Communication

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Photoactivatable and Water Soluble FRET Dyes with High Uncaging Cross Section

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The superb sensitivity of fluorescence detection has enabled its broad applications. To apply fluorescence microscopy to follow the trajectory, speed, and timing of molecular and cellular movements in biological systems, photoactivatable fluorophores (caged dyes) serve as powerful imaging probes providing information of high spatiotemporal resolution.[1−4] Recent rapid progress in imaging technology raises new demands for photoactivatable dyes with improved properties including, among others, good water solubility and biocompatibility, photostability of parent fluorophores, and high photolytic efficiency by both one photon and multiphoton excitations.

We recently developed a new class of caged coumarins with several major improvements.[5] The photolytic efficiencies of these 1-(2-nitrophenyl)ethyl (NPE)-caged 7-hydroxy coumarin 3-carboxamides are exceptionally high, with one photon uncaging cross sections (product of the uncaging quantum yield Q_u and extinction coefficient ϵ) above 6000 $M^{-1}cm^{-1}$ at 365 nm and two photon uncaging cross sections over 0.37 Goeppert-Mayer (GM, 1GM = 10^{-50} cm^4s/photon) at 740 nm.[5,6] Mechanistic studies suggests that the high uncaging efficiency of NPE-caged coumarins is mediated through the process of "substrate assisted photolysis", in which the photonic energy absorbed by coumarin chromophore is utilized to cleave the NPE cage.[5] Such a process appears to be unique to coumarin dyes because the UV uncaging efficiencies of other caged fluorophores are nearly 2 orders of magnitude lower.[1,7−9]

To extend this development to prepare caged fluorophores emitting different colors, we designed a new caged green dye, CCC-1 (***C***aged ***C***oumarin-***C***alcein, **1**, Figure 1A), based on the principle of fluorescence resonance energy transfer (FRET). CCC-1 contains a NPE-caged coumarin 3-carboxamide and a calcein 6-carboxamide connected by a cyclohexyl linker. There is an extensive spectral overlap between coumarin emission and fluorescein excitation, a property that has been exploited to design FRET sensors of reporter enzymes.[10,11] Among green emitting xanthene dyes, we chose calcein as the energy acceptor because it has good water solubility and long cytoplasmic retention time, two desirable features for labeling and long-term tracking of biological specimens.[12]

CCC-1 was synthesized from coumarin and fluorescein derivatives in 7 steps (Supplementary Figure 1, Supporting Information). After coupling calcein diacetate **6** with NPE-caged coumarin **4**, we separated two regio-isomers containing either calcein 5-carboxamide or calcein 6-carboxamide (Supplementary Figure 1, compounds **7** and **8**, respectively). To generate CCC-1, we attempted to remove *tert*-butyl ester and phenoxy acetate sequentially by acid and base hydrolysis. While deprotection of *tert*-butyl ester with trifluoroacetic acid (TFA) proceeded to completion in less than an hour, subsequent alkaline hydrolysis of phenoxy acetate was accompanied by the concomitant degradation of coumarin lactone even under carefully controlled conditions. We eventually found that phenoxy acetate could be removed by prolonged treatment of the sample with TFA. This one step procedure provided CCC-1 in quantitative yield.

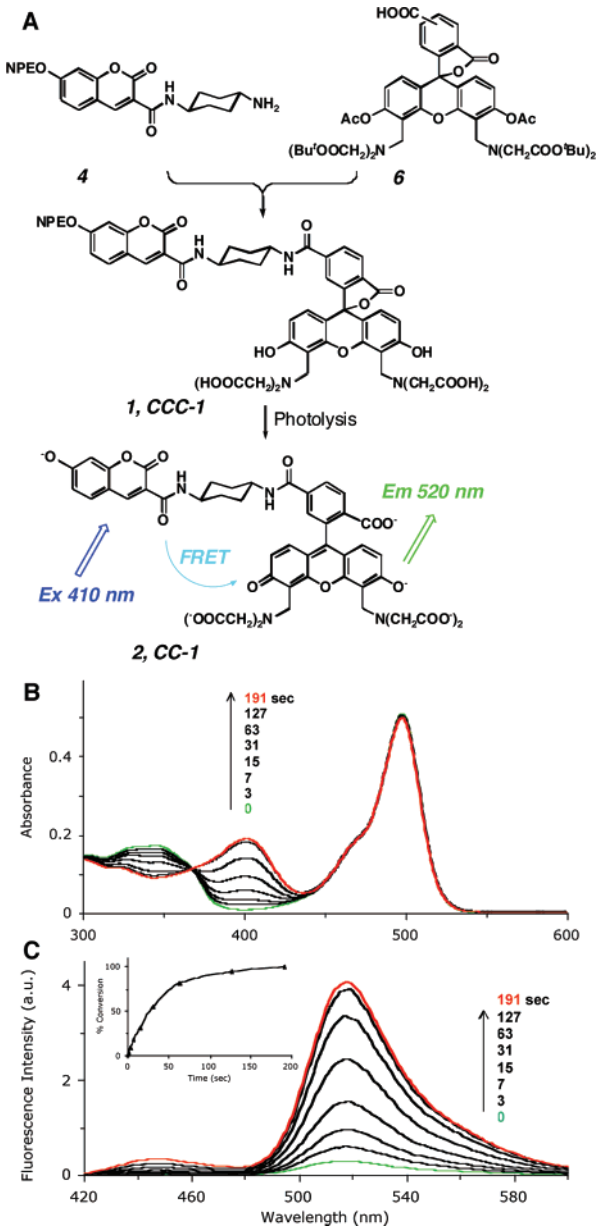

Figure 1. (A) Design and synthesis of CCC-1. (B) Absorption of CCC-1 before (0 s) and after increasing doses (in seconds) of UV illumination (365 nm). (C) Fluorescence emission of CCC-1 (Ex 410 nm) before (0 s) and after UV illumination. The insert shows the conversion of CCC-1 as measured from the fluorescence intensity increase at 520 nm. All spectra were taken in an aqueous buffer containing 20 mM Mops, 100 mM KCl, pH 7.3.

10.1021/ja071427+ CCC: $37.00

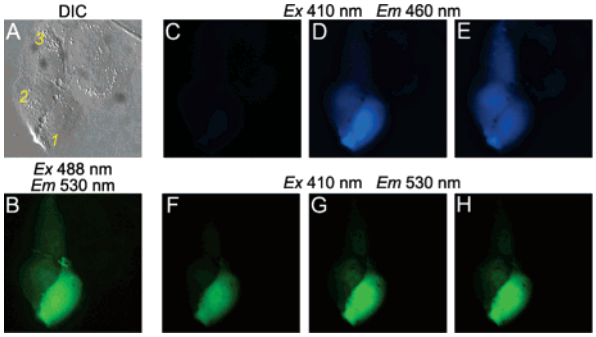

Figure 2. Uncaging and imaging of CCC-1/Dextran in living cells. CCC-1/Dextran was injected into selected MDCK cells (cell 1 in A) expressing Cx43-Venus. Cells were then loaded with NPE-HCCC2/AM. (A, B) Differential interference contrast (A) and green fluorescence (B) images of cells. (C−E). Coumarin (HCCC2) fluorescence of cells before (C), shortly after (D), and ∼2 min after (E) a localized uncaging of cell 1. (F−H). Corresponding green emission (Ex 410 nm) of CCC-1/Dextran before (F), shortly after (G), and ∼2 min after (H) uncaging.

CCC-1 shows two major absorption peaks at 346 and 497 nm, with corresponding ϵ of 28 000 and 80 000 $M^{-1}cm^{-1}$ (Figure 1B). These peaks result from $\pi-\pi^*$ electronic transitions of NPE-caged coumarin and calcein 6-carboxamide, respectively. Photolysis of CCC-1 at 365 nm changed the absorption spectrum in a UV light dose-dependent manner: the absorption near 346 nm decreased progressively and a new peak around 405 nm appeared. The new absorption peak corresponds to the coumarin 3-carboxamide moiety of the photolytic product, CC-1 (**2**, Figure 1A). During the photolysis, the absorption from the calcein moiety near 500 nm changed little (Figure 1B).

Fluorescence measurements were consistent with the absorption data. Prior to uncaging, excitation of CCC-1 at 497 nm generated an emission spectrum similar to that of calcein, showing the peak emission at 520 nm (Supplementary Figure 2, Supporting Information). Excitation of CCC-1 at 410 nm produced a similar spectrum but of much lower intensity (Figure 1C). This weak emission was from the direct excitation of the calcein 6-carboxamide of CCC-1, whose absorption extends below 400 nm. Coumarin emission intensity of CCC-1 between 420 and 470 nm was very low, consistent with our previous measurement showing that the fluorescence quantum yield of NPE-caged coumarin was only 0.25%.[5] UV illumination progressively increased fluorescence intensity of the sample. The amplitude of increase was much more pronounced above 480 nm, suggesting efficient energy transfer from coumarin to calcein (Figure 1C). At the end of photolysis, emission intensity at 520 nm increased more than 14 times using 410 nm excitation. From the time course of uncaging (Figure 1C, insert), we calculated Q_u of CCC-1 to be about 0.3 at 365 nm, and the uncaging cross section to be 6000 $M^{-1}cm^{-1}$, over a hundred times higher than that of a caged fluorescein.[9]

The photolyzed product, CC-1, has a FRET efficiency of about 95%, estimated by comparing the emission intensity of CC-1 at 450 nm with that of an equal concentration of a standard sample of coumarin 3-carboxamide (HCC1/Me[5]). CCC-1a, CCC-1's regio-isomer containing calcein 5-carboxamide (Supplementary Figure 1), could also be photolyzed efficiently at 365 nm. Its photolyzed product, CC-1a, exhibits stronger blue emission (420−470 nm) than CC-1 and has a FRET efficiency of about 88% (Supplementary Figure 3, Supporting Information). The variation in the FRET efficiency between CC-1 and CC-1a probably reflects differences in the relative orientation and/or the distance of two interacting dipoles (coumarin and calcein) in these two regio-isomers.

To apply CCC-1 for bio-labeling, we exploit the chemistry of iminodiacetate of forming cyclic anhydride.[13] CCC-1 contains a pair of iminodiacetates which serves as a convenient handle for bioconjugation. After activating CCC-1 with acetic anhydride, we coupled it with dextran amines to prepare CCC-1/Dextran (Supplementary Figure 4, Supporting Information). To evaluate its imaging properties in cells, we injected CCC-1/Dextran into MDCK cells expressing a gap junction protein, connexin 43. Injected cells were identified by their strong green fluorescence exciting around 488 nm (Figure 2B). Because CC-1 has high FRET efficiency and shows little blue emission, we explored using CCC-1/Dextran and a caged coumarin in same experiments for multicolor imaging. We loaded the cells with a caged and cell permeable coumarin, NPE-HCCC2/AM.[5] Prior to uncaging, none of the cells showed blue fluorescence (Figure 2C), and the injected cell (cell 1, Figure 2A) only showed a weak green emission by 410 nm excitation (Figure 2F). We then selectively illuminated cell 1 with a narrow beam of UV light to photolyze NPE-HCCC2 and CCC-1/Dextran. This generated HC-CC2 and CC-1/Dextran to cause a jump in fluorescence intensity in both the blue and FRET channel (Figure 2D and 2G). HCCC2 (MW 449 D) rapidly diffused to neighboring coupled cells 2 and 3 through gap junction channels,[14] and the coumarin fluorescence intensity in these coupled cells approached equilibrium about 2 min later (Figure 2E). In contrast, because CC-1/Dextran (MW ≈ 10 KD) is too big to go through connexin channels, it stayed in cell 1, so the FRET signal remained unchanged (Figure 2H).

In summary, we have developed a new caged green dye that is water soluble, has high uncaging and FRET efficiency, and has friendly chemistry for bioconjugation. Moreover, we can localize the caged FRET dye by exciting it near 490 nm prior to photolysis. This is especially desirable for experiments demanding highly localized photoactivation (for example, by two photon uncaging[6]), which requires knowing the distribution of the label in three dimensions. These probes thus offer new opportunities to image molecular and cellular dynamics.

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Supporting Information Available: Synthesis and characterization of products and methods for bioconjugation and imaging. This material is available free of charge via the Internet at http://pubs.acs.org.

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