Conditionally Activatable Visible-Light Photocages

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ABSTRACT: The proof of concept for conditionally activatable photocages is demonstrated on a new vinyltetrazine-derivatized coumarin. The tetrazine form is disabled in terms of light-induced cargo release, however, bioorthogonal transformation of the modulating tetrazine moiety results in fully restored photoresponsivity. Irradiation of such a “click-armed” photocage with blue light leads to fast and efficient release of a set of caged model species, conjugated via various linkages. Live-cell applicability of the concept was also demonstrated by the conditional release of a fluorogenic probe using mitochondrial pretargeting.

INTRODUCTION

The past decade has brought remarkable advances in light-related techniques, allowing them to grow from simple means of observation to a precision tool in biology and medical sciences.1−3 Its noninvasive nature and remote action, together with its easy control and fast and cost efficient operation make these techniques very appealing. These processes became possible by the development of photoresponsive materials that efficiently convert light into chemical energy. Among photoresponsive materials, photolabile protecting groups (PPGs) or photocages (PCs) play an increasing role in both chemical biology studies and in therapeutic applications.4−8 These photosensitive groups may be used to mask the biological function of small-molecular effectors,9−15 proteins,16−18 nucleotides,19,20 or drugs,21−23 rendering them inactive. Upon light induced removal of these photolabile moieties by irradiation with a suitable wavelength, the activity of the caged substrate is restored. Manipulation of biological systems via photocaging has already revolutionized chemical biology. Nevertheless, the full potential of photocaging is yet to be exploited. To extend the use of these photoresponsive elements especially in the context of chemical biology, several limitations should be addressed, such as UV light activation,23−26 poor water solubility,27,28 and the lack of potential for targeting.29−31 In addition to the impact on chemical biology, photocaging-based drug delivery systems, especially photoactivated chemotherapy (PACT) could also benefit from the development of such improved photocages possessing specific targeting elements.25,32

In recent years, a few notable examples were presented as “clickable” photocages targeting various intracellular compartments.31,33,34 However, in these instances, click-chemistry (i.e., copper-catalyzed azide alkyne cycloaddition) was only used to facilitate the assembly of the organelle-targeting photocage, rather than to serve as the key element of the targeting process.35−37 To the best of our knowledge, such clickable photocages where the clickable moiety is also the targeting element are not yet reported. Redefining the role of the clickable function, however, is rather an incremental step toward improved photocages. Exploiting the modulation power that certain biocompatible click handles (i.e., bioorthogonal functions) exert on chromophores gives an extra twist to the story. On the basis of our extensive work on the development of bioorthogonal fluorogenic (turn on) probes,38−40 we hypothesized that a similar concept can be applied to modulate the photoresponsivity of photocages. According to our foreseen concept termed “conditional photocaging”, such switchable constructs become photocages solely by “arming” via a chemical transformation of the quencher moiety in a specific chemical reaction (i.e., a bioorthogonal reaction). Following this highly specific bioorthogonal ligation step to the target, the caged, biologically active molecule can be released upon light irradiation (“activation”). Nonspecifically bound or free (disabled) constructs, however, remain inactive even on exposure to light.

Lately, several accounts have been reported on the development of so-called click-and-release systems that rely
on the spontaneous elimination of caged compounds upon a bioorthogonal reaction (i.e., inverse electron demand Diels–Alder, IEDDA, reaction of tetrazines, and strained alkenes). Although it seems similar at first sight, our approach is conceptually different. Our click-and-uncage constructs are based on the quenched activity of the photocage, which is reinstated after the reaction of the quencher moiety. Moreover, the further necessity of light irradiation enables an extra level of temporal and spatial control over the release of the caged active species. During the course of this work, Vázquez et al. reported on the bioorthogonal modulation of the 1O₂ sensitizing potential of BODIPY derivatives allowing conditional photodynamic applications (i.e., PDT). The above hypothesized bioorthogonal modulation of photocages would enable the oxygen independent, complementary concept of conditional photoactivated chemotherapy (PACT).

Herein, we demonstrate the proof of concept of conditional photoactivation by disclosing the development and study of a bioorthogonal moiety- (tetrazine-) modulated, visible-light sensitive click-and-uncage platform with various caged compounds. Besides in vitro experiments, live-cell applicability of the concept is also demonstrated through the pretargeting-dependent conditional photorelease of a fluorogenic probe.

■ RESULTS AND DISCUSSION

Prompted by the above considerations, we turned our attention to coumarin-based photocages and tetrazine quenched fluorogenic probes. We assumed that both the fluorescence and the light-induced bond dissociation originate from the same excited state, thus, we hypothesized that similarly to fluorescence, the photoresponsivity of photocages can also be modulated by the bioorthogonal and quencher tetrazine moiety. We have recently observed transforming the tetrazine in a bioorthogonal reaction. It was also observed that the vinylene linkage shifts the absorption wavelength of the related coumarin with ca. 60 nm toward the red range resulting in visible light absorption. Therefore, we designed compound 1, which combined elements of coumarinyl photocages and bioorthogonally activatable vinylene linked coumarinyl-tetrazine fluorogenic probes.

Cage 1 was accessed through a synthetic route starting from 3-bromo-7-diethylamino-4-hydroxymethylcoumarin using the previously established procedure for the synthesis of vinyltetrazinylated frames and further conjugated with three different amino acids as model caged molecules (Scheme 1). Boc-phenylalanine, Fmoc-lysine, and Boc-tyrosine-tBu-ester were readily converted to their corresponding caged derivatives resulting in ester (2), carbamate (3), and carbonate (4) linked species, respectively. In accordance with our previous observations, absorption spectra of all derivatives were redshifted compared to plain coumarin-caged congeners, with absorption maxima around 475 nm (tetrazine form) and medium molar absorption coefficients in acetonitrile-HEPES 2:1 (pH 7.0). As expected, fluorescence of the tetrazine derivatives was found to be practically zero. Reaction with a strained alkyne, BCN ((1R,8S,9s)-Bicyclo[6.1.0]non-4-yn-9-ylmethanol) resulted in blue-shifted absorption maxima (around 445 nm) and, very importantly, a ca. 1000-fold increase in bright green emission intensity at around 535 nm.

Next, we have compared the photouncaging features of the tetrazines and their respective BCN-conjugated congeners. On the basis of the near quantitative fluorescence quenching, we anticipated that the photodissociation is also suppressed. Gratifyingly, when the samples were irradiated with blue LED (463 nm, for details, please refer to the Supporting Information, SI), neither the release of the caged amino acids nor photodestruction could be observed in case of the unarmed (tetrazine) constructs. Irradiation, “activation” of the BCN-conjugated, “clicked and armed” forms under the same...
conditions, however, led to rapid release of all three amino acids, as seen by HPLC–MS (see Figure 1 for the traces, Figures S5–S7 for the HPLC chromatograms). Moreover, the tetrazine forms were found quite photostable, and no release of the amino acids could be detected after 30 min of irradiation. Comparison of the different linkages between the photocage and the amino acids suggests that carbonate 4-BCN was the most photolabile with an uncaging quantum yield of 3.5%, followed by ester 2-BCN and carbamate 3-BCN. The uncaging quantum yields and efficiencies are summarized in Table 1. Solvent-dependency of the uncaging of 2-BCN was also elaborated (Table S1). These results showed that higher water content results in increased photochemical quantum yields, enhancing the quantum yield by suppressing recombination (see below).50

We also wished to provide theoretical evidence for the experimental results. To this end, the low-lying excited states of a vinylene linked tetrazine-coumarin model system and its cyclooctyne conjugate were studied. We used the acetonic acid ester of 9 for the calculations. The −NEt2 group was replaced with −NMe2 to decrease the number of conformers being considered. The results showed that the vinylene linkage participates in the π-system of the chromophore, which explains the red-shifted absorbance. Furthermore, it was revealed that the S1 state of the vinylene-linked tetrazine-coumarin corresponds to the dark n → π* excitation of the tetrazine-vinylcoumarin system (HOMO → LUMO transition of the model compound), see Figure 2), while the S2 state is predominantly formed by promoting an electron from the highest π orbital of the vinylcoumarin to the lowest-lying π* orbital of the tetrazine-vinylcoumarin system (HOMO → LUMO+1 transition of the model). The probabilities of both the S0 → S1 and S0 → S2 transitions are high, which suggests that the molecule gets into its S2 state upon irradiation with blue light, followed by a rapid internal conversion to the dark S1 and then to the ground state. The photoreaction presumably also takes place on the S0 surface, thus the presence of the tetrazine ring precludes both the reaction and the radiative decay of the excited state. After conjugation with cyclooctyne, the n → π* type state no longer exists and the π → π* state of the vinylcoumarin (HOMO → LUMO transition of the cyclooctyne-conjugated model compound) becomes the lowest

Figure 1. Scheme of the conditional uncaging and degradation and release profiles of the photocages determined by HPLC.

### Table 1. Spectroscopic Properties and Photochemical Quantum Yields of the Compounds in MeCN-HEPES 2:1 (pH 7.0)

| Compound | λ<sub>max</sub> (nm) | ε<sub>max</sub> (M<sup>-1</sup> cm<sup>−1</sup>) | Φ<sub>deg</sub> (%) | Φ<sub>bac</sub> (%) | Φ<sub>deg</sub> (%) | Φ<sub>unc</sub> (%) | ε<sub>unc</sub> × Φ<sub>u</sub> (M<sup>−1</sup> cm<sup>−1</sup>) |
|----------|---------------------|------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 1/1-BCN  | 463/436             | 527/531          | 45 800/44 700   | 61              | 0.44            | 0.74            | 107             |
| 2/2-BCN  | 470/442             | 536/538          | 44 100/42 800   | 69              | 0.10            | 0.42            | 31              |
| 3/3-BCN  | 468/440             | 534/535          | 42 600/40 800   | 38              | 3.50            | 4.40            | 875             |

*Fluorescence quantum yield. *Uncaging (release) quantum yield. *Degradation quantum yield. *Measured in HEPES buffer. Quantum Yields were Determined Only for the Clicked Derivatives. *See also Table S1.
singlet excited state enabling both the fluorescence and the bond-dissociation.

As discussed above, not only do the constructs become photoresponsive after the click reaction, but their fluorescence is also restored (1000× increase, see Figure S1 in the SI). Such an inherently fluorogenic system is itself suitable to indicate the localization of the conjugated constructs; however, it does not provide any evidence of the uncaging process. In order to investigate the applicability of our concept in living systems, we wished to visualize both the pretargeting and uncaging processes through the liberation of a fluorogenic substrate that does not interfere with the activation/excitation of the coumarin cage. The use of rhodols as quenched fluorogenic markers is quite rare despite the fact that they are bright, easily accessible, and very importantly, require only one acyl/carbamoyl functionalization of the phenolic OH to render it fully quenched. Taking spatial separation of the coumarin and the rhodol moieties into consideration in order to suppress recombination, we have designed compound 5 (Figure 3). The well-established dimethylethylenediamine-carbamoyl self-immolative linker provides sufficient spatial separation and fast release kinetics (SI section 4). Moreover, the carbamoyl-derived rhodol is practically nonemissive. LED irradiation of construct 5 and its “click-armed” 5-BCN congener was monitored by fluorescence spectroscopy and HPLC–MS. Both experiments revealed that unarmed construct 5 is not photoresponsive, while its click-armed BCN conjugate allows liberation of the rhodol upon LED activation.

Gratifyingly, uncaging of the rhodol resulted in an overall 1000× increase of fluorescence intensity at the rhodol channel (λex = 515 nm) after 15 min of irradiation. Fluorescence spectroscopy monitoring of the uncaging process revealed further information regarding the kinetics of the self-immolative destruction of the linker, i.e., following photolysis of the linkage between the coumarin and the linker. The self-immolation process requires a few more extra minutes to go to completion (Figures 3c,d and S3 for further details on the kinetics).

On the basis of the excellent ability of 5 for monitoring the uncaging process, we selected mitochondria as an intracellular target due to its well established targetability with the triphenylphosphonium (TPP) moiety. In order to achieve specific organelle localization, we synthesized TPP-BCN (Scheme S3) for delivering a bioorthogonal platform into the mitochondria. Conditional uncaging was investigated using confocal fluorescence microscopy imaging of A-431 (skin

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**Figure 2.** Low-lying excited states of the model tetrazine and its BCN-clicked product.

**Figure 3.** (a) Structure of 5, (b) scheme for the conditional uncaging of 5, and (c) emission spectra of the uncaging of 5-BCN upon various irradiation and wait time (1 μM in PBS, λex = 515 nm); the arrows indicate subsequent irradiation of the sample (d) fluorescence intensity of 5-BCN at 566 nm, the blue lines represent the irradiation time, and (e) photographs of the samples under ambient and UV light.
cancer) cells either with or without pretreatment with TPP-BCN. We also investigated the effects of extracellularly preassembled TPP-5. In each case, the cells were treated with the photocaged-constructs for 1 h (200 nM) and then imaged directly without removal of unreacted tetrazines (no-wash condition). As can be seen in Figure 4, only cells pretargeted with TPP-BCN show clear colocalization with MitoTracker Deep Red (present in all experiments), confirming successful bioorthogonal-targeting of the photocage inside the mitochondria. It can also be seen that the green emission of the coumarin upon excitation with the blue laser (488 nm) is only visible in the case of pretargeting, demonstrating the fluorogenicity of the coumarin photocage upon bioorthogonal conjugation. In contrast, preassembled derivative TPP-5 was not taken up by the cells, indicating the often overlooked importance of the 2-step assembly of active species inside cells. Possibly due to its large size and increased molecular weight, the preclicked triphenylphosphonium-containing conjugate is unable to cross the cell membrane.

Live-cell photouncaging of the fluorogenic rhodol was investigated using the built-in blue metal halide lamp (FITC bandpass filter cube) of the microscope (excitation: 460-500 nm). Each field-of-view was irradiated for 5 s, and then the images were taken at least 1 min after irradiation. To clearly see the highly localized effect of uncaging, we obtained 3 × 3 tile scans before and after irradiation of the central area (Figure 5). The cells treated only with tetrazine 5 showed a small fluorescence enhancement in the yellow (rhodol) channel that pretargeted with TPP-BCN show clear colocalization with MitoTracker Deep Red (present in all experiments), confirming successful bioorthogonal-targeting of the photocage inside the mitochondria. It can also be seen that the green emission of the coumarin upon excitation with the blue laser (488 nm) is only visible in the case of pretargeting, demonstrating the fluorogenicity of the coumarin photocage upon bioorthogonal conjugation. In contrast, preassembled derivative TPP-5 was not taken up by the cells, indicating the often overlooked importance of the 2-step assembly of active species inside cells. Possibly due to its large size and increased molecular weight, the preclicked triphenylphosphonium-containing conjugate is unable to cross the cell membrane.

Figure 4. Confocal images of the colocalization of (a) cells treated only with tetrazine 5 for 1 h (200 nM); (b) cells pretargeted with TPP-BCN (10 μM) for 1 h, then with 5 (200 nM); and (c) cells treated with TPP-5 (200 nM). The colors refer to the corresponding emission channels (green: coumarin with 488 nm excitation, red: MitoTracker Deep Red (10 nM) with 638 or 552 nm excitation, and yellow: rhodol with 552 nm excitation). The brightness of the insets is enhanced for better visibility.

Figure 5. Tile scan experiments before (upper image) and after (lower image) irradiation of the central area (marked with the dotted circle) with the built-in blue lamp (460–500 nm, 5 s) of the microscope. (a) Cells treated only with tetrazine 5 for 1 h (200 nM); (b) cells pretargeted with TPP-BCN (10 μM) for 1 h, then with 5 (200 nM). The colors refer to the corresponding emission channels (yellow: rhodol, red: MitoTracker Deep Red). The white squares indicate the magnified area of the images. Further images are shown in SI Section S7.
is dispersed evenly throughout the cells. By contrast, the cells pretargeted with TPP-BCN displayed bright fluorescence after irradiation that is mostly located inside the mitochondria. Similar results were obtained by visualizing the uncaging process in real time, using the built-in laser (488 nm with continuous imaging at both the red and the yellow channels, see the SI Videos and Figure S22). Importantly, the confined irradiation area combined with the subcellular pretargeting can serve as dual control for highly localized manipulation as demonstrated by our fluorogenic click and uncage platform.

■ CONCLUSIONS

In summary, we have demonstrated the proof of concept study of a bioorthogonal click reaction activatable photocage system. Experimental evidence and theoretical calculations suggested that the presence of the bioorthogonal tetracene motif efficiently quenches the excited state of the coumarin necessary for photolysis resulting in disabled photoresponsivity (both in terms of photocaging and fluorescence). Transformation of the tetracene moiety in a bioorthogonal click-reaction fully restores its sensitivity for light. Since bioorthogonal reactions enable highly specific targeting of cells or cellular structures, such conditionally activatable photocages provide an extra level of spatial and temporal control for the release of the caged compounds. This was demonstrated in live cells using a fluorogenic, conditionally activatable construct that solely became light sensitive when the cells were pretargeted with a mitochondria directed, complementary bioorthogonal function. These results confirm the applicability of our concept in biological systems and also clearly demonstrate the advantage of pretargeting and bioorthogonal chemistry. The applicability of this system in photoactivated chemotherapy involving the conditional release of drugs is currently under investigation in our laboratory and results will be reported in due course.

■ ASSOCIATED CONTENT

◆ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.0c07508.

Experimental details, synthetic procedures, spectroscopic characterization, physical data determination, details on the imaging experiments, further images, and viability assessment (PDF)

Video showing the uncaging process in 64x speed without TPP-BCN (AVI)

Video showing the uncaging process in 64x speed with TPP-BCN (AVI)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

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