Communication

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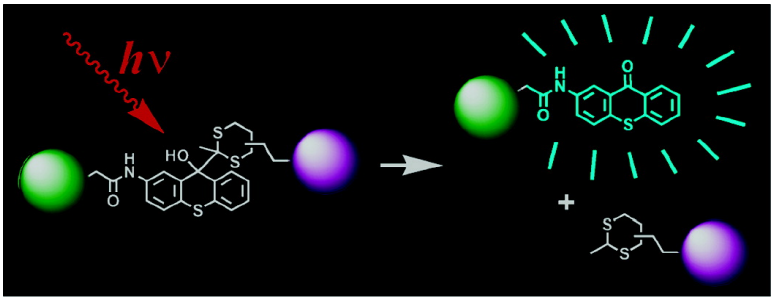

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Release and Report: A New Photolabile Caging System with a Two-Photon Fluorescence Reporting Function

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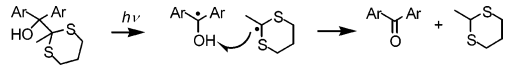
Photoinduced release of caged biological effectors has become a powerful research tool in molecular life sciences. For the past four decades, since it was first introduced by Barltrop,[1] the *o*-nitrobenzyl group in various modifications has been used for such caging, and the arsenal of photoremovable groups is rapidly growing.[2] It was later realized that it is important not only to release a biological effector but also to quantify its release and spatial distribution in real time by fluorescence microscopy. One existing solution involves tethering a fluorophore to a quencher via a photolabile linker, so that in the armed state most of the fluorescence is quenched.[3] Another approach is to cage a peripheral hydroxy group of a generic fluorophore.[4] The caveat is that the fluorophore's UV absorption remains higher than that of the caging group, interfering with the photorelease.

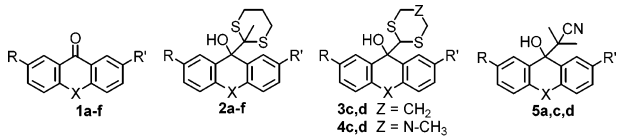
We suggest that disrupting the conjugation at the core of the fluorophore's π-system via the formation of a covalent C−C bond could be more advantageous for the design of the next generation *release and report* system. In this Communication we describe a novel photolabile system with a fluorescence reporting function based on diaryl ketones.

Addition of nucleophiles, including lithiated dithianes, to conjugated ketones disrupts conjugation, resulting in a blue shift of the absorption maxima and dramatic decrease in fluorescence intensity. We found that carbonyl compounds masked this way can be *readily* released by direct irradiation via homolytic C−C fragmentation followed by disproportionation of the two radicals. In cases when the diaryl ketone is fluorescent, the release is quantified by the emission intensity.



The best quantum yields of fragmentation were achieved when at least one of the two deconjugated aromatic moieties had heteroatom substituents. Radical leaving groups other than 1,3-dithia heterocycles were also used, such as isobutyronitrile (as in **5a**,**c**,**d**). Both the ketone and the dithiane component were outfitted with *handles* to furnish photolabile *latches* capable of linking or immobilizing biological effectors. We have previously synthesized a series of dithianes suitable for this function.[5]

We tested several aromatic ketones for photorelease and found that derivatives of 2-amidoxanthone and -thioxanthone are most suitable for our goals. One of the additional important criteria taken into consideration is that thioxanthones are long known to have large two-photon absorption cross sections and thus can be monitored by two-photon fluorescence microscopy. Recently it was noted that there is a need for fluorophores with absorption maxima of 380−420 nm to facilitate the use of powerful near-IR femtosecond sources in the range 760−840 nm.[6] Our fluorophores of choice, derivatives of 2-amidothioxanthones, have UV absorption bands in the vicinity of 400 nm, with strong emission at 458 nm. It is known that 2-alkoxy or hydroxy substituents enhance thioxanthone's fluorescence.[7] In screening thioxanthone derivatives for strong emission, we found that 2-amidothioxanthones have even higher fluorescence quantum yields, $\phi_{FL} = 0.64$ for the 2-butanoylamido fluorophore **1d** (MeCN, $\lambda_{max\ abs} = 398$ nm, $\lambda_{max\ em} = 458$ nm) and $\phi_{FL} = 0.79$ for the 2,7-diacetamido fluorophore **1e** (MeCN, $\lambda_{max\ abs} = 410$ nm, $\lambda_{max\ em} = 460$ nm). The 2-amido derivative of xanthone, **1b**, also showed strong emission: $\phi_{FL} = 0.73$, $\lambda_{max\ abs} = 354$ nm, and $\lambda_{max\ em} = 440$ nm.



Nucleophilic additions to the carbonyl groups of ketones **1a**−**f** reduce their fluorescence by 2 orders of magnitude. Yet, the remaining *p*-amidodiphenyl sulfide moiety has tailing absorption well above 300 nm, allowing for photodeprotection in a wide spectral area. The quantum yield of the fragmentation is wavelength dependent: for **2d** it is 0.56 at 365 nm and nearly unity at 320 nm. The relative quantum yield of fragmentation is highest for the 2-methyldithiane adducts **2**. Adducts of dithiane (**3**), dithiazine (**4**), and isobutyronitrile (**5**) cleave with about 30−60% relative quantum efficiency of the methyldithiane adducts.

Laser flash photolysis of **2d** (355 nm Nd:YAG) showed a weak absorption band below 400 nm, which we assigned by analogy to the 2-amidothioxanthenol radical.[7b] The lifetime of these species was 1.7 μs, and no further processes were detected, which puts an upper bound on the estimated time scale of this fragmentation.

Bulk photoreaction of **2d** at 320 nm (0.49 mW cm^{-2}) in acetonitrile solution was monitored by fluorescence of the product, i.e., ketone **1d**. Within less than 2 min, 90% conversion was achieved at this wavelength. Irradiation with the U-360 broad bandpass filter produced the same result in 10 min. Adduct **2d** has very weak fluorescence; the overall emission at 458 nm had increased by more than 2 orders of magnitude.

Below we exemplify the general concept of the thioxanthone-based *release and report* system with the release of the model compounds—dithianes or dithiazines—immobilized on TentaGel beads or PAMAM dendrimers. A typical synthetic procedure is outlined in Scheme 1. 2-Aminothioxanthone **6**[8] was acylated with glutaric anhydride and reacted with an excess of the nucleophile (lithiated dithiane is shown) to furnish **2f**, which was converted into the *N*-hydroxysuccinimide ester, **2f-NHS**, and incubated in an orbital shaker with either 90 μm TentaGel-NH_2 beads or PAMAM-NH_2 dendrimer. According to NMR and elemental analysis, approximately 99 out of 128 surface amino groups of the fifth generation dendrimer were actually immobilized after 60 h of gentle shaking.

A 10 mg sample of the **2f**-TentaGel beads was irradiated with a

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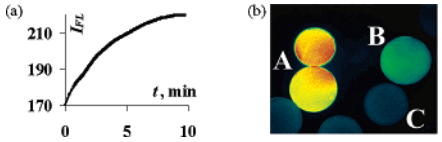


Figure 1. Fluorescence monitoring of the release from TentaGel beads: (a) overall curve of fluorescence recovery in 10 mg of TentaGel beads irradiated with a U-360 nm broad band filter, arbitrary units; (b) pseudocolor image of the beads (excited at 405 nm); see text.

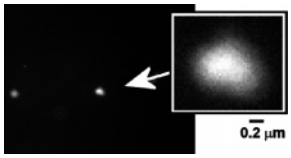
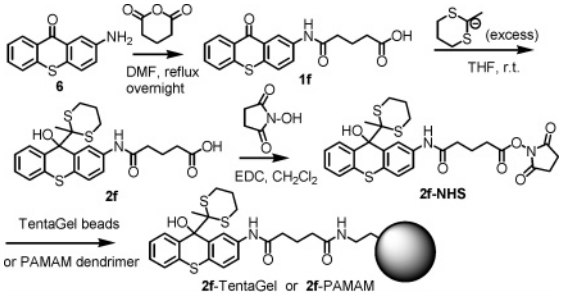


Figure 2. **2f**-PAMAM after 2 min irradiation in a Rayonet photoreactor.

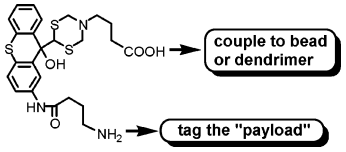
Scheme 1. Synthesis and Immobilization of **2f** on 90 μm TentaGel-NH_2 Beads or Fifth Generation PAMAM Dendrimer



U-360 broadband filter, and the total fluorescence was monitored (Figure 1a, arbitrary units). The resulting beads were mixed with the original **2f**-TentaGel beads and the blank TentaGel beads as a control for comparison. Figure 1b shows an image with all three bead types present: the blank beads (**C**, mean intensity 61.9), the intermediate **2f**-TentaGel beads (**B**, mean intensity 78.3), and the brightest irradiated beads (**A**, mean intensity 111.0).

Irradiation of **2f**-PAMAM produced a 17-fold increase in fluorescence intensity. The image of a brightly lit dendrimer molecule, Figure 2, was obtained by multiple dilutions in glycerol (used to slow diffusion[9]). The calculated diffusion path in glycerol during a 0.2 s CCD camera exposure time is 140 nm, which is in keeping with an ~200 nm bright inner spot (Figure 2, inset).

Such a massive photorelease of the payload molecules in the vicinity of a 6−7 nm dendrimer offers a way to create high local concentrations of the released compounds and at the same time image and quantify the release. A complementary approach is to release the effector, tagged with 2-amidothioxanthone, while immobilizing the radical leaving group. We implement this approach with dithianes or dithiazines outfitted with appropriate handles, such as amino acid-based dithiazines,[10] shown below, allowing for utilization of the well-developed peptide synthesis chemistry.



The photoreleased fragments, tagged by amidothioxanthone, can be monitored and quantified by two-photon fluorescence microscopy. Laser flash photolysis of ketone **1d** at 532 nm showed strong laser-induced fluorescence (LIF) with emission closely matching the spectrum generated at 355 nm (Figure 3a). The quadratic dependence of the LIF intensity on the relative laser power is shown in Figure 3b. The two-photon-induced fluorescence lifetime was also the same, ~4.5−4.8 ns.

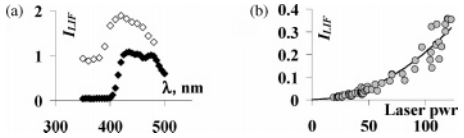


Figure 3. Ketone **1d**: (a) single- and two-photon LIF spectra exited at 355 nm (◇) and 532 nm (◆) respectively; (b) quadratic dependence of the LIF intensity on the power of the 532 nm laser pulses.

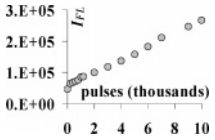


Figure 4. Two-photon-induced fragmentation of **2d** at 532 nm.

We further found that adduct **2d** itself possesses a considerable two-photon absorption cross section, allowing for the two-photon excitation to be used not only for fluorescence monitoring of the released ketone but also to effect the actual uncaging. Figure 4 shows fragmentation of **2d** (1 mM solution in acetonitrile), monitored by steady-state fluorescence of the generated ketone **1d** as a function of laser pulses. After 10K shots, fluorescence increased 5-fold.

Photobleaching of the reporter ketone was tested with a 405 nm-filtered, focused output of a medium-pressure mercury lamp (13 mW cm^{-2}, approximately 9.5 × 10^{19} photons/h). After 4 h of continuous irradiation of the 10^{-4} M solution, the fluorescence intensity decreased by only 7.1%.

To conclude, we have developed a new and efficient photocaging system capable of nearly instantaneous quantification of the released payloads by single- and two-photon fluorescence. The uncaging itself can also be initiated via a two-photon process.

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Supporting Information Available: Synthetic procedures, NMR spectra, and photochemical and photophysical data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) Stokes' hydrodynamic radius of the **1f** dendrimer is 3.2 nm, as calculated from the PFG NMR diffusion coefficient of 3.42 × 10^{-7} cm^2/s measured in DMSO-d_6. According to the Einstein equation, during the 200 ms exposure time of the CCD camera, a dendrimer of this size would travel about 9 μm in acetonitrile, which necessitated the use of a more viscous solvent, glycerol, for fluorescence imaging.
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