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Highly Activatable and Rapidly Releasable Caged Fluorescein Derivatives

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The cellular dynamics of living cells can be investigated by using functional small organic molecules, such as fluorescence probes.[1] One approach is to use caged compounds, in which critically important functional groups for bioactivity are protected with caging groups (photoremovable protecting groups),[2] which can be removed upon brief illumination with ultraviolet (UV) light to release the original bioactive molecules, such as neurotransmitters, second messengers, and drugs. It is then possible to supply these molecules with high temporal and spatial resolution in a noninvasive manner by controlling both the time and application site of the excitation light. Caged fluorophores have proved to be very useful for investigating cell lineage or for fluorescence labeling of cellular proteins.[3] Desirable properties for a caged fluorophore include fast release of the fluorophore and a large enhancement of the fluorescence in response to brief irradiation, in order to minimize cell damage. Among many caged fluorophores, caged fluorescein is one of the most popular dyes because fluorescein has many advantages for bioimaging, including high fluorescence quantum efficiency, high extinction coefficient around 490 nm, and high water solubility under physiological conditions. However, traditional caged fluoresceins have various drawbacks. Since almost all of the reported caged fluoresceins are bis-caged lactone forms, full activation of them requires deprotection of two photoremovable protecting groups. Thus, full fluorophore release and a large fluorescence increase require quite prolonged irradiation with high-intensity UV light. Moreover, photobyproducts derived from caging groups may show cytotoxicity, as in the case of 2-nitrobenzyl derivatives. Therefore, for several reasons, it would be preferable to use a single caging group in a molecule. Krafft et al. reported a mono-caged fluorescein ether fixed in the lactone form which had a single caging group, but the photoactivated product was less fluorescent than fluorescein.[4] There is thus a need for a rapidly releasable and highly activatable caged fluorescein for bioimaging.

In this study, we report novel caged fluorescein derivatives which showed rapid uncaging and a large fluorescence enhancement. We first synthesized mono-2-nitrobenzyl-caged fluorescein (MonoNB-FL) according to Scheme S1. Since MonoNB-FL has a single protecting group, we expected that it would be more rapidly activated than commercially available bis(5-carboxymethoxy-2-nitrobenzyl)-caged fluorescein (BisCMNB-FL, Figure 1A). As expected, MonoNB-FL showed a faster increase of fluorescence than BisCMNB-FL upon 20 s irradiation with UV light at around 350 nm in 100 mM sodium phosphate buffer (pH 7.4), as shown in Figure 2B. However, MonoNB-FL has a high fluorescence quantum efficiency ($\Phi = 0.117$) before photoactivation. Generally, nitro-containing dyes have almost no fluorescence due to quenching of the singlet excited state by photoinduced electron-transfer (PeT) process.[5] However, in this case, PeT from the xanthene moiety to the nitrobenzyl group is less favorable because the electron density of the xanthene moiety is lowered by the electron-withdrawing effect of the nitrobenzyl group. Although MonoNB-FL was easily releasable, its high background fluorescence before photoactivation would result in a low signal-to-noise ratio, and therefore it is not expected to be suitable for bioimaging.

Next, we tried lowering the fluorescence quantum efficiency before photoactivation by using TokyoGreen instead of fluorescein. We have shown that the fluorescent properties of a wide range of

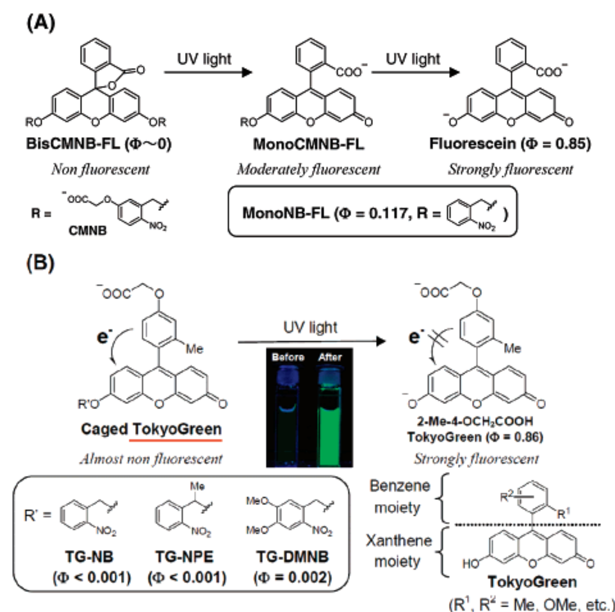

Figure 1. (A) Uncaging scheme of caged fluorescein. (B) Uncaging scheme and structures of caged TokyoGreen. The pictures were taken before and after UV irradiation of an aqueous solution of TG-NPE (1 μM).

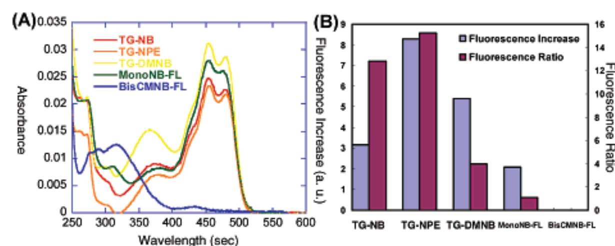

Figure 2. (A) Absorption spectra of caged compounds (1 μM) in 100 mM sodium phosphate buffer, pH 7.4, containing 0.1% DMF as a cosolvent. (B) Fluorescence increase and fluorescence ratio values at the emission maximum wavelength (caged fluorescein: 516 nm, caged TokyoGreen: 513 nm) upon UV irradiation at around 350 nm for 20 s. Fluorescence increase and fluorescence ratio denote the increase and the ratio, respectively, of the fluorescence intensity after 20 s UV irradiation with respect to the fluorescence before irradiation at the emission maximum wavelength.

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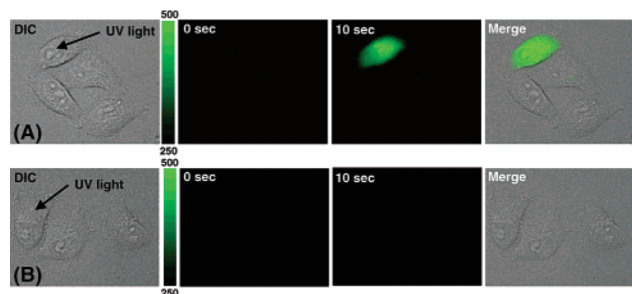

Figure 3. Cell application of (A) TG-NPE AM and (B) BisCMNB-FL AM. Differential interference contrast (DIC), fluorescence images before (0 s) and after (10 s) irradiation, and merge.

fluorophores, including fluorescein, rhodamine, and BODIPY, can be controlled precisely by utilizing the concept of PeT.[6] TokyoGreens are fluorescein derivatives in which the carboxylic group of fluorescein is replaced with a methyl or methoxy group, and their fluorescence quantum yield can be precisely controlled by adjusting the oxidation potential of their benzene moiety.[7,8] On the basis of the TokyoGreen platform, we have designed and synthesized three caged TokyoGreens (Figure 1B) according to Scheme S2. In these derivatives, that is, the 2-nitrobenzyl (TG-NB), 1-(2-nitrophenyl)ethyl (TG-NPE), and 4,5-dimethoxy-2-nitrobenzyl (TG-DMNB) derivatives, the oxidation potential of the benzene moiety is finely tuned to show a drastic change of fluorescence upon cleavage of the caging group. Before photoactivation, caged TokyoGreen should be almost nonfluorescent because of quenching of the singlet excited state via the PeT process, while removal of the caging group by UV illumination should result in a large fluorescence enhancement, as the PeT process becomes less favorable. As expected, the fluorescence quantum efficiency was decreased to less than 1/100 of that of MonoNB-FL in the cases of TG-NB and TG-NPE and less than 1/50 in the case of TG-DMNB. Low background fluorescence was thus achieved by rational molecular design based on the TokyoGreen platform. All three derivatives showed almost the same spectral features, except that TG-DMNB had a higher extinction coefficient at around 300−400 nm, as reported elsewhere (Figure 2A).[9] Next, aqueous solutions of the caged compounds (1 μM in 100 mM sodium phosphate buffer, pH 7.4) were illuminated with UV light in a cuvette of a monochromator system. In practical applications, a rapid increase of fluorescence upon brief irradiation is critical to minimize cell damage. Figure 2B shows the fluorescence increase and ratio values at the emission maximum wavelength upon brief irradiation (330−370 nm, 1.88 mW/cm^2 at 350 nm, 20 s). The fluorescence increase was the largest with TG-NPE, followed by TG-DMNB, TG-NB, MonoNB-FL, and BisCMNB-FL; indeed, BisCMNB-FL showed no fluorescence change in this setting. The fluorescence ratio was the largest for TG-NPE, followed by TG-NB, TG-DMNB, and MonoNB-FL. That of BisCMNB-FL could not be determined. The uncaging quantum efficiency of TG-NPE was determined to be 0.03 by means of HPLC analyses, being slightly smaller than that of BisCMNB-FL (Supporting Information). In cuvette experiments, the caged TokyoGreens were clearly superior to commercially available caged fluorescein in terms of fluorescence enhancement. Among our compounds, TG-NPE appeared to be the best, affording the greatest fluorescence activation upon brief UV irradiation.

To determine the effectiveness of caged TokyoGreen in a biological system, we examined the fluorescence labeling of single living cells. HeLa cells were incubated with TG-NPE AM (1 μM), the membrane-permeable acetoxymethyl ester of TG-NPE, and a selected cell was irradiated with UV light (330−385 nm) from a 100 W high-pressure mercury lamp through the objective lens of a fluorescence microscope. After irradiation for 10 s, the irradiated cell became brightly fluorescent (Figure 3A). In contrast, similar UV irradiation (10 s) of BisCMNB-FL AM (1 μM) loaded cells did not result in any detectable fluorescence increase (Figure 3B). Even after longer irradiation (up to 600 s) or the use of a higher BisCMNB-FL AM concentration (10 μM), the fluorescence intensity did not increase as much as that of TG-NPE AM. No cytotoxicity of TG-NPE AM was observed under these experimental conditions with the use of the Calcein AM/EthD-1 assay. Thus, caged TokyoGreen was concluded to be superior to traditional caged fluoresceins. Furthermore, a macromolecular TG-NPE-dextran conjugate was prepared, and it was confirmed that caged TokyoGreen also worked in this context, in both cuvette and cell systems (Supporting Information).

In conclusion, we have designed and developed novel caged fluorescein derivatives (caged TokyoGreens) which are rapidly activatable upon brief irradiation and show a large fluorescence enhancement. In a biological application, fluorescence labeling of a single living cell was far more efficient with TG-NPE AM than with a traditional caged fluorescein. Moreover, in this TokyoGreen-based molecular design, a wide variety of photoremovable protecting groups susceptible to different wavelengths of uncaging light could be used to create designed-to-order caged fluorophores for various experimental conditions. We believe caged TokyoGreen represents a breakthrough in caged fluorophore technology.

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Supporting Information Available: Synthesis and characterization of caged TokyoGreens, and experimental detail of photoirradiation experiments in cuvette and cell assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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