Photoactivatable fluorescein derivatives with azidomethyl caging groups for tracing oligonucleotides in living human cells†

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A new photocaged fluorescent compound, azidomethyl fluorescein, was successfully utilized to monitor the dynamics of oligonucleotides in living human cells.

Light-directed activation of photocleavable compounds has emerged as a powerful tool for generating concentration jumps of biomolecules in complex molecular environments and has provided important information on the reaction mechanisms of biomolecules *in vitro* and *in cell* because it can be precisely controlled in amplitude, location, and timing, thus imposing spatiotemporal control on the system under study.[1] The most common technology to conduct light-regulation of functionalized oligonucleotides involves the installation of a photoprotecting group that can be completely removed by light irradiation onto a biologically active molecule. This process, termed "caging", has been successfully employed in light-controlled activation of small molecule inducers of gene expression, fluorophores, peptides, and proteins.[2] The cage technology has also been applied to functionalized oligonucleotides such as catalytic oligonucleotides,[3] aptamers,[4] antisense DNA,[5] siRNA,[6] molecular beacon,[7] and fluorogenic DNA for bioimaging.[8]

Various photolabile protecting groups have been reported. These are derivatives of the *o*-nitrobenzyl group,[9] the coumarin group,[10] the *p*-hydroxyphenacyl group,[11] and the ketoprofen group.[12] Because each type of photocleavable group has specific advantages and limitations for use in a given application, there has continued to be considerable interest in the development of new photocleavable groups, which may offer additional versatility.

Here we report for the first time a photocleavable azidomethyl group for protecting hydroxyl groups. Notably, this group is the smallest group ever reported among photolabile protecting groups. The new photocleavable group was applied to a caged fluorescent compound. Caged fluorescent compounds are very useful for investigations of the cellular dynamics of oligonucleotides in living cells. The behavior of intracellular RNAs can be monitored with high temporal and spatial resolution using antisense oligonucleotides that bind their complementary target RNAs and fluoresce when triggered by light. However, to our knowledge, only one example of a caged fluorescent oligonucleotide has been reported.[8] Tang *et al.* reported a fluorogenic oligonucleotide triggered by light containing a 2-nitrophenyl photocleavable linker-tethered quencher (dabsyl) group.[8] They succeeded in uncaging the dabsyl group and lighting up living cells. This probe produces a fluorescent signal according to the resonance energy transfer (RET) mechanism, in which a pair of quencher and fluorescence dyes is normally used. The maximum signal/background (S/B) ratio reached 50 : 1.[13] However, higher S/B ratios are required to observe gene expression in living cells. Various fluorogenic compounds, in which fluorescence modulation is caused by photoinduced electron transfer or absorption change is triggered by a chemical reaction accompanying a transformation of chemical structure, have been developed recently.[14] The S/B ratios for these types of molecules could exceed those of the RET mechanism.[13] We reported very recently a reduction-triggered fluorescence (RETF) probe having an azidomethyl-protected fluorescein as the fluorogenic molecule that showed a response with a high S/B ratio.[15] The azidomethyl group can be removed not only by reduction but also by ultraviolet light irradiation.

In this study, we report a new class of caged fluorescein derivatives using an azidomethyl group as a photoremovable protecting group (Scheme 1). In the first step of the reaction, a nitrogen molecule is eliminated from the azide group by photoirradiation. Consequently, the azide group is transformed to a nitrene group. The nitrene could abstract a proton from a water molecule to form an amino-hemiacetal. The resulting amino-hemiacetal group is quickly hydrolyzed in an aqueous environment to give an unmasked phenol group and the probe emits a fluorescence signal. These compounds offer significant stability before ultraviolet light irradiation and a rapid enhancement of fluorescence after the irradiation under biological conditions.[15] Moreover, the oligonucleotides labeled with these compounds successfully lit up even in living human cells.

Monoazidomethyl fluorescein (**MAF**) **1** and bis-azidomethyl fluorescein (**BAF**) **2** were synthesized according to methods we have reported previously.[15] Briefly, **MAF 1** was synthesized by treatment of commercially available fluorescein with one equivalent of methyl bromoacetate, followed by azidation in three steps. **BAF 2** was synthesized by azidation from iodofluoroscein in three steps. **MAF 1** offers a quantitative fluorescence signal to give a single product after deprotection of the azidomethyl group (Φ = 0.221) (See supporting information for photolysis quantum yield). On the other hand, **BAF 2** offers a more potent fluorescence signal after deprotection of the two azidomethyl groups (Φ = 0.664). These compounds could not be introduced into the oligonucleotide by solid phase synthesis because of the instability of the azide group under the deprotection conditions. Therefore, postmodification of compounds was carried out. To prepare **ODN 1**, the monoazidomethyl fluorescein NHS ester was reacted with

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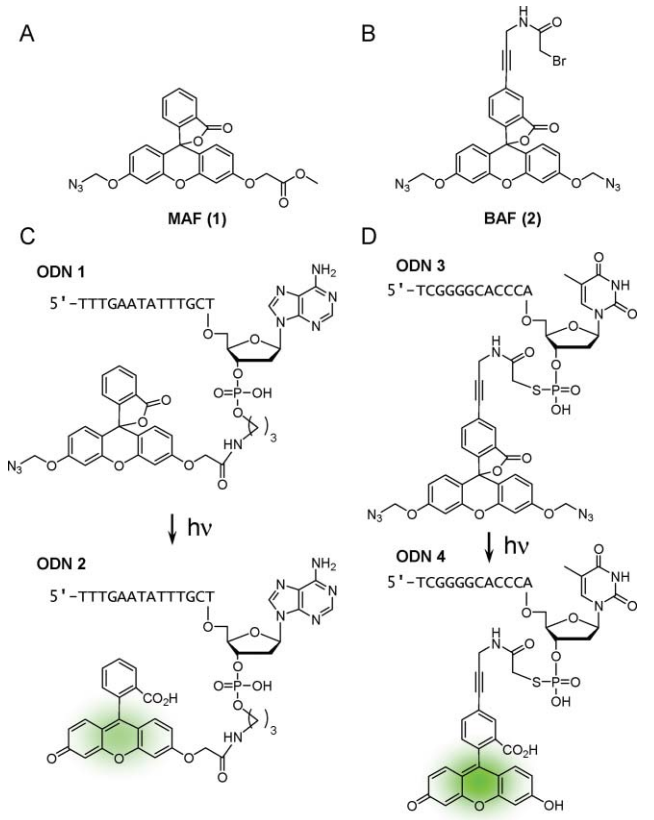

Scheme 1 Structures of (A) monoazidomethyl fluorescein (MAF) and (B) bis-azidomethyl fluorescein (BAF). Uncaging scheme of (C) MAF and (D) BAF-labeled oligodeoxynucleotide.

3′ amino-modified 15-mer oligonucleotide, while the bromoacetyl group of the bis-azidomethyl fluorescein was reacted with the phosphorothioate group on the 13-mer oligonucleotides to prepare **ODN 3** (Scheme 1). The probe structures were confirmed by ESI–TOF mass spectrometry (see supporting information). The probe can be stably stored without decomposition of the azidomethyl-protected fluorophore in aqueous solution but not under acidic conditions.[15]

We tested the photochemical properties of **MAF 1** and **ODN 1**. The solution was scanned across the range 350–550 nm during UV–vis spectroscopic analysis or 500–650 nm with excitation at 490 nm during fluorescence analysis before irradiation and after every 1 min of irradiation ($\lambda_{irr} = 365$ nm, 200 mW cm^{-2}) to monitor the progress of the photocleavage reaction. First, the absorption spectra for 5 μM solutions of **MAF 1** and **ODN 1** in 20 mM Tris–HCl buffer (pH 7.2) were recorded, as shown in Fig. 1. **MAF 1** showed no absorbance at wavelengths longer than 350 nm in 20 mM Tris–HCl (pH 7.2), whereas the uncaged products showed absorption bands at 455 and 490 nm after UV irradiation. The absorption at 450 nm increased over time, as shown in the inset of Fig. 1. The signal at 450 nm reached saturation within 8 min as a result of deprotection of the azidomethyl group. Similarly, **ODN 1** showed no absorbance before UV irradiation. On the other hand, the absorption band at 450 nm increased over time and reached saturation within 8 min. Hence, the efficiencies of photodeprotection were almost the same between the small molecule and labeled ODN. Next, the fluorescence properties of

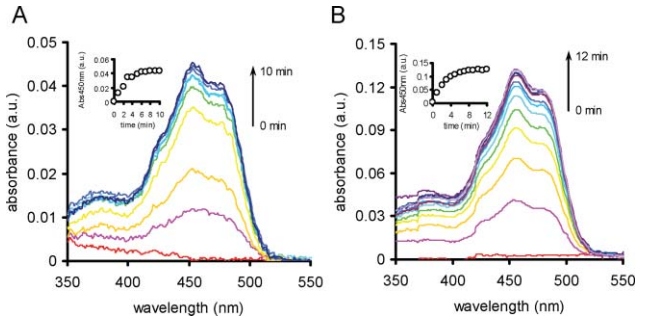

Fig. 1 UV-VIS spectrum of 5 μM of **MAF 1** (A) or **ODN 1** (B) in 20 mM of Tris-HCl (pH 7.2) buffer at increasing UV irradiation times. Photoirradiation was carried out every 1 min (λirr = 365 nm, 200 mW cm^{-2}) using SP-7 Spot Cure (USHIO).

MAF 1 and **ODN 1** were also examined (Fig. 2). No significant fluorescence was observed for **MAF 1** and **ODN 1** upon excitation at 490 nm. After ultraviolet light irradiation, **MAF 1** and **ODN 1** showed strong fluorescence emission at around 520 nm and reached saturation within 8 min. The emission was enhanced almost 1350-fold and 630-fold for **MAF 1** and **ODN 1**, respectively. This property is considerably superior to the RET quenching system and is clearly helpful for imaging in cells.

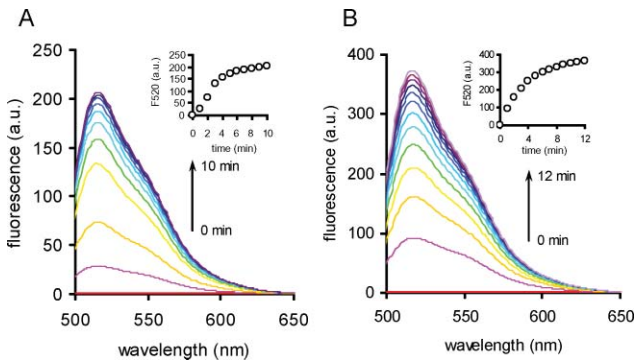

Fig. 2 Fluorescence spectrum of 5 μM of **MAF 1** (A) or **ODN 1** (B) in 20 mM of Tris-HCl buffer (pH 7.2) at increasing UV irradiation times (λirr = 365 nm, λex = 490 nm).

Photo-irradiation to **MAF 1** and **ODN 1** was carried out in a similar manner. However, the absorption of **MAF 1** is lower than that of **ODN 1**. HPLC analysis showed **ODN 1** was transformed to the corresponding fluorescent product in 85% yield (Fig. S1). On the other hand, analysis using thin layer chromatography (TLC) was carried out to clarify the reaction of **MAF 1**. TLC indicated that some unknown byproduct without fluorescence appeared thorough the photo-irradiation (Fig. S2). This might explain the lower absorption of **MAF 1** after the reaction.

Tracing biological substances using photocaged fluorogenic compounds in living cells is an important technique for biological research. To determine the applicability of azidomethyl-protected fluorescein for this purpose, we carried out the photocaging reaction and traced the resulting fluorescence signal in living human cells. In the *in cell* experiment, we used the **BAF 2**-labeled **ODN 3** because **BAF 2** offered a higher quantum yield than **MAF 1** after deprotection. Hela cells were cultured on 35 mm Petri dishes with glass bottoms. **ODN 3** (1 μM) was mixed with transfection reagent, and then transfected into cells according to the standard

protocol provided by the manufacturer. Imaging experiments were performed using a laser scanning confocal microscope. Pinpoint photoactivation with a diameter of about 1.5 μm was performed to monitor the dynamics of oligonucleotides in a single cell, using ~0.8 mW cm^{-2} of 405 nm laser light through the objective. For simultaneous imaging of photoactivated fluorescein, the 488 nm line of an argon laser was used for fluorescein excitation and its emission was collected using a 500/600 nm band-pass filter. As shown in Fig. 3, the region around the irradiation point became brightly fluorescent after 25 s of irradiation, whereas no signal was observed from the cells before irradiation. Tang *et al.* showed that a longer irradiation time of 2 h was required for photoactivation of fluorogenic oligonucleotides using a 0.5 mW cm^{-2} UV lamp with 365 nm.[8] Therefore, our molecule is more reactive to photoactivation and milder to living cells than previous methods. The fluorescence intensities of three regions of interest (ROIs 10, 11 and 13) were observed. The fluorescent intensities of ROIs (10 and 11) close to the point of irradiation increased with time. In contrast, the intensity of ROI 13, far from the irradiation point, showed no significant increase. These results show that the photodeprotected product (**ODN 4**) did not diffuse around the irradiation point.

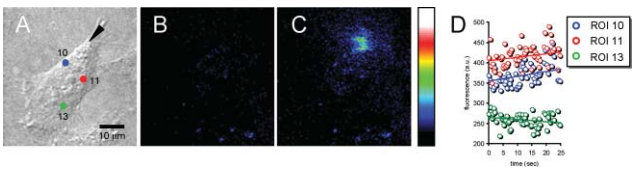

Fig. 3 Photoactivation of BAF-labeled oligonucleotide (**ODN 3**) in living Hela cell. (A) Phase contrast image. Black arrow shows the photo-activated point. (B and C) Fluorescent images before (0 s; B) and after irradiation (25 s; C). (D) Plots of fluorescent intensities of all ROIs.

In conclusion, we have designed and synthesized new caged fluorescein derivatives containing azidomethyl-protecting groups that are rapidly activated upon brief irradiation and show a strong increase in fluorescence. Moreover, the azidomethyl fluorescein-labeled oligonucleotide was successfully photoactivated and the resulting fluorescence signal was monitored in living human cells. Future work will be aimed at making antisense oligonucleotides that bind their complementary target RNAs and chasing the behavior of specific RNA molecules in living cells.

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