A Journal of the Gesellschaft Deutscher Chemiker

Angewandte International Edition GDCh Chemie

www.angewandte.org

Accepted Article

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To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.202001830

Link to VoR: https://doi.org/10.1002/anie.202001830

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10.1002/anie.202001830

RESEARCH ARTICLE

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Expanding the Versatility of Microbial Transglutaminase Using α-Effect Nucleophiles as Noncanonical Substrates

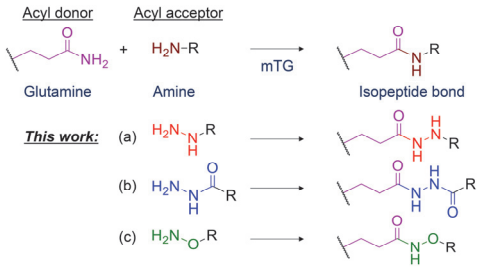
Tak Ian Chio,[a] Breanna R. Demestichas,[a] Brittany M. Brems,[b] Susan L. Bane,*[a] and L. Nathan Tumey*[b]

Abstract: The substrate promiscuity of microbial transglutaminase (mTG) has been exploited in various applications in biotechnology, in particular for the attachment of alkyl amines to glutamine-containing peptides and proteins. Here we expand the substrate repertoire to include hydrazines, hydrazides, and alkoxyamines, resulting in the formation of isopeptide bonds with varied susceptibilities to hydrolysis or exchange by mTG. Furthermore, we demonstrate that simple unsubstituted hydrazine and dihydrazides can be used to install reactive hydrazide handles onto the side chain of internal glutamine residues. The distinct hydrazide handles can be further coupled with carbonyls, including *ortho*-carbonylphenylboronic acids, to form site-specific and functional bioconjugates with tunable hydrolytic stability. The extension of the substrate scope of mTG beyond canonical amines thus substantially broadens the versatility of the enzyme, providing a new approach to facilitate novel applications.

Introduction

A number of enzyme-based methods have been developed to incorporate novel functionalities into biomolecules.[1] These strategies make use of the ability of the enzymes to accept diverse substrate analogues. One prominent member of this enzymatic toolbox is transglutaminase (TG), of which the simpler single-domain and calcium-independent variants of microbial origin are commonly used.[2] Microbial transglutaminase (mTG) catalyzes a transamidation reaction between certain surface-exposed glutamine (Gln) and lysine (Lys) residues on protein substrates, crosslinking the two via an isopeptide bond. In addition to Lys, mTG is also known to recognize a wide range of primary amine substrates, thus providing the basis for mTG's utility to equip proteins with assorted functional moieties.[3] The enzyme, for instance, has been employed to generate protein-polymer and antibody-drug conjugates (ADCs) for pharmaceutical applications.[4]

The catalytic mechanism of mTG involves a nucleophilic attack of the target acyl donor, Gln, by the enzyme's active site cysteine to form a thioester intermediate. Subsequent nucleophilic attack of the thioester by an amine substrate serving as the acyl acceptor results in the formation of the transamidated product. Given the mechanistic role of the amine as a nucleophile and the broad substrate tolerance of the enzyme, we envisaged that the scope of the acyl acceptor substrate could be extended to include other nucleophiles, such as α-effect amines.



Scheme 1. Transamidation reaction catalyzed by microbial transglutaminase (mTG) using an amine or an α-effect nucleophile – (a) hydrazine, (b) hydrazide, or (c) alkoxyamine as the acyl acceptor substrate.

Indeed, evidence of hydrazine and hydrazide acting as TG substrates can be found in early studies in the 1970s that aimed to elucidate the mechanism of hydrazine-/hydrazide-functionalized agents as inhibitors of mammalian tissue TG.[5] Detection of isotopic labeling by C14-isonicotinic acid hydrazide (isoniazid) was observed for N-benzyloxycarbonyl-L-glutaminylglycine (ZQG, a common model acyl donor substrate for TG) and a variety of proteins using guinea pig liver TG. Hydrazinonaphthalazine (hydralazine) reportedly led to the same result. Meanwhile, hydroxylamine is used as a substrate in the classical hydroxamate assay used to measure TG activity.[6] Despite these early indications that TG can recognize α-effect nucleophiles, contemporary use of mTG in bioconjugation chemistry has been exclusively focused on alkyl amine substrates. Herein, we explore the expansion of the applied substrate palette to include α-effect nucleophiles (Scheme 1) and we show that the versatility of mTG can be bolstered by using these "rediscovered" substrates.

Results and Discussion

Coupling of α-effect nucleophiles to peptides/proteins by mTG

We first set out to verify mTG's substrate tolerance to α-effect nucleophiles, as evidence from previous studies were primarily based on mammalian TG. The ZQG acyl donor substrate was incubated with mTG and benzoic hydrazide, the latter being isostructural to isonicotinic acid hydrazide and used as our initial α-effect amine-functionalized substrate. Analysis by ESI-MS found a product with an m/z corresponding to ZQG incorporated with benzoic hydrazide (Figure 1a; Figure S1). The structure of

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the isolated product was determined by NMR (Figure S2), which indicated that the terminal amino group of the hydrazide (not the amide-like nitrogen) was the site of conjugation to the acyl group of Gln in ZQG.

Having demonstrated that benzoic hydrazide is an effective mTG substrate in a model dipeptide, we next examined whether it could serve as a substrate in a more typical site-specific protein conjugation. Given the growing prevalence of mTG in the assembly of site-specific ADC therapeutics,[4b, 4c] a trastuzumab antibody targeting the oncogenic human epidermal growth factor receptor 2 (HER2) was used as our model protein. In particular, we used a mutant, herein referred as Tras LC-Q, that has an LLQG tag engineered at the C-terminus of the light chain. Since the endogenous Gln residues in native immunoglobulin G antibodies lack reactivity to mTG, enzymatic conjugation is known to occur site-specifically at the engineered Gln on the light chain.[4c] Indeed, treatment of Tras LC-Q with benzoic hydrazide under fairly typical mTG coupling conditions resulted in a single conjugation per light chain, but not the heavy chain (Figure 1a; Figure S3). Meanwhile, the native trastuzumab, which does not carry the engineered Gln, was inert when subjected to the same conditions (Figure S4), supporting the specificity of the enzymatic reaction.

Kinetics of mTG-catalyzed conjugation of benzoic hydrazide to Tras LC-Q was then compared with its amine counterpart, phenethylamine. Both substrates demonstrated very similar rates of conjugation (Figure 1b; Figure S8), suggesting that the enhanced nucleophilicity of the hydrazide did not result in an increase in the rate of displacement of the thioester acyl-enzyme intermediate.

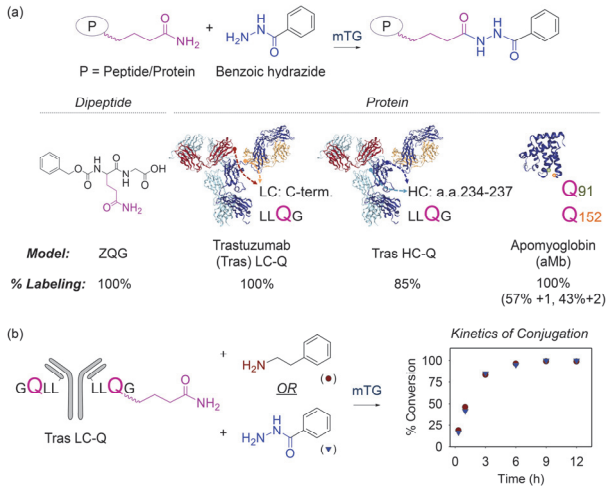


Figure 1. Coupling of α-effect nucleophiles to peptides/proteins by mTG. (a) mTG-catalyzed conjugation of benzoic hydrazide to ZQG, Tras LC-Q, Tras HC-Q, or aMb. For aMb, two products with modification on one (+1) or two (+2) sites were detected. PBD entries 1HZH and 1YMB were used as representative illustrations of the trastuzumab mutants and aMb, respectively. Each chain of the antibody is color-coded, with red and yellow corresponding to the light chain (LC) and light blue and dark blue corresponding to the heavy chain (HC). The pinpointed sites are the approximate locations of the reactive Gln on the protein based on the PBD entries. C term. = C-terminus; a.a. = amino acid. Similar protein conjugations with other α-effect amine substrates (phenylhydrazine and *o*-benzylhydroxylamine) are shown in Figure S5, S7, & S9. (b) Comparison of the kinetics of enzymatic conjugation of phenethylamine (circle) vs. benzoic hydrazide (triangle) to Tras LC-Q.

To determine whether antibody conjugation with the hydrazide substrate can be extended to locations beyond the C-terminus of Tras LC-Q, we tested another trastuzumab mutant, referred as Tras HC-Q, which has the LLQG tag engineered at the internal residues 234-237 of the heavy chain's C_H2 domain.[4c] Specific incorporation of benzoic hydrazide to the heavy chain of Tras HC-Q was observed (~85%), demonstrating that the recognition motif installed on other sites of the antibody are amenable to conjugation with α-effect amines by mTG (Figure 1a; Figure S5). We also attempted to modify the native Gln295 residue of a deglycosylated trastuzumab.[4b] However, no incorporation of benzoic hydrazide was observed on the antibody under our experimental conditions (Figure S6).

To further demonstrate the scope of the mTG-promoted reaction with noncanonical substrates, we employed another protein, apomyoglobin (aMb), which has been previously shown to undergo mTG-mediated modification by amines at Gln91 (fully modified) and Gln152 (partially modified).[7] Consistent with this study, incubation of aMb with mTG and benzoic hydrazide yielded two product peaks on ESI-MS (Figure 1a; Figure S7), with the major and minor products harboring one and two modifications, respectively. Taken together, our results thus far showcased the ability of mTG to recognize an α-effect amine-functionalized substrate, which can be coupled enzymatically to reactive Gln-containing peptides and proteins.

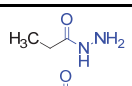
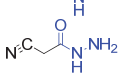
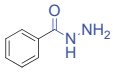
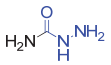
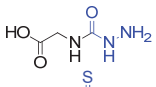
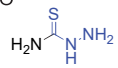
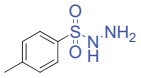
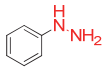
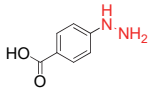
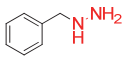
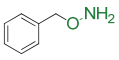
Conjugation efficiency and optimization for coupling of assorted α-effect nucleophile substrates

We next surveyed the structure-reactivity relationship for a series of hydrazide derivatives, including alkyl hydrazides (**1a-b**), semicarbazides (**1d-e**), thiosemicarbazides (**1f**) and sulfonyl hydrazides (**1g**), as well as a few hydrazines (**1h-j**) and alkoxyamines (**1k-l**). The efficiency of conjugation of the tested substrates was calculated based on the loading on the light chain of Tras LC-Q as determined by ESI-MS after antibody reduction (Figure S9). Encouragingly, many of the assorted α-effect nucleophile substrates (**1b-d**, **1h**, **1l**) exhibited high efficiency of conjugation (>80%). Some were less efficient substrates under our initial conditions (Table 1; Condition 1). Compounds with a nearby carboxylic acid exhibited comparatively lower extent of conjugation than their parent molecule (compare **1d** vs. **1e** and **1h** vs. **1i**). This is consistent with previous observations that having a carboxylic acid group proximal to the amine hampers conjugation,[8] suggesting that the structure-reactivity relationship for amine substrates may be transferrable to α-effect nucleophiles.

In order to increase the yield for the less efficient substrates, we performed a systematic optimization of a number of parameters, including pH, temperature, and the equivalents of mTG and substrate (Figure S10). Unexpectedly, reduced temperature (4°C) significantly improved the loading as compared to 22°C and 37°C (Figure S10b). We speculate that this may be due to improved enzyme stability. A negligible effect was observed when increasing the enzyme concentration (Figure S10c), similar to results from a previous study.[4d] Meanwhile, increased substrate concentration contributed positively to loading (Figure S10d). Using thiosemicarbazide as the representative substrate for this optimization, the loading was increased from ~16% to ~95% (Table 1). The optimized conditions enabled complete or near-complete loading for many

of the substrates that were less efficiently conjugated under the parent conditions (Table 1; Condition 2). The exception in our list is *p*-toluenesulfonyl hydrazide (**1g**), which exhibited only 10% loading even under the optimized conditions, suggesting that (aryl) sulfonyl hydrazides are inherently poor substrates for mTG.

Table 1. Percent mTG-mediated conjugation of assorted α-effect nucleophiles to Tras LC-Q

	Substrate	Structure	Condition 1[a]	Condition 2[b]
1a	Propanoic hydrazide		57	84
1b	Cyanoacetohydrazide		94	ND
1c	Benzoic hydrazide		100	ND
1d	Semicarbazide		86	100
1e	(Hydrazinecarbonyl) glycine		67	95
1f	Thiosemicarbazide		16	95
1g	*p*-Toluenesulfonyl hydrazide		0	10
1h	Phenylhydrazine		99	ND
1i	4-hydrazinobenzoic acid		0	ND
1j	Benzylhydrazine		77	85
1k	*o*-Ethylhydroxylamine		62	100
1l	*o*-Benzylhydroxylamine		98	ND

[a] Condition 1: 1 mg/mL Tras LC-Q, pH = ~7.4, 6.5 wt. eq. mTG, 5% DMSO, 50 eq. substrate (per reactive Gln), 22°C. [b] Condition 2: 1 mg/mL Tras LC-Q, pH ~7.4, 65 wt. eq. mTG, 0% DMSO, 450 eq. substrate (per reactive Gln), 4°C. ND = not determined. Note: The weight equivalent (wt. eq.) of mTG corresponds to the ratio of the wt. of the formulated mTG (which contains ~1% mTG by wt.) to the wt. of the antibody used in the sample.

Stability of the isopeptide bond analogues toward mTG-mediated hydrolysis and exchange

For the typical mTG-catalyzed transamidation of Gln with an alkyl amine, the resultant linkage is an amide bond (known as an isopeptide bond). On the other hand, transamidation with α-effect amines result in the formation of isopeptide analogues, which are akin to classical peptide bond surrogates employed for peptide backbone modifications (e.g. hydrazinopeptide, aminoxypeptide, and azapeptide).[9] The unique chemical and structural properties of these pseudopeptide linkages can contribute to enhanced proteolytic stability, hence their application in peptidomimetics.[10] The stability improvement associated with these "α-effect" pseudopeptide linkages thus prompted us to explore whether the isopeptide bond analogues formed from the α-effect amine substrates (Table 1) may confer a similar protective ability against proteolysis, specifically by mTG itself. Although TG is known for its bond-forming reaction, it is also able to catalyze the reverse reaction.[11] In the absence of excess amine substrates, the enzyme can hydrolyze the isopeptide linkage, thereby irreversibly converting the original Gln residue to a glutamic acid. Alternatively, in the presence of an excess of another amine substrate, the enzyme can promote transamidation, or the exchange of one amine for another. We therefore examined whether the isopeptide analogues generated by mTG and α-effect amine substrates can prevent this reversal or exchange reaction, thus offering an advantage over traditional amine substrates.

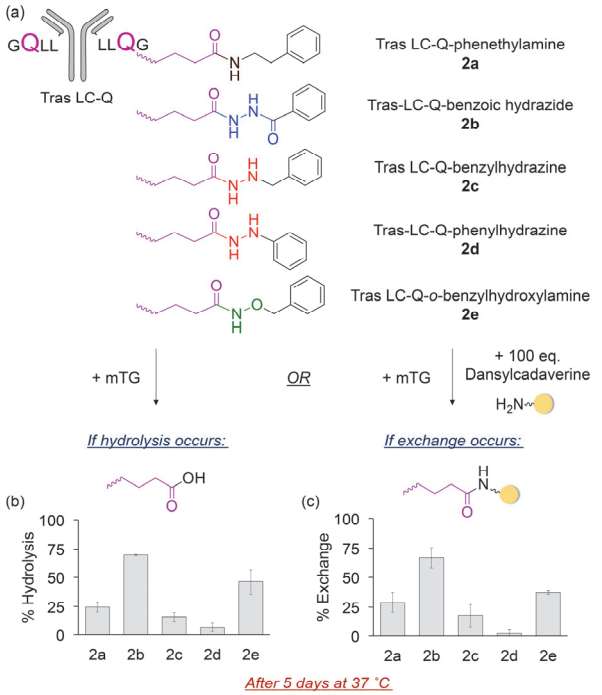


Figure 2. Stability of the isopeptide bond analogues toward hydrolysis or exchange by mTG. (a) Scheme illustrating the Tras LC-Q conjugates prepared for stability evaluation and the resultant structures should hydrolysis or exchange occur. 2a-2e were incubated in 0.1 M PBS, pH 7.4 at 37 °C for 5 days in the presence of (b) mTG or (c) mTG and dansylcadaverine to assess their susceptibilities to hydrolysis or exchange, respectively. The bar graphs illustrate the average and the standard deviation from two independent experiments.

To compare the stability of the different isopeptide derivatives, we prepared a series of conjugates of Tras LC-Q with phenethylamine (**2a**), benzoic hydrazide (**2b**), benzylhydrazine (**2c**), phenylhydrazine (**2d**), and *o*-benzylhydroxylamine (**2e**) (Figure 2a). The conjugates were then subjected to incubation with mTG in phosphate-buffered saline (PBS), pH 7.4 at 37 °C and their integrity was monitored over time (up to five days) by ESI-MS. Reversibility was detected based on the appearance of a lower molecular weight peak

corresponding to the deamidated species. The extent of hydrolysis of the conjugates was observed in the order, from most to least: benzoic hydrazide > *o*-benzylhydroxylamine > phenethylamine > benzylhydrazine > phenylhydrazine (Figure 2b; Figure S11). The same trend was observed when excess of a different amine substrate, dansylcadaverine, was incubated along with the conjugate and the enzyme, though instead of the deamidated species, the transamidated product with dansylcadaverine was observed (Figure 2c; Figure S11). Notably, in both scenarios of enzymatically induced hydrolysis or exchange, the two conjugates of Tras LC-Q with hydrazine substrates, benzylhydrazine (**2c**) and phenylhydrazine (**2d**), exhibited the least turnover, with the latter showing ≤ ~10% deamidation after five days under these forcing conditions. Curiously, under identical conditions but in the absence of mTG, **2d** and, to a small extent, **2c**, underwent hydrolysis over the five-day incubation (Figure S12). The reason for this deviation is unclear given the general regard of the hydrazide linkage to be relatively stable.[12]

The stability evaluation based on Tras LC-Q conjugates **2a**-**2e** suggests that the isopeptide subtype generated by conjugation of hydrazine substrates are less susceptible to reversibility or exchange by mTG than the classical isopeptide linkage. As enzymatic reactivity of the acyl donor substrate may differ depending on nearby residues and its local conformation,[7, 13] we note that the trend may differ depending on the site and the protein. Nevertheless, the overall differential stabilities of the isopeptide bond derivatives toward hydrolysis or exchange by mTG present a new design consideration when using the enzyme as a tool for bioconjugate assembly.

Functionalization of a peptide/protein with a side chain hydrazide group

In an early study examining unsubstituted hydrazine as a diamine substrate, the authors observed two products after incubating it along with ZQG and guinea pig liver TG.[14] One of the products was a dimer crosslinked via both ends of hydrazine. Another product, of interest to us, was a monomer in which one end of the hydrazine was conjugated, transforming Gln into γ-glutamic acid-hydrazide. Formation of this monomer species could be favored over the dimer by increasing the concentration of hydrazine, which presumably competes with the monomer product as substrate. Therefore, in the presence of TG and sufficient excess of hydrazine, the Gln side chain could be converted into a hydrazide functional group. This previous work thus inspired us to explore the application of unsubstituted hydrazine as a TG substrate to introduce a side chain hydrazide moiety in peptides and proteins.

We first verified that hydrazine also acts as a substrate for mTG, resulting in the conversion of ZQG to Z(Q-hydrazide)G as was found with mammalian TG (Figure S13). Furthermore, continuing with the rationale of using difunctional hydrazines to convert the carboxamide group of Gln into a hydrazide, the simplest dihydrazides, namely carbohydrazide and thiocarbohydrazide, were also explored as substrates (Figure 3a). In both cases, ZQG was converted to the respective Z(Q-carbohydrazide)G and Z(Q-thiocarbohydrazide)G as confirmed by ESI-MS (Figure S14-15).

Next, the ability to install the various hydrazide functional groups on a protein was examined, again using Tras LC-Q. Full modification of the engineered Gln on the light chain was observed using unsubstituted hydrazine as substrate (Figure 3a; S18). For carbohydrazide, in addition to the modified light chain, a species with a mass equivalent to a dimer of two light chains crosslinked via both ends of a carbohydrazide was observed on ESI-MS (Figure S16). As expected based on the previous study,[14] formation of the functionalized monomer can be maximized by increasing the carbohydrazide concentration (Figure S16-17). Meanwhile, derivatization with thiocarbohydrazide can be achieved using the optimized conditions in Table 1 (Condition 2) (Figure S18).

Since the reactive Gln in the LLQG tag of Tras LC-Q is positioned within the C-terminus, we sought to illustrate further that the hydrazide functionalization could be implemented on an internal Gln residue. To that end, we treated the aMb protein with mTG along with unsubstituted hydrazine, carbohydrazide, or thiocarbohydrazide. Indeed, as determined by ESI-MS, aMb could be derivatized by all three substrates, with full conversion to the respective hydrazide group on one predominant site particularly when paired with hydrazine and carbohydrazide substrates (Figure S19). As aforementioned, Gln91 of aMb has previously been shown to be the major mTG-reactive site.[7] Therefore, the enzymatic modification likely stemmed from conjugation to Gln91, supporting the ability to achieve hydrazide functionalization at internal amino acid residues. A method to install a hydrazide group internally on the side chain would complement existing strategies that typically introduce the reactive group on the C-terminus of the protein backbone.[15] Such a method could find use in protein semisynthesis, where the hydrazide is well known for serving as a stable thioester precursor for native chemical ligation.[16] Notably, internal installation of this thioester synthon on the side chain could enable synthetic access to unique branched and lariat protein structures distinct from the canonical linear scaffold.

Application of the installed hydrazide handles in bioconjugation

Aside from potential application in protein semisynthesis, the mTG-derived hydrazides could be used as a bioorthogonal handle for coupling with aldehydes or ketones.[17] For example, hydrazones have been widely employed as pH-sensitive linkers in triggered release systems. To demonstrate similar applications of mTG-derived hydrazides, Tras LC-Q was derivatized with an alkyl hydrazide (**3a**), carbohydrazide (**3b**), or thiocarbohydrazide (**3c**) and allowed to react with 10 eq. of an aldehyde fluorophore (coumarin aldehyde) at pH ~7.4 in the presence of 4-amino-phenylalanine as a reaction catalyst (Figure 3a).[18] Reducing SDS-PAGE analysis showed that only the modified light chain was fluorescently labeled, consistent with the chemoselective nature of the reaction (Figure 3b, lanes 1-4). Tras LC-Q-hydrazide (**3a**) and -carbohydrazide (**3b**) had similar coupling efficiencies (~70% based on ESI-MS; Figure S21). On the other hand, Tras LC-Q-thiocarbohydrazide (**3c**) had a relatively low conjugation yield (~30%) under the experimental conditions. We thus focus our discussion on **3a** and **3b**, though we note that the sulfur-containing thiocarbohydrazide handle (**3c**) may be useful for other applications.

Periodic reimaging of the gel stored in acidic destaining solution (10% acetic acid, 50% methanol) showed that the

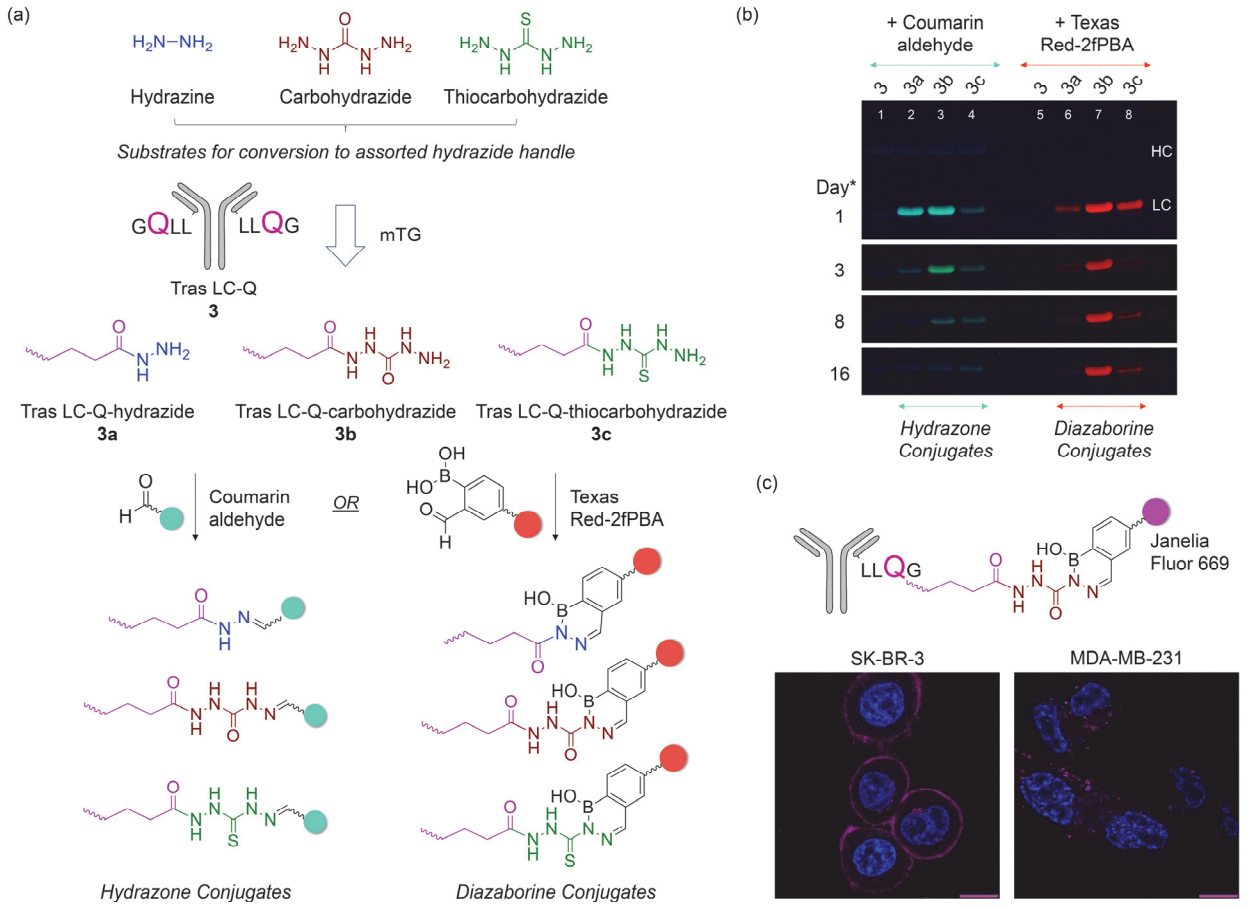


Figure 3. Introduction of assorted reactive hydrazides on the Gln side chain of Tras LC-Q and their applications in bioconjugation. (a) Scheme illustrating the use of unsubstituted hydrazine and dihydrazides as mTG substrates to install assorted hydrazide handles and their subsequent coupling to an aldehyde- or 2fPBA-functionalized fluorophore. (b) Reducing SDS-PAGE and in-gel fluorescence of the carbonyl coupling reactions. *The numbers on the side correspond to the number of days that the gel was kept in an acidic destaining solution (10% acetic acid, 50% methanol). All in-gel fluorescence images were captured using the same exposure time. The image of the gel after Coomassie staining is shown in Figure S23. HC = heavy chain; LC = light chain. (c) Fluorescence microscopic images of SK-BR-3 or MDA-MB-231 cells that were treated with the antibody-fluorophore conjugate of **3b** and Janelia Fluor 669-2fPBA. The cells were counterstained with Hoechst 33342 nuclear dye. A diode 405 nm laser and a 633 nm laser were used for excitation. Scale bar = 10 μm. The structures of all the fluorophores used in this figure are shown in Figure S20.

fluorescence of the bands corresponding to the conjugates of **3a** and **3b** decreased over time (Figure 3b, lanes 2 & 3), indicating hydrolysis of the hydrazone conjugates in the acidic milieu. Importantly, fluorescence of the acyl hydrazone conjugate (lane 2) diminished more quickly than the carbohydrazone (lane 3), suggesting that the carbohydrazone linkage is more hydrolytically stable. Our observation is in line with the reportedly shorter half-life of acyl hydrazone compared to semicarbazone,[19] the latter sharing resemblance to a carbohydrazone. The different hydrazide handles (alkyl hydrazide vs. carbohydrazide) therefore rendered variation in the rates of hydrolysis of the resultant hydrazone conjugates. This can be exploited to fine-tune the cleavage rate, which is an important design factor for ADCs containing such hydrolytic linkages.[20]

Although the reversible hydrazone construct has been leveraged to enable controlled payload delivery, it is not ideal for other applications where the stability of the linkage is essential. Moreover, hydrazone formation is known to be slow unless catalyzed or performed under acidic conditions.[17] Recent developments in a variation of the classical aldehyde/ketone condensation involving placement of a boronic acid *ortho* to an aromatic aldehyde or ketone offer opportunities to improve both product stability and reaction kinetics.[21] Intramolecular Lewis acid catalysis from the *ortho*-boronic acid increases the condensation rate by several orders of magnitude compared to simple carbonyls. In particular, the reaction between 2-formylphenylboronic acid (2fPBA) and a hydrazide rapidly results in a hydrazone intermediate that further cyclizes into a boron-nitrogen heterocycle.[22] Stability of this 2,3,1-benzodiazaborine (DAB) product can vary by changing the nature of the hydrazide, with certain substituted hydrazides and semicarbazides capable of forming stable DABs that are suited to serve as robust coupling scaffolds.[23] To extend the utility of the mTG-modified protein-hydrazides in areas that are limiting for conventional hydrazone conjugates, bioconjugation using the 2fPBA-based subtype of carbonyl chemistry was also examined.

Tras LC-Q-hydrazide (**3a**), -carbohydrazide (**3b**), and -thiocarbohydrazide (**3c**) were allowed to react with 5 eq. of a 2fPBA-functionalized fluorophore, Texas Red-2fPBA (Tx Red-2fPBA), at pH 7.4 (Figure 3a). Reducing SDS-PAGE analysis showed that, once again, only the modified light chain was fluorescently labeled (Figure 3b, lanes 5-8; Figure S22). In-gel fluorescence of the DAB conjugate of Tras LC-Q-hydrazide (lane 6) decreased over time when the gel was kept in acidic destaining solution, consistent with our previous finding that the DAB product of 2fPBA and an alkyl hydrazide is acid-labile.[22] On the other hand, the fluorophore-labeled conjugate of Tras LC-Q-carbohydrazide (**3b**) showed a negligible difference over time (up to two weeks), demonstrating remarkable stability (Figure 3b, lane 7). Notably, the DAB conjugate of **3b** persisted even when the hydrazone conjugate with coumarin aldehyde had disassembled. (compare lane 2-3 with lane 7). This demonstrates that the carbohydrazide-2fPBA ligation results in a DAB linkage with stability that exceeds the hydrolytic limit of typical hydrazones.

To ensure that the bioconjugate generated via the chemoenzymatic process retains the function of the biomolecule, Tras LC-Q-carbohydrazide coupled to a 2fPBA-functionalized fluorophore (Janelia Fluor 669-2fPBA) was prepared (Figure S24) and tested for its functional activity in antigen binding. SK-BR-3 (HER2+) and MDA-MB-231 (HER2−) breast cancer cells were treated with the antibody-fluorophore conjugate (AFC). Clear fluorescence localization on the cell membrane of SK-BR-3 cells, but not MDA-MB-231 cells, was observed by fluorescence microscopy (Figure 3c), illustrating the unperturbed function of the AFC in binding its HER2 target on the cell surface.

Taken together, these results demonstrate the application of the mTG-derived hydrazides in bioorthogonal conjugation with carbonyls. Importantly, they also highlighted the impact that the choice of the hydrazide and the participating carbonyl can have on the hydrolytic stability of the resultant linkage. Appreciating this tunability, our method of hydrazide functionalization affords a handful of hydrazide handles to be elected for installation. Furthermore, as a complement to strategies that commonly append a carbonyl group on the biomolecule,[24] the alternative introduction of the hydrazide functional group on the protein enables rational selection of the carbonyl partner to cater to specific applications.

Conclusion

In summary, we have demonstrated that hydrazines, hydrazides, and alkoxyamines can be used as acyl acceptor substrates for mTG, thus significantly broadening the substrate repertoire of this promiscuous enzyme. Transamidation with these noncanonical substrates produces diverse analogues of the isopeptide bond. Their disparate propensities to bond cleavage/exchange by mTG prompt new considerations when using the enzyme to assemble bioconjugates, particularly those that may encounter mTG or its homologues in their functioning milieu. In addition to providing diversity in the linkages generated via direct conjugation, the expanded substrate repertoire also offers a way to directly introduce a reactive functional group for chemoenzymatic conjugation. Unsubstituted hydrazine and dihydrazides, which are widely accessible and inexpensive, can be used as substrates for mTG to install assorted hydrazide functionalities in peptides and proteins. The introduced hydrazide handle can subsequently participate in bioorthogonal ligation with conventional aldehydes/ketones or (the more recently developed) *ortho*-carbonylphenylboronic acids to form dynamic or stable bioconjugates. Importantly, the distinguished side chain position at which the hydrazide is presented enables functionalization of an internal amino acid residue, which complements existing methods that typically introduce the hydrazide at the C-terminus or the complementary carbonyl group on the protein. The diversification of the substrate scope of mTG with α-effect amines therefore further unravels the versatility of this important enzyme and invites the possibility of continued expansion of the applied substrate palette with other nucleophilic moieties.

Acknowledgements

Funding was provided by the NIH Grant R15 CA227747, the SUNY Research Foundation Accelerator Fund (S.L.B.), and the Research Foundation of the State of New York (L.N.T.). The engineered antibodies used in this work were a gift from Pfizer, Inc. The authors wish to acknowledge the Dr. G. Clifford & Florence B. Decker Foundation for the generous donation of equipment that were used in these studies. The Regional NMR Facility at Binghamton University is supported by the NSF (CHE-0922815). SK-BR-3 and MDA-MB-231 cells were a gift from Prof. Tracy Brooks and Prof. Ming An, respectively. The authors would like to thank Dr. Han Gu for the syntheses of (hydrazinecarbonyl)glycine and Texas Red- and Janelia Fluor 669-2fPBA; Saptarshi Ghosh for the synthesis of coumarin aldehyde; and David Tuttle for gel imaging assistance.

Keywords: α-effect nucleophile • click chemistry • enzyme catalysis • isopeptide • microbial transglutaminase

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