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A Bioorthogonal Near-Infrared Fluorogenic Probe for mRNA Detection

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Supporting Information

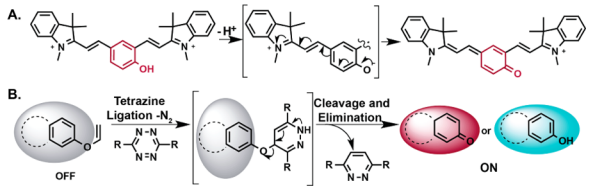
ABSTRACT: There is significant interest in developing methods that visualize and detect RNA. Bioorthogonal template-driven tetrazine ligations could be a powerful route to visualizing nucleic acids in native cells, yet past work has been limited with respect to the diversity of fluorogens that can be activated via a tetrazine reaction. Herein we report a novel bioorthogonal tetrazine uncaging reaction that harnesses tetrazine reactivity to unmask vinyl ether caged fluorophores spanning the visible spectrum, including a near-infrared (NIR)-emitting cyanine dye. Vinyl ether caged fluorophores and tetrazine partners are conjugated to high-affinity antisense nucleic acid probes, which show highly selective fluorogenic reactivity when annealed to their respective target RNA sequences. A target sequence in the 3′ untranslated region of an expressed mRNA was detected in live cells employing appropriate nucleic acid probes bearing a tetrazine-reactive NIR fluorogen. Given the expansion of tetrazine fluorogenic chemistry to NIR dyes, we believe highly selective proximity-induced fluorogenic tetrazine reactions could find broad uses in illuminating endogenous biomolecules in cells and tissues.

There has been increasing interest in the use of bioorthogonal reactions, such as tetrazine ligations, for the detection and imaging of biomolecules.[1] A powerful strategy involves the use of fluorogenic probes to reveal biomolecule localization in living cells.[2] Benefiting from recent advances in tetrazine synthetic method development,[3] several tetrazine fluorogenic probes have been synthesized and applied to cellular imaging.[2b,c,3b,4] Appended tetrazines can quench several classes of fluorophores, which upon completion of the tetrazine bioorthogonal reaction, increase in fluorescence up to 100−1000-fold. These highly fluorogenic probes have been used for diverse applications such as no-wash live-cell imaging,[2b,c] endogenous oncogenic miRNA detection,[5] and systemic fluorescence imaging in vivo.[2a] However, existing tetrazine reactive fluorogenic probes are believed to be quenched by through-bond energy transfer (TBET) or Förster resonance energy transfer (FRET) between the donor fluorophore and acceptor tetrazine, the latter of which has an inherent absorbance typically between 510 and 550 nm. Although far-red and near-infrared (NIR) fluorophores have significant advantages in live cell applications, such as lower background signal, deeper tissue penetration, and less tissue damage,[6] they remain challenging to quench by tetrazines via energy transfer based mechanisms, likely due to poor spectral overlap.[4,7] Herein we report the design of a tetrazine reactive NIR fluorogenic probe through an alternative quenching mechanism and its application to detect mRNAs.

In addition to TBET and FRET mechanisms, fluorogenic probes can be quenched by internal charge transfer (ICT) processes.[2e,8] Specific reactive functional groups can act as a trigger by interrupting the pull-push conjugated π-electron system[9] or by holding the fluorophores in a non-ionizable form.[10] Recently, several bioorthogonal reactions with a "click to release" feature[11] have been reported with applications in bioluminescence imaging,[10a] fluorophore uncaging,[12] endogenous oncogenic miRNA detection,[5] antibody-drug-conjugate release,[11] and protein uncaging.[13] We envisioned that a bioorthogonal click to release strategy to unmask quenched ICT fluorophores could be harnessed to expand the diversity of tetrazine reactive fluorogenic probes.

The phenol group is common in various fluorophore skeletons such as coumarins and xanthene dyes. Interestingly, an NIR-emitting quinone cyanine dye has been reported consisting of a phenoxide anion in resonance with a cyclohexadienone anion (Scheme 1A).[9a,14] We hypothesized that a vinyl group, a reported dienophile for tetrazine reactions,[15] could be used to cage this phenoxide and quench fluorescence through ICT. To achieve bioorthogonal release of the phenoxide, we utilized a phenyl vinyl ether as a novel dienophile that can undergo a tetrazine uncaging reaction (Scheme 1B). Vinyl ethers are known to react with tetrazines through a cascaded ligation−elimination process, resulting in pyridazine and a free hydroxyl group in almost quantitative yield.[16] Unlike the canonical tetrazine ligation, the tetrazine uncaging reaction unmasks the phenoxide to regenerate the fluorophore.

Scheme 1. (A) Resonance Structures of the Phenoxide Anion within a Cyanine Scaffold and (B) Schematic of Fluorogenic Probe Design Using a Tetrazine−Vinyl Ether Uncaging Reaction



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To verify our fluorogenic probe design, we first synthesized model vinyl ether cyanine compound **1**. Starting from the commercially available compound 4-hydroxyisophthalaldehyde, **1** can be obtained in three steps as a light yellow solid. The reaction between **1** and dipyridyl tetrazine **2** proceeds with high efficiency, resulting in two products **3** and **4** in 93% and 95% yield, respectively (Figure 1A). When **4** is added to PBS buffer,

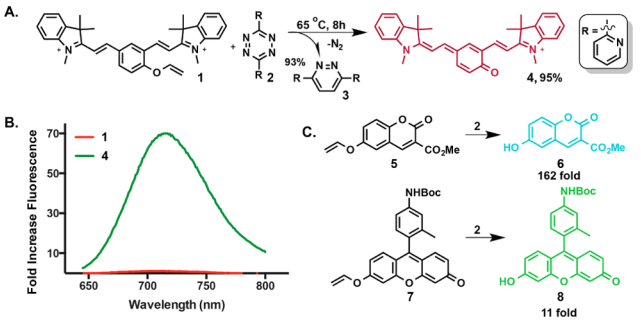

Figure 1. Vinyl ether fluorogenic tetrazine uncaging reaction. (A) Reaction of vinyl ether caged cyanine dye **1** with tetrazine **2** to recover **4**. (B) Fluorescence spectrum of caged **1** and its corresponding uncaged cyanine compound **4**. Fluorescence was normalized against compound **1**. (C) Alternate vinyl ether caged fluorophores and their relative fluorescence turn-on upon uncaging.

the solution turns cyan immediately, and a 70-fold fluorescence increase is observed compared to caged precursor **1** (Figure 1B). We also investigated the absorption spectra of **1** and **4**. Compound **4** has a strong absorption peak at 620 nm, however after being caged by the vinyl ether, the absorbance peak exhibits a hypsochromic shift to 550 nm, and excitation at 620 nm results in negligible fluorescence (Figure S4A,B). The significant shift in absorption provides support that the vinyl ether ICT strategy for fluorescence quenching is highly effective.[2e]

To better understand the breadth of our approach, we investigated the quenching ability of vinyl ether cages on other fluorophore skeletons. Coumarin and fluorescein vinyl ether derivatives **5** and **7** are straightforward to synthesize in two purification steps from commercially available materials. Coumarin derivative **5** does not absorb at 420 nm, while its uncaged parent, **6**, exhibits a significant absorbance at this wavelength (Figure S5A). Therefore, after reaction of **5** with tetrazine to produce **6**, excitation at 420 nm led to a remarkable 162-fold increase in fluorescence (Figures 1C and S5B). In contrast, fluorescein vinyl ether **7** exhibited a relatively modest 11-fold turn-on after uncaging (Figures 1C and S6B). This is consistent with the absorption spectrum of **5**, which displayed a weakened, but present, peak intensity at 480 nm (Figure S6A).

Once we confirmed the fluorogenic nature of our tetrazine reactive vinyl ether probes, we investigated the reaction kinetics of a model system (Scheme S1). As we expected, due to the use of unstrained vinyl ether dienophiles, the reaction is relatively sluggish (Figure S2) compared to alternative tetrazine mediated uncaging reactions[11] at the micromolar concentrations typically useful in direct live cell labeling applications.[17] However, this shortcoming becomes an advantage when applied to proximity-induced reactions such as those templated by nucleic acids. There has been increasing interest in applications of bioorthogonal chemistry utilizing proximity-induced reactions, which benefit from a dramatic increase in the effective concentration of the two reaction partners by biomolecule promoted spatial proximity.[5,18] Nucleic acid templated reactions are of high specificity and sensitivity. Reactions between antisense probes only proceed at low concentrations (nM) when in the presence of the corresponding template. For these reasons, such reactions have been widely used for nucleic acid imaging and detection.[19] However, there have been limited examples of RNA-templated fluorogenic reactions with NIR fluorescent probes, despite the advantages of far-red probes in cellular imaging.[20] We therefore sought to design a NIR imaging probe, utilizing the vinyl ether cage, that would be useful for detecting cellular RNA of interest.

Initially we designed a series of *N*-hydroxysuccinimide (NHS) esters for oligonucleotide modification. Vinyl ether NHS compounds **VE-Co** and **VE-Fl** were obtained by post-functionalization from previous model compounds **5** and **7**, and **VE-Cy** was obtained through a slightly modified synthetic route (Figure 2A). By using a classical NHS coupling protocol, a

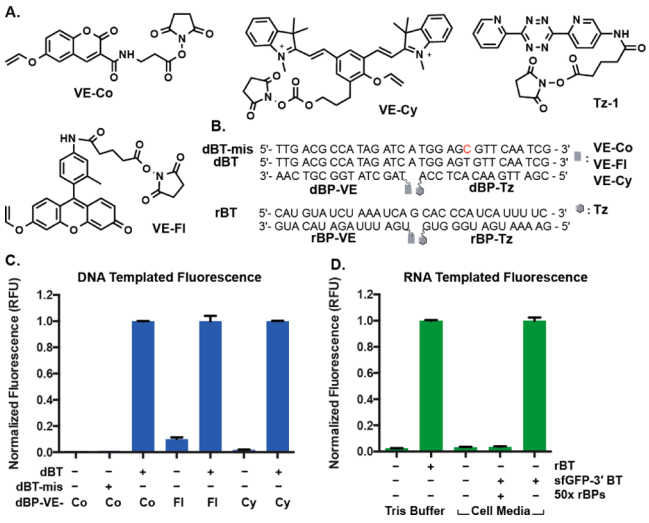

Figure 2. Nucleic acid templated fluorogenic tetrazine uncaging reactions. (A) Vinyl ether and tetrazine NHS conjugates used to synthesize oligonucleotide probes shown in part B. (B) Sequence and alignment of DNA (**dBT** and **dBT-mis**) and RNA (**rBT**) templates with their corresponding oligo probes. (C) Fluorescence increase from DNA-templated tetrazine uncaging reactions. Buffer reactions were carried out for 8 h at 37 °C in 100 mM Tris pH 7.4 containing 200 mM $MgCl_2$ in the presence of 100 nM **dBP-Tz** and **dBT** or **dBT-mis** as well as **dBT-VE** fluorophore as indicated above. (D) Fluorescence increase from RNA-templated tetrazine uncaging reactions. All reactions were carried out in a similar fashion as described in part C, using either **rBT** or in vitro transcribed **sfGFP-3′ BT** as the template with **rBT-Tz** and **rBT-VE-Cy** as the reactive probes. Control RNA binding pobes lacking tetrazine or VE-Cy modifications (50x rBPs) were employed to block fluorogenic probe binding in column 4. When using the **sfGFP-3′ BT** template, F-12K cell medium with 10% FBS was used in lieu of $MgCl_2$ supplemented buffer.

panel of nucleic acid binding probes bearing either vinyl ether protected fluorophores (**dBP-VE** and **rBP-VE**) or tetrazines (**dBP-Tz** and **rBP-Tz**) were produced and characterized by ESI-TOF-MS (Figure 2B). Antisense binding probes and their matching templates were chosen and designed based upon previous optimization of oligonucleotide promoted tetrazine ligation.[19a] In the presence of the complementary nucleic acid binding template **dBT**, the reaction between coumarin vinyl ether probe **dBP-VE-Co** and tetrazine probe **dBP-Tz** took place immediately, as observed by fluorimetry. The first-order rate constant was measured as $(2.1 \pm 0.03) \times 10^{-4}\ s^{-1}$, with a reaction

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half-life of 54.9 min (Figure S3). After the templated tetrazine uncaging reaction completed, a >100-fold fluorescence increase was observed (columns 1 and 3, Figures 2C and S7). We also validated the specificity of our templated tetrazine uncaging reaction by testing reactivity with a template bearing a single mismatch, **mis-dBP** (Figure 2B). Almost no increase in fluorescence was detected after nucleic acid binding probes were incubated for 8 h with **mis-dBP** (column 2, Figures 2C and S7). We also investigated the fluorogenicity of the alternative fluorescein and cyanine oligo probes. We were delighted to find that after 8 h of incubation, the green fluorescein analogue, **dBP-VE-Fl**, and NIR cyanine analogue, **dBP-VE-Cy**, also exhibited turn-on ratios similar to the previously described model reactions (columns 4−7, Figure 2C).

We next explored the application of our probes to detect mRNA. Conventional methodologies for visualizing mRNA include the use of antisense probes,[21] aptamers,[22] fusion proteins that recognize specific RNA secondary structures,[23] and enzymatic labeling strategies.[24] While many methods for mRNA detection exist, mainstream methods suffer from obstacles such as high background due to lack of fluorogenic probes or require synthetically modified RNA targets.[25] There is therefore a need to develop robust methodologies suitable for detection of RNA in cells and tissues.[26]

Our small fluorogenic NIR RNA-templated tetrazine uncaging probes seemed aptly fit to address many of the current challenges in RNA detection. Chiefly, fluorogenic templated RNA labeling does not intrinsically require genetically encoded targets or large reporter proteins and can be used to target naturally encoded RNA sequences, so long as they remain accessible to antisense probe binding. To ensure our oligo probes remained stable in physiological media, and to improve cell permeability, we utilized 2′-*O*-methylation and phosphorothioated RNA probes.[27] Additionally, these modifications are known to increase binding affinity and specificity to RNA targets.[27,28] We designed our probes with predicted melting temperatures between 51 and 54 °C, such that they would remain bound to the target of interest, ensuring that fluorescent signal could not arise from reacted probes that dissociate from their RNA target. To experimentally confirm lack of dissociation and turnover at physiological temperatures (37 °C), the melting curves of each RNA probe binding to the designated RNA template was measured. As predicted, no significant melting transitions were observable below 50 °C (Figure S1).

In order to assess our RNA-based probes (**rBP-VE** and **rBP-Tz**), we investigated the vinyl ether tetrazine uncaging reaction in the presence of a 31 nucleotide synthetic RNA binding template, **rBT**. As expected, the reaction proceeded smoothly, yielding an excellent NIR fluorescence signal after 8 h (Figure 2D). We next tested the binding and fluorogenic turn-on with a full length (946 nt) *in vitro* transcribed mRNA transcript, **sfGFP-3′ BT**. This mRNA encodes for the fluorescent protein sfGFP bearing two probe RNA recognition sequences (**rBT** sequences) within the 3′ UTR. To assess the viability of our approach *in vivo*, the mRNA-templated reaction was performed in physiologically relevant F-12K cell media containing 10% FBS; As expected, we observed a remarkable signal increase (columns 3 and 5, Figure 2D) in the presence of the mRNA template. To validate applications requiring detection of the relative levels of specific RNAs in cells, we titrated different concentrations of **sfGFP-3′ BT** in the presence of a common concentration of probes which yielded a linear fluorescence response as expected (Figure S8). In order to confirm the fluorescence signal was due to hybridization and subsequent tetrazine reaction, we added 50 equiv of our 2′-OMe-RNA probes lacking the tetrazine and vinyl ether partners as competitive inhibitors. After 8 h, there was no significant signal increase compared to controls, demonstrating that fluorescence is directly related to recognition of the mRNA target (column 4, Figure 2D).

As a proof of concept of our probe's practicality for cellular RNA detection, we chose to detect the previously tested sfGFP mRNA expressed in living cells. We first transiently transfected CHO cells with a plasmid encoding for **sfGFP-3′ BT**. After overnight transfection of the plasmid, we subsequently transfected our cells with 25 nM of the oligo probes **rBP-VE-Cy** and **rBP-Tz** for 4 h. We then employed confocal microscopy to directly image the cells without a washing step (Figure 3A). In

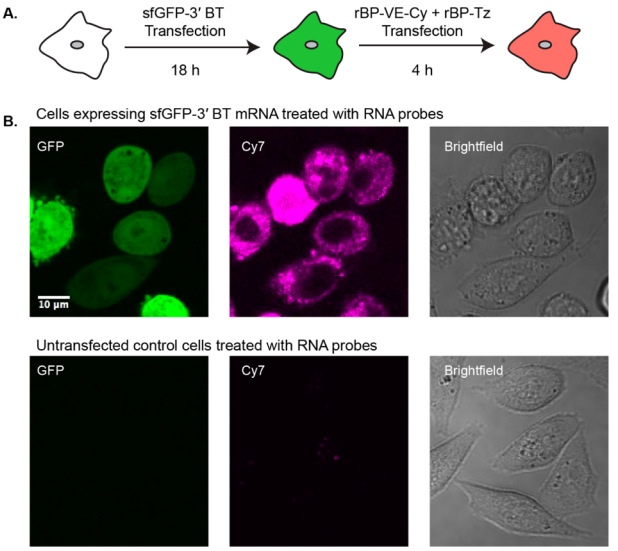

Figure 3. Live cell detection by fluorogenic tetrazine uncaging oligo probes. (A) Cartoon depicting transfection and imaging protocol for live cell imaging. (B) CHO cells treated with 25 nM fluorogenic NIR antisense probes in the presence (top row) or absence (bottom row) of prior transfection with **sfGFP-3′ BT** plasmid containing the complementary target sequence in the 3′ UTR.

cells expressing the target mRNA, we observed significant fluorescence signals, with predominant localization throughout the cytoplasm. Nuclear export has been estimated to occur in under an hour[29] and therefore we were unsurprised to see little labeling in the nucleus after 4 h (Figure 3B). Cells expressing the target mRNA and treated with **rBP-VE-Cy** but not **rBP-Tz**, cells not expressing the target mRNA, and cells expressing an mRNA lacking 3′rBT (pcDNA3-EGFP) exhibited significantly less NIR fluorescence 4 h after transfection with **rBP-VE-Cy** and **rBP-Tz** (Figures 3B and S9).

In this work, we have developed novel fluorogenic oligonucleotide probes whose fluorescence is triggered by a vinyl ether tetrazine uncaging reaction templated by DNA and RNA sequences. While traditional fluorogenic tetrazine probes rely on TBET or FRET mechanisms, our novel methodology cages the fluorophores through an ICT quenching pathway. Using this alternative fluorogenic mechanism, we have been able to employ a wide range of fluorophore scaffolds, including NIR-emitting cyanine dyes that are not well-quenched by tetrazines via energy-transfer mechanisms. We have demonstrated the use of these probes to detect RNA within both an in vitro and cellular context. We believe these NIR fluorogenic bioorthogonal probes

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will have future applications in RNA detection in cells and tissues as well as RNA diagnostics.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b01625.

Detailed experimental procedures, additional data controls, and characterization data, including Tables S1 and S2, Figures S1−S9, and Scheme S1 (PDF)

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Notes

The authors declare no competing financial interest.

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