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Ideal Bioorthogonal Reactions Using A Site-Specifically Encoded Tetrazine Amino Acid

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Supporting Information

ABSTRACT: Bioorthogonal reactions for labeling biomolecules in live cells have been limited by slow reaction rates or low component selectivity and stability. Ideal bioorthogonal reactions with high reaction rates, high selectivity, and high stability would allow for stoichiometric labeling of biomolecules in minutes and eliminate the need to wash out excess labeling reagent. Currently, no general method exists for controlled stoichiometric or substoichiometric labeling of proteins in live cells. To overcome this limitation, we developed a significantly improved tetrazine-containing amino acid (Tet-v2.0) and genetically encoded Tet-v2.0 with an evolved aminoacyl-tRNA synthetase/$tRNA_{(CUA)}$ pair. We demonstrated *in cellulo* that protein containing Tet-v2.0 reacts selectively with cyclopropane-fused *trans*-cyclooctene (sTCO) with a bimolecular rate constant of 72,500 ± 1660 M^{-1} s^{-1} without reacting with other cellular components. This bioorthogonal ligation of Tet-v2.0-protein reacts *in cellulo* with substoichiometric amounts of sTCO-label fast enough to remove the labeling reagent from media in minutes, thereby eliminating the need to wash out label. This ideal bioorthogonal reaction will enable the monitoring of a larger window of cellular processes in real time.

The development of bioorthogonal reactions and strategies to apply them in the study of biopolymers has transformed our ability to study and engineer biomolecules. The early successes of this technology inspired nearly two decades of research toward building faster and more selective reactions.[1] The broadly defined bioorthogonal reaction is a selective reaction between functional groups in the presence of biological entities. Great progress has been made at increasing the rate and selectivity of bioorthogonal reactions, but the vast majority of reactions still cannot be used inside living cells because: (i) high molecular concentrations in cellular environments increase off target side-reactions, (ii) the functional groups introduced compromise the cellular environment and/or catalytic processes, and (iii) the cell interior is challenging to access efficiently with the necessary functionalized molecules.[1] A few chemoselective reactions have cleared the more stringent *in cellulo* hurdle, but their sluggish reaction rates prevent utility.[2] The ideal bioorthogonal reaction which functions *in cellulo* with quantitative yields at low concentrations and with exquisite chemoselectivity is said to represent the Holy Grail of chemical synthesis.[3]

The goal of the ideal bioorthogonal reaction should be to label molecules *in cellulo* faster than the rate constants of cellular processes but without side reactions or degradation of reagents that prevent complete ligation. To compete effectively with cellular processes, ideal bioorthogonal reactions need (i) fast kinetics (>10^4 M^{-1} s^{-1}) to react completely on biological time scales of seconds to minutes and to function at biological concentrations (μM to nM) of both biomolecule and label, (ii) high selectivity to ensure only the target biomolecules are modified, (iii) functional groups stable enough to enable the labeling of quantitative portions of biomolecules *in vivo*, and (iv) small structural components as to not adversely affect the structure and function of the biomolecule under investigation.

As defined, ideal bioorthogonal reactions would enable access to new scientific inquiry because they could turn on or trap typical biological events *in vivo* at rates comparable to enzymatic reactions (typically 10^3–10^6 M^{-1} s^{-1}). In addition, many applications, such as delivery of visual probes in organisms for nuclear medicine, single molecule spectroscopy, and fluorescent imaging, demand extremely fast reaction rates because low concentrations of labeling reagents are required.[1a,c,4] The ideal bioorthogonal reaction presented here will allow short reaction times even at substoichiometric concentrations of labeling reagents. The use of stoichiometric concentrations of labeling reagent reduces background signal and side reactions from excessive unreacted label.

An exciting class of bioorthogonal ligations, inverse-electron demand Diels–Alder (IED-DA), posts rate constants up to 10^6 M^{-1} s^{-1} between tetrazines and strained *trans*-cyclooctenes (TCO).[5] Current functional groups that provide these exceptional rates lack the *in vivo* stability and selectivity to meet the requirements of the ideal bioorthogonal reaction. More stable TCO-containing amino acids have been site-specifically incorporated into proteins by using genetic code expansion and react *in vivo* with dipyrimidal-tetrazines, showing labeling rates of 5200 M^{-1} s^{-1}.[2b,6] Unfortunately, when the reaction rate is increased by adding electron-withdrawing groups to the tetrazine or strain to TCO, these components lose significant *in vivo* selectivity. The commonly used 3-phenyl-*s*-tetrazine and 3,6-(dipyridin-2-yl)-*s*-tetrazine are extremely reactive with strained

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alkenes but can act as electrophiles for cellular thiols.[7] A strained version of *trans*-cyclooctene, sTCO, (cyclopropane-fused trans-cyclooctene) is also not compatible with genetic code expansion as an amino acid because its isomerization *in vivo* results in a half-life of 0.67 days.[6,8] If instead, a modestly active tetrazine amino acid is encoded into the protein, the short half-life of sTCO is acceptable because the sTCO-attached labeling reagent will be consumed prior to significant decomposition.

We site-specifically encoded the first tetrazine amino acid (Tet-v1.0) into proteins showing this functionality is compatible with genetic code expansion (Figure 1A).[2b,6] The *in cellulo*

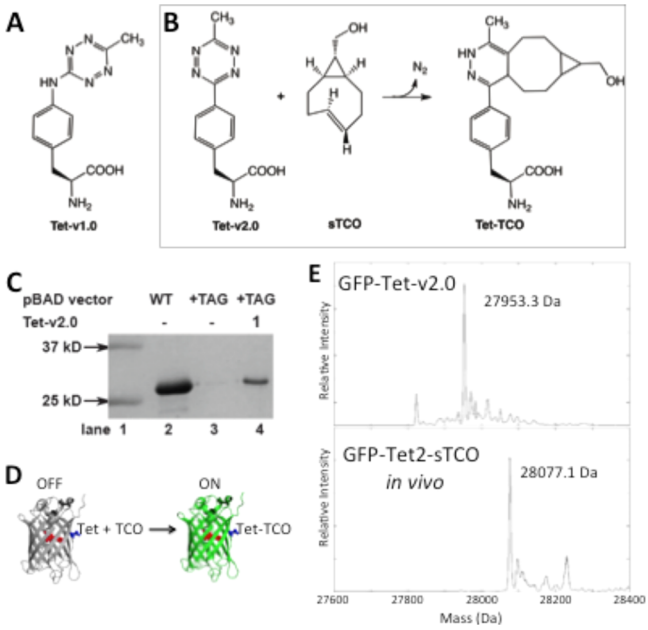

Figure 1. Genetic incorporation of Tet-v2.0, into proteins and labeling with sTCO. (A) Structure of Tet-v1.0 (B) Reaction of Tet-v2.0 with sTCO to form the stable conjugate Tet-sTCO. (C) SDS-PAGE analysis of site-specific incorporation of Tet-v2.0 in response to the amber codon. Lane 2 shows expression levels of GFP-wt from pBad-GFP-His_6. Lanes 3 and 4 show the Tet-v2.0 dependent production of GFP-Tet-v2.0 (D) Excitation at 488 nm produces low fluorescence for GFP-Tet, while the reaction forming GFP-Tet-TCO produces full fluorescence for GFP. (E) ESI-Q MS analysis of GFP-Tet-v2.0 shows a single major peak at 27,953.3 ± 1 Da. *In cellulo* reaction of GFP-Tet-v2.0 with sTCO shows a single major peak at 28,077.1 ± 1 Da consistent with the expected mass increase from specific and quantitative reaction with sTCO. Each sample did show +22 ± 1 and −131 ± 1 Da peaks consistent with the mass of a sodium adduct and the removal of N-terminal methionine. No other peaks were observed that would correlate with background incorporation of natural amino acids.

reaction rate of Tet-v1.0 with sTCO was faster than most bioorthogonal ligations at 880 M^{-1} s^{-1}, but was not fast enough to probe biological processes as an ideal bioorthogonal reaction. A maximum synthetic yield of 3% and low levels of hydrolysis at the amine linkage are additional weaknesses of Tet-v1.0 that ultimately limit its utility. To overcome these shortcomings and push the limits of *in vivo* bioorthogonal reaction rates, we generated a second tetrazine amino acid (Tet-v2.0) using a robust synthetic route. We genetically incorporated Tet-v2.0 into proteins and characterized the reactivity of Tet-v2.0-GFP *in cellulo* to show that it qualifies as an ideal bioorthogonal ligation.

We predicted that removing the amine linkage of Tet-v1.0 would increase the tetrazine reaction rate and prevent hydrolysis at that junction. Replacing the strongly electron-donating secondary amine linkage with the weakly donating phenyl substituent is expected to significantly accelerate the IED-DA reaction.[9] Using a nickel triflate catalyst for generating tetrazines from nitriles,[10] we were able to produce 4-(6-methyl-*s*-tetrazin-3-yl)phenylalanine (Tet-v2.0) in two steps in a 57% yield from commercially available starting materials (SI Scheme 1). Tet-v2.0 proved to be highly stable in PBS exhibiting no degradation over 10 days, in contrast to 3-phenyl-*s*-tetrazine and 3,6-(dipyridin-2-yl)-*s*-tetrazine which show 50% loss after 1 day (SI Figure 11).[7] To investigate if Tet-v2.0 is stable in the presence of thiols, we monitored by NMR 1 mM Tet-v2.0 and 1 mM 2-mercaptoethanol in PBS buffer. This reaction showed no change in Tet-v2.0 over 5 days, but we did notice 2-mercaptoethanol was converted to the disulfide more quickly in the presence of Tet-v2.0 compared to controls. Increasing the concentration of Tet-v2.0 and 2-mercaptoethanol while removing oxygen allowed us to confirm that Tet-v2.0 can catalyze disulfide formation by cycling through the 1,4-dihydro-1,2,4,5-tetrazine amino acid form (SI Scheme 3). This shows that tetrazine amino acids can serve as an electrophile at high concentrations of thiols, but under biological conditions, the oxidized tetrazine redox state dominates and is available for IED-DA reactions.

In order to genetically incorporate Tet-v2.0 into protein and test its *in vivo* activity with sTCO (Figure 1B), we evolved an orthogonal *Methanococcus jannaschii* (*Mj*) tyrosyl tRNA synthetase (RS)/$tRNA_{CUA}$ pair capable of incorporating Tet-v2.0 in *E. coli* (see SI for details).[11] RS plasmids from surviving clones were transformed into cells with a plasmid containing a GFP gene interrupted with an amber codon.[12] Ninety-six colonies assessed for Tet-v2.0-dependent expression of GFP contained seven clones that had significant GFP-Tet-v2.0 expression in the presence of Tet-v2.0 and no detectable GFP fluorescence over background in the absence of Tet-v2.0 (SI Figure 4). Sequencing revealed that all seven RS sequences were unique (SI Table 2).

To facilitate robust expression of site-specifically encoded Tet-v2.0 containing proteins, the top performing Tet-RS was cloned into a *pDule* vector that contains one copy of *Mj* $tRNA_{CUA}$ to create *pDule-Tet2.0*.[11,12] Expression of a GFP gene interrupted by an amber codon at site 150 in the presence of *pDule-Tet2.0* was efficient and dependent on the presence of Tet-v2.0 (Figure 1C). Using 1 mM Tet-v2.0, 13.0 mg of GFP-Tet-v2.0 was purified per liter of medium, while GFP-wt yielded 161 mg/L under similar conditions (no GFP was produced in the absence of Tet-v2.0). To demonstrate that Tet-v2.0 can be stably incorporated into recombinant proteins using *pDule-Tet2.0*, we compared the masses of GFP-Tet-v2.0 to GFP-wt using ESI-Q mass analysis. The native GFP-wt has the expected mass of 27827 ± 1 Da and GFP-Tet-v2.0 exhibits the expected mass increase to 27,955 ± 1 Da, verifying that Tet-v2.0 is incorporated at a single site (Figure 1E and SI Figure 5A). Overall, the results of protein expression, MS analysis, and SDS PAGE demonstrate the cellular stability and efficient, high fidelity incorporation of Tet-v2.0 into proteins using a pDule system.

Previously, we showed that tetrazine amino acids quench GFP fluorescence when encoded close to its chromophore, and fluorescence returns when reacted with TCO-labels (Figure 1D). This increase in fluorescence exhibited by GFP-Tet-v2.0 upon reaction enables quantification of labeling reactions and reaction rates *in vitro* and *in vivo*. Incubation of GFP-Tet-v2.0 (1.25 μM) with 13 μM sTCO in PBS buffer showed a complete return of fluorescence in <10 s indicating that GFP-Tet-v2.0-sTCO was

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formed. ESI-Q of the desalted reaction mixture confirmed the quantitative conversion of GFP-Tet-v2.0 (expected 27,954.5 Da; observed 27,955.7 ± 1 Da) to GFP-Tet-v2.0-sTCO (expected 28,078.7 Da; observed 28,078.3 ± 1 Da) (SI Figure 5). This demonstrates, the reaction between GFP-Tet-v2.0 and sTCO is quantitative *in vitro* and that all GFP-Tet-v2.0 was in the reactive oxidized form.

To determine if this bioorthogonal ligation is also quantitative *in cellulo*, *E. coli* cells containing expressed GFP-Tet-v2.0 were incubated with 3.3 μM sTCO in PBS buffer. Complete fluorescence returned in <10 s, indicating that GFP-Tet-v2.0-sTCO had been formed. After incubation at room temperature for 24 h, the cells were lysed, and GFP-Tet-v2.0-sTCO-His_6 was affinity purified and analyzed by ESI-Q MS. The resulting molecular mass matched the expected molecular mass of GFP-Tet-sTCO (Figure 1E). This verifies that the *in cellulo* reaction is facile, quantitative, and produces a stable conjugated product.

An ideal bioorthogonal reaction requires an *in cellulo* rate of $>10^4$ M^{-1} s^{-1} to reach completion in seconds to minutes at biological concentrations (μM to nM) of both biomolecule and label. To determine if reactions of Tet-v2.0 on a protein are fast enough to meet these rates, the reaction of GFP-Tet-v2.0 with sTCO was measured. The kinetics of the reaction were performed under pseudo-first-order conditions as verified by a single exponential fit for return of product fluorescence. The *in vitro* second-order rate constant for GFP-Tet-v2.0 with sTCO was calculated to be 87,000 ± 1440 M^{-1} s^{-1} (Figure 2A). Surprisingly the site-specific Tet-v2.0-protein reaction with sTCO is 2 orders of magnitude faster than Tet-v1.0.

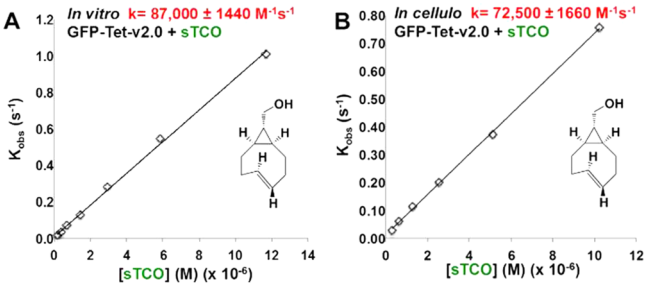

Figure 2. *In vitro* and *in cellulo* rate constant determination for reaction of GFP-Tet-v2.0 with sTCO. (A) Kinetics of GFP-Tet-v2.0 with sTCO *in vitro* resulted in a rate constant of k = 87,000 ± 1440 M^{-1} s^{-1} in a PBS buffer at pH 7 at 21 °C. (B) Kinetics of GFP-Tet-v2.0 with sTCO *in cellulo* resulted in a rate constant of k = 72,500 ± 1660 M^{-1} s^{-1}. For both experiments, unimolecular rate constants were calculated by fitting the rate of product formation to a single exponential at different concentrations of sTCO, and the bimolecular rate constant was determined using the observed unimolecular rate constants (k_{obs} = k[TCO]).

To date, no bioorthogonal rate constants $>10^3$ M^{-1} s^{-1} have been measured *in cellulo*.[1b,c] To determine the rate constant for this reaction inside live cells, *E. coli* expressing GFP-Tet-v2.0 was washed in PBS buffer, and reacted with sTCO. The *in cellulo* bimolecular rate constant for this reaction is 72,500 ± 1660 M^{-1} s^{-1} and is fast enough to meet the needs of the ideal bioorthogonal ligation (Figure 2B). This *in cellulo* reaction rate will allow 95% labeling in <1 min at 1 μM Tet-v2.0-protein and sTCO label. The short reaction time is enabled by a $t_{1/2}$ of 12−14 s. Ideal bioorthogonal reaction rates eliminate the need for time-consuming washing steps prior to cell analysis and allow for immediate monitoring of cellular events since the labeling reaction is rapidly completed at stoichiometric concentrations of label.

To verify that the Tet-v2.0-protein/sTCO reaction rate is sufficient to effectively use substoichiometric concentrations of label in live cells, we reduced the amount of sTCO added to *E. coli* cells containing GFP-Tet-v2.0 (Figure 3A). For comparison,

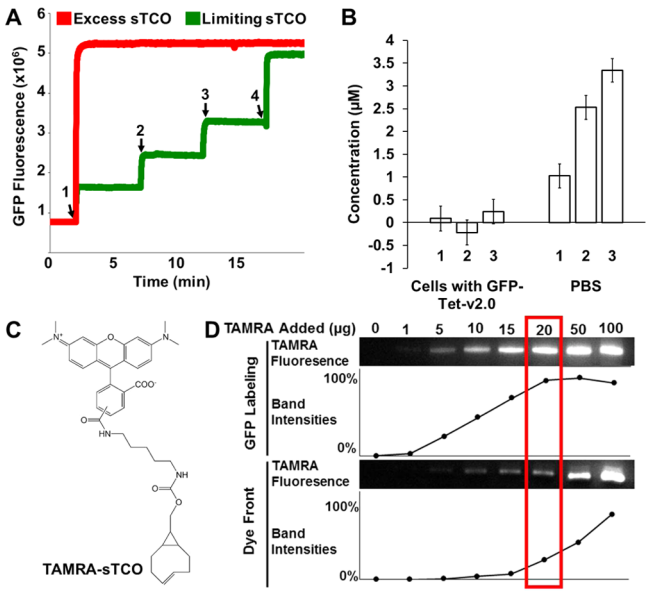

Figure 3. Substoichiometric characterization of GFP-Tet-v2.0 reaction with sTCO. (A) Red trace shows fluorescent change from sTCO added in excess. Green trace shows fluorescent change from the first three additions of 1/5 equiv of sTCO and the fourth addition of excess sTCO. (B) Concentrations of sTCO in medium were determined for samples removed after sTCO additions 1−3. Concentrations of sTCO were determined for identical additions of sTCO to buffer alone. (C) Structure of TAMRA-sTCO. (D) Substoichiometric labeling of *E. coli* lysate containing expressed GFP-Tet-v2.0 with TAMRA-sTCO. Lysate incubated with TAMRA-sTCO was separated on SDS-PAGE and imaged fluorometrically. Displayed regions correspond to GFP and dye front migration with their relative band intensities. The red box highlights the point of 100% protein labeling.

traditional labeling conditions using an excess of sTCO show complete labeling in ∼1 min (red trace). The green trace shows four additions of sTCO to cells containing GFP-Tet-v2.0. The first three sTCO additions are 1/5 the molar amount of GFP-Tet-v2.0, and the fourth addition is an excess of sTCO. The substoichiometric labeling reproducibly showed complete labeling within 1 min. When reacting sTCO with Tet-v2.0-protein substoichiometrically *in vivo*, all sTCO-label should bind to Tet-v2.0-protein *in vivo* leaving none in extracellular solution. To verify that this was the case in our experiment, we assayed samples of the solution for sTCO after fluorescence plateau from each sTCO addition (points 1−4, Figure 3A). Following substoichiometric additions of sTCO, (points 1−3) negligible concentrations of sTCO were detected in solution (Figure 3B). This contrasts with the stepwise increase in concentration of sTCO detected in solution when identical amounts of sTCO were added to PBS buffer in the absence of Tet-v2.0-protein. This feature of Tet-v2.0 thus eliminates the need for a wash out step when labeling protein *in vivo* if sTCO is conjugated to a fluorescent dye.

To demonstrate that the wash out step of a conjugated dye is nonessential when reaction rates of this magnitude are employed,

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a tetramethyl-rhodamine (TAMRA)-linked sTCO label was synthesized (Figure 3C). TAMRA-sTCO was incubated with purified GFP-Tet-v2.0 *in vitro*, and analysis by SDS-PAGE demonstrated a reaction between GFP-Tet-v2.0 and TAMRA-sTCO (SI Figure 9). Fluorescence imaging of the gel showed a band present only when TAMRA-sTCO and GFP-Tet-v2.0 were present. Labeling of protein in living cells with low concentrations of dyes is often slow and incomplete because dye diffusion into cells at these concentrations and time scales is limiting.[13] Conjugated TAMRA dyes have previously been shown to enter mammalian cells, but slower bioorthogonal reaction rates required higher concentrations of TAMRA-labels and longer reaction times.[6,14] As suggested by others, improved fluorescent dyes are needed to overcome the rate-limiting steps of cellular uptake with fast bioorthogonal ligations.[13] To circumvent this problem for this substoichiometric demonstration, we reacted TAMRA-sTCO with *E. coli* lysate containing GFP-Tet-v2.0 at quantities of TAMRA-sTCO ranging from 5 to 500% of the total GFP-Tet-v2.0 concentration. The lysate was analyzed by SDS-PAGE and showed two rhodamine fluorescence bands; a ∼27 kDa band corresponding to GFP-Tet-v2.0 conjugated to TAMRA-sTCO and a dye front migrating band corresponding to unreacted TAMRA-sTCO (Figure 3D). As expected, the fluorescent TAMRA-GFP band increased incrementally in intensity with additions of TAMRA-sTCO until the intensity plateaued at ∼100% labeled GFP-Tet-v2.0 (20 μg TAMRA-sTCO Figure 3D). While TAMRA-sTCO was added to the full lysate, only Tet-v2.0-GFP was labeled, and TAMARA dye did not accumulate at the dye front of the gel until GFP-Tet-v2.0 was completely labeled (Sup Figure 10). After this point, TAMRA fluorescence at the dye front increased rapidly with the amount of TAMRA-sTCO added as would be expected from a reaction with excess label. Together these data indicate that efficient substoichiometric reactions of protein-Tet-v2.0 with TAMRA-sTCO are possible in the presence of cellular components.

In summary, we have developed an *in cellulo* bioorthogonal reaction based on a genetically encodable tetrazine amino acid that meets the demands of an ideal bioorthogonal ligation. Tet-v2.0 is a small amino acid and can be easily moved to many locations on a protein as to not perturb protein structure and function. The on-protein bimolecular rate constant of 87,000 ± 1440 M^{-1} s^{-1} gives this robust reaction the speed it needs to compete with cellular processes.

The same attributes that make this reaction ideal open the door to a variety of applications. The bimolecular rate constant is a significant improvement over previous *in vivo* biorthogonal ligations. This speed affords complete labeling of Tet-v2.0-protein in minutes even with low concentration of the sTCO label or concentrations below that of the protein being labeled. A substoichiometric *in vivo* biorthogonal ligation has applications toward drug-antibody conjugates where it could minimize the clearance time of drugs or radioactive labels targeted to specific cells. Additionally, the high rate combined with *in cellulo* reactivity enable one to probe various pathways on a biologically relevant time scale. To our knowledge, this is the first demonstration of a bioorthogonal ligation with sufficient selectivity and a high enough reaction rate to substoichiometrically label proteins in live cells, thereby eliminating the need to wash out excess label prior to imaging. At this point, the ability of the fluorescent probe to enter the cytosol is the limiting factor to *in cellulo* substoichiometric labeling. Combining the flexibility of genetic code expansion with the diversity of labels in live cells allows for numerous creative applications that modulate cellular function.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b03275.

Experimental data and supplementary figures (PDF)

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Notes

The authors declare no competing financial interest.

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