layer of the pyramid indicates that the z confinement was approximately 1000 nm.

c. Sensitizer Comparison. A major goal of this work is to compare the reaction efficiencies for various sensitizers. Both the TPE cross section, δ, and fluorescence quantum yield, ϕ, must be considered since the former relates to the number photoexcitation rate at constant power and the latter to the intersystem cross-

Table 1

photo-sensitizer	2-P cross section (10^{-50} cm^4 s) at 800 nm	fluorescence quantum yield	laser exposure (μs)
rose bengal	10	0.01	300
eosin	10	0.20	400
erythrosin	10	0.02	400
fluorescein	10	0.80	*a*
rhodamine B	200	0.5	*a*
coumarin 519	10	0.6	*a*

[a] Not observed.

ing efficiency to form the reactive triplet state. In the simplest picture, at constant laser power, the reaction efficiency should be proportional to δ/ϕ. Known TPE values were used for fluorescein and rhodamine B,[19] while those for erythrosin, eosin, and coumarin 519 were measured as described in the Experimental Section. The approximate TPE cross sections and exposure times are tabulated in Table 1. These numbers have errors of approximately 30−50% and are thus provided only as a guideline for comparison of efficiency.

Rose bengal, eosin Y, and erythrosin have approximately the same two-photon absorption cross sections and displayed very similar reaction efficiencies, despite having fluorescence quantum yields varying by approximately a factor of 20. This result shows that the reaction efficiency is a complex function, depending on a combination of factors including TPE cross section, fluorescence quantum yield, and electron affinities. For example, eosin has substantial bromine substitution whereas rose bengal and erythrosin are iodine-substituted: bromine has a larger electron affinity than iodine (78.2 vs 71.3 kcal/mol) and thus leads to enhanced efficiency by more facile electron abstraction from the co-initiator. This effect then compensates for the decreased triplet state production of eosin, resulting in similar polymerization efficiencies.

Both rhodamine B and coumarin 519 have large TPE cross sections, yet neither successfully induced TPE polymerization. Presumably the lack of reaction is due to inappropriate triplet state chemistry for reaction with TEA. More specifically, neither of these chromophores have significant halide substitution and thus have decreased enhanced electron affinities relative to the substituted fluorescein derivatives.

Conclusions. We have investigated the two-photon excited polymerization of acrylamide using several xanthene sensitizers. We have achieved minimum feature sizes in the range of 500 nm. TPE fabrication is expected to have excellent biocompatibility since polymerization in aqueous environments is readily achieved.

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