Bioconjugation

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Locked by Design: A Conformationally Constrained Transglutaminase Tag Enables Efficient Site-Specific Conjugation

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Abstract: *Based on the crystal structure of a natural protein substrate for microbial transglutaminase, an enzyme that catalyzes protein crosslinking, a recognition motif for site-specific conjugation was rationally designed. Conformationally locked by an intramolecular disulfide bond, this structural mimic of a native conjugation site ensured efficient conjugation of a reporter cargo to the therapeutic monoclonal antibody cetuximab without erosion of its binding properties.*

Bioconjugation reactions involving natural or synthetic macromolecules, particularly proteins, have emerged as powerful tools for tailoring their architectures and engineering functional properties.[1] By using diverse ligation strategies, a wide range of proteins have been fluorescently labeled or radiolabeled, self-assembled, or tethered with functional molecules such as polyethylene glycol (PEG), porphyrins, peptides, peptide nucleic acids, and drugs.[1b] Among these constructs, bioconjugates combining a functional payload with a monoclonal antibody counterpart have attracted keen research interest in recent years.[2] In these engineered antibody–drug conjugates (ADCs), the antibody specifically directs the uptake and release of a highly potent toxin into a cancer cell.[3]

Generally, the production of well-defined antibody conjugates is extremely challenging in view of the required selectivity, specificity, and reactivity under physiological conditions.[1a] Although the assembly of toxins or other functional units on an antibody scaffold should, in an ideal case, proceed in a controllable manner with respect to stoichiometry and regiospecificity,[1b] the current chemical strategies for protein modification mostly rely on conjugation with surface-exposed lysine or cysteine residues.[4] Obviously, this often leads to heterogeneous populations of conjugates with variable cargo distribution, which may result in diversified characteristics and inconsistent in vivo performance.[4g,5]

This challenge can be addressed by, among other approaches,[6] applying enzyme-mediated conjugation.[7] In this respect, microbial transglutaminase (MTG) and sortase A have successfully been used for the site-specific ligation of antibodies with cytotoxic/labeling cargoes through the formation of isopeptide[8] or peptide bonds,[9] respectively.

Transglutaminases (TGs, E.C.2.3.2.13) catalyze acyl transfer between the ε-amino group of lysine and the γ-carboxamide of glutamine; they tolerate a broad spectrum of amine substrates designed to resemble the side chain of lysine.[10] However, stringent constraints are specified for the glutamine substrate. To be accepted by MTG, the Gln residue must be located within a defined amino acid sequence motif possessing a particular conformation and sufficient flexibility.[10b] Unfortunately, a lack of information concerning the composition and architecture of the conjugation sites in natural transglutaminase substrates significantly hampers the application of these enzymes to the generation of protein conjugates. In recent years, efforts have been made to optimize MTG with respect to stability, activity, and specificity.[11] A number of approaches have been explored to elucidate the substrate specificity of MTG by using fluorescent derivatives of the artificial substrate *N*-benzyloxycarbonyl-L-glutaminylglycine as the acyl donor and various primary amines as acyl acceptors.[12] Moreover, the application of phage display and mRNA display has revealed glutamine consensus sequences for MTG that can be used for enzyme-mediated conjugation and for the production of homogenous ADCs.[8c,13]

We hypothesized that transglutaminase recognition sites that are derived from the natural MTG substrates and mimic the natural structural environment may be efficiently recognized by the enzyme, since it is well known that patterns of tertiary structure can be more important for protein–protein interactions than just the amino acid sequence.[14] In this study, molecular design based on the crystal structure of a natural protein substrate for a bacterial transglutaminase resulted in an engineered structural mimic of an MTG native

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conjugation site. This so-called MTG-tag ensured covalent linkage between reporting molecules and 1) a model peptide or 2) a full-length monoclonal antibody with exquisite conjugation efficiency.

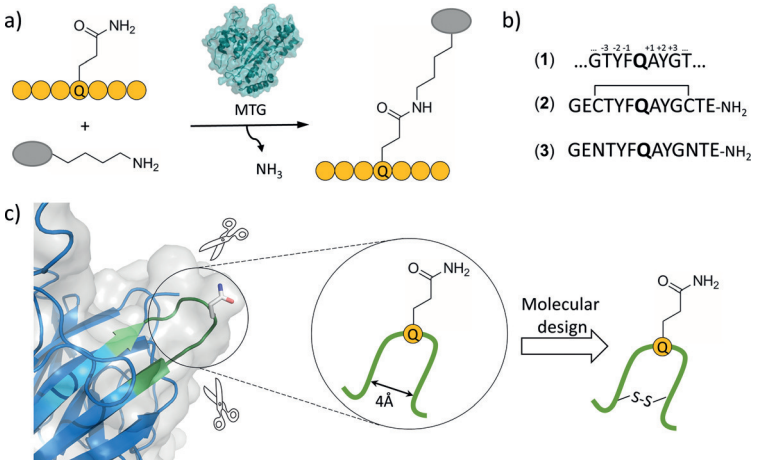
Bacterial transglutaminases, although they perform efficiently on partially or fully denatured proteins, act in a sequence- and conformation-dependent manner on folded species.[10b,f] Indeed, a preliminary study showed that none of the 64 glutamines in the monoclonal antibody cetuximab, an EGFR-binding antibody that is in clinical use for the treatment of colorectal cancer, is efficiently recognized as an MTG substrate residue for site-specific conjugation with a primary amine counterpart (Figure S14 in the Supporting Information). These observations are fully consistent with previous findings.[10c,d,15] To date, neither the sequences nor the structural environment of natural MTG substrate sites are known. Therefore, it is not possible to follow a common grafting approach, where a sequence- and structure-resembling fragment of a natural MTG recognition motif is inserted into a target protein. We recently reported three natural substrate proteins of MTG.[16] Among them, the 37 kDa dispase autolysis inducing protein (DAIP), which triggers autolysis of neutral metalloproteases, has attracted our attention.[16c] Sequencing from the amino terminus of the protein followed by sequencing of the whole genome of *Streptomyces mobaraensis* strain 40847 revealed the DAIP-encoding gene. With this information in hand, the parent DAIP, which contains five glutamine residues, as well as derivatives possessing multiple Gln-to-Asn exchanges, were recombinantly produced in *E. coli* cells. Successive MTG-promoted ligation with monobiotinylcadaverine resulted in the identification of three MTG modification sites (Q39, and Q298, and Q345) that enable exceptionally efficient enzyme performance.[16d]

To gain further insight into the MTG substrate recognition pattern, we solved the crystal structure of DAIP at 1.7 Å resolution. We found that DAIP possesses a beta propeller structure with both MTG substrate sites located on the protein surface, specifically, in surface-exposed loops. However, they involve different Gln-neighboring residues and loop conformations. In particular, the Q298 MTG site is defined by a type II β-turn that connects two beta strands (Scheme 1, sequence **1**), while Q39 is located in a loop with an extended conformation, and the structure around Q345 is not resolved. Interestingly, **1** shows no similarity with consensus sequences for microbial transglutaminase that have been identified by phage-display screening of linear peptides.[13c] Indeed, it possesses neither aromatic amino acids nor arginine residues preceding the reactive glutamine residue at position −3 (Scheme 1 b). Moreover, this sequence lacks the double-Q pairs that have been found to be preferred substrate sites.[17]

Having taken the recognition sequence **1** as a starting point, we designed oligopeptide **2**. In order to precisely mimic the functional Q298 loop in DAIP, which is bordered by G295 and G302 with a C_α distance of about 4 Å, flanking cysteine residues were introduced to rigidify the β-turn upon oxidation into a covalent disulfide (Scheme 1, peptide **2**). To define the position for a structure-stabilizing cystine, we conducted structural superposition of **2** with a classical disulfide-constrained peptidic molecule. Typically, disulfide-bridged peptides of that size display Cys–Cys C_α distances of about 4 Å (e.g., 3.8 Å in open-chain sunflower trypsin inhibitor, PDB: 1JBN).[18] Interestingly, we also observed MTG-mediated modification of reduced and S-alkylated substrate **2** (Figures S4–S7 in the Supporting Information). Hence, as a control and to investigate the role of loop fixation on the efficiency of MTG-mediated conjugation, peptide **3** was synthesized, with cysteine residues replaced by asparagines to mimic that situation. Since the core sequence is rather hydrophobic, GE and TE pairs were added as flanking residues to enhance the water solubility of peptides **2** and **3**.

Following the assembly of **2** and **3** on solid support with successive oxidative folding for the Cys-containing peptide **2** (Section S1.2 and Figures S1, S2, S8, S9 in the Supporting Information), MTG-catalyzed ligation was studied in proof-of-concept experiments with these peptides and the monobiotinylcadaverine derivative **4** as an amine counterpart (Figure 1 a) in aqueous buffer at pH 7 (Figures S3, S10–S12, S23–S26 and Section S1.5 in the Supporting Information). HPLC monitoring of the enzyme-catalyzed reaction to give conjugates **5** and **6** verified the anticipated effect of the induced conformational constraint on the accessibility of the Gln residue since substrate **2** demonstrated significantly faster conversion than substrate **3** (Figure 1 b). Indeed, in the presence of a molar excess of **4** (5 equivalents with respect to the peptide), no **2** was found in the reaction mixture after 60 min (Figure S11), which was not the case for **3** after 3 h.

Encouraged by these findings, we decided to apply our structural design that relies on a conformationally locked recognition motif to the regioselective conjugation of an antibody with a cargo of interest. For the initial experiments, we used



Scheme 1. a) Transglutaminase-mediated conjugation of glutamine (Q) with an amine-donor substrate. b) Amino acid sequence of a natural transglutaminase recognition sequence (**1**) taken from the substrate protein DAIP and the engineered sequences (**2** and **3**) used in this study for conjugation. c) Schematic depiction of the design of an engineered transglutaminase recognition sequence (MTG-tag) that mimics the functional Q298 loop in DAIP.

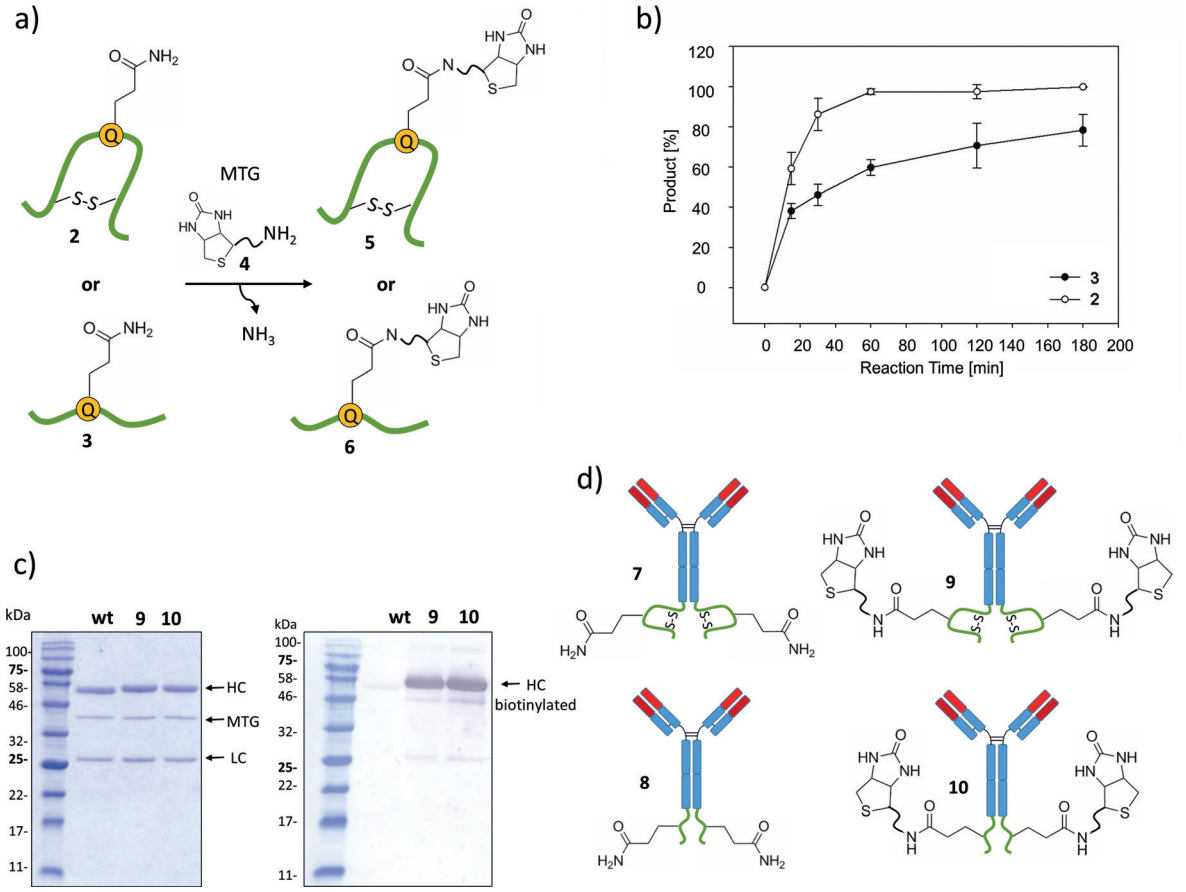


Figure 1. MTG-mediated conjugation of engineered model peptides and full-length monoclonal antibodies. a) Engineered model peptides **2** and **3**, which contain a transglutaminase recognition motif, were biotinylated in an MTG-catalyzed reaction with monobiotinylcadaverine (**4**, MBC). b) Biotinylation kinetics for the conformationally constrained peptide **2** (○) compared to the linear peptide **3** (●). Error bars representing standard deviation were calculated from triplicates. See also Figures S11, S12. c) SDS-PAGE gel and western blot analysis of biotinylated cetuximab variants with C-terminal MTG-tags (**9** and **10**). Biotinylation was visualized by using streptavidin-conjugated alkaline phosphatase and NBT/BCIP (right panel). d) An overview of cetuximab variants with C-terminal MTG-tags used for MTG-promoted biotinylation (**7** and **8**) and the respective conjugated antibodies (**9** and **10**).

cetuximab variants equipped with engineered MTG-tags containing the disulfide-locked (**7** Figure 1 d) and the disulfide-lacking (**8** Figure 1 d) recognition motifs (Figure S13). The occurrence of a disulfide bridge in **7** was verified by Ellmann's test (Figure S22). This construct, which possesses two cysteines in the tag sequence, displayed identical Cys content to **8**, which contains two Asn residues instead of two Cys residues, thus indicating that no additional thiols were present. Since MTG-catalyzed intramolecular crosslinking via the C-terminal lysine (K447) was observed in a preliminary study involving the wild-type sequence, this residue was deleted (Figure S20). As a model cargo, monobiotinylcadaverine **4** was used. Co-incubation of **7** or **8** with **4** in the presence of MTG was carried out for 3 h at ambient temperature, followed by SDS-PAGE analysis with subsequent western blot. Under denaturing conditions, biotinylated cetuximab heavy chains (**9**, **10**; Figure 1 c) were visualized by the addition of a streptavidin-conjugated alkaline phosphatase followed by treatment with substrates 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT) to reveal binding, thus verifying successful conjugation for both antibodies (Figure 1 c). The conjugation efficiencies for the MTG-catalyzed reaction of **7** and **8** with fluorescently labeled cadaverine were estimated to be 85% and 86%, respectively (Figure S21). To examine the specificity of the MTG-tag-promoted conjugation, the heavy chain of construct **10**, generated under denaturing conditions of SDS-PAGE (Figure S15), was further in-gel reduced and the resulting free thiols were alkylated with iodoacetamide and treated with trypsin.[19] Digestion products were analyzed by MALDI-TOF-MS (Figure S16–S18). Condensation with substrate **4** was observed exclusively at the glutamine residue within the MTG-tag (corresponding to Q453 in the modified heavy chain). However, since this procedure ensured a sequence coverage of only 56% in our hands, unspecific biotinylation, presumably at the modification-prone residue Q295 (as well as other glutamines), should not be excluded.[10d,15]

To investigate whether C-terminal modification of the antibody upon the introduction of an MTG-tag jeopardizes the bioactivity of parent EGFR-specific cetuximab, cellular binding assays were conducted by using EGFR-overexpressing EBC-1 cancer cells and EGFR-negative CHO-K1 cells as

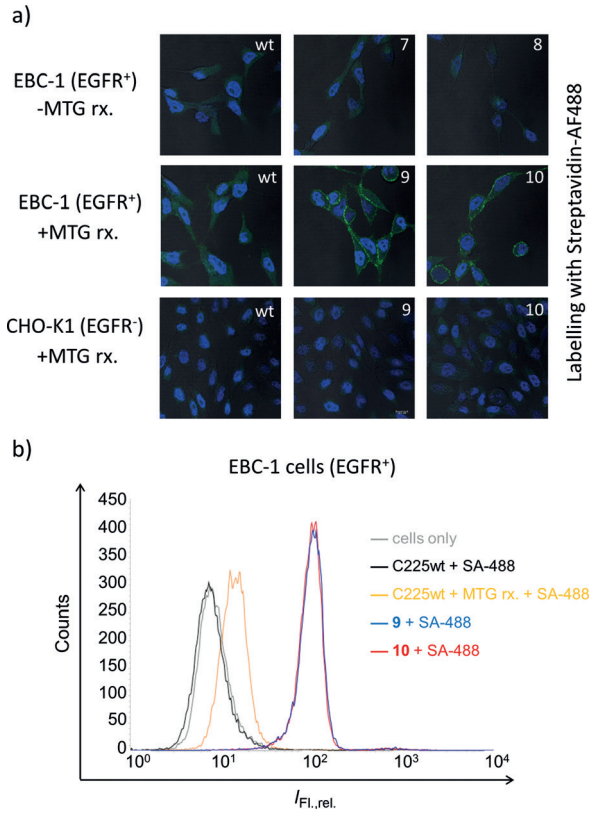


Figure 2. Binding of biotinylated cetuximab studied by cell assays and flow cytometry. a) Cell binding assays for the non-biotinylated constructs **7**, **8** and wt cetuximab (upper panel), and biotinylated cetuximab variants **9** and **10** compared to wt cetuximab in EGFR-positive EBC-1 cells and EGFR-negative CHO-K1 cells (+/- MTG rx.: with/without MTG-catalyzed reaction, middle and lower panels). Cells were mounted using ProLong Diamond Antifade Mountant with DAPI (blue) and the biotin label visualized with streptavidin–AF488 (green). Cells were visualized by using a confocal microscope. Merged pictures are shown with differential interference contrast (DIC). b) Flow cytometry of EGFR-positive EBC-1 cells incubated with biotinylated cetuximab variants **9** and **10** (blue and red). Cells bound to biotinylated cetuximab were fluorescently labelled through the addition of streptavidin (SA)–AF488 (see Figure S19).

controls (Figure 2a). The cells were incubated with purified constructs **7-10**, as well as wildtype (wt) cetuximab, in the presence (conjugates **9**, **10**, and wt) and absence (antibodies **7**, **8**, and wt) of MTG (Figure 2a, middle and upper lines, respectively). Visualization of binding to the cell surface was achieved by merging recorded microscopic images showing the fluorescence of DAPI-stained cell nuclei (blue) and biotin labeling with streptavidin-conjugated Alexa Fluor 488 (AF488, green). Although the intense green fluorescence for conjugates **9** and **10** clearly indicates binding to the target cells, some background fluorescence detected for the parent antibody suggests minor unspecific biotinylation. No efforts have yet been made to further improve the labeling selectivity through tuning the antibody/enzyme ratio, reaction time, temperature, or ligand concentration. As expected, no green fluorescence was observed for EGFR-negative control cells (lower panels) or for non-conjugated antibodies **7** and **8**.

Additionally, the binding properties of the produced conjugates were assessed by flow cytometry by using the same cell lines followed by incubation with streptavidin–AF488 and recording of the AF488 fluorescence (Figure 2b). The intense fluorescence shown by EBC-1 cells in the presence of conjugates **9** and **10** confirmed the results obtained by fluorescence microscopy. Interestingly, weak unspecific binding was detected for parent cetuximab as well (Figure 2b, orange line), albeit with an intensity that one order of magnitude lower. This effect is very likely due to antibody labelling at non-specific sites.[10d,15] Indeed, it is well known that Q295 is readily modified by MTG, particularly in the absence of the natural IgG1 glycosylation at the neighboring Asn297.[10c]

To conclude, based on the crystal structure of a natural protein substrate of a bacterial transglutaminase, we engineered an MTG-tag that enables fast and quantitative transglutaminase-catalyzed conjugation at a low molar excess of the amine-donor substrate. This novel tag possesses a peculiar amino acid composition that bears no similarity to oligopeptides identified to date via phage-display. Having locked the conformation of the tag through the introduction of an intramolecular disulfide bond, we confirmed that the model disulfide-containing peptide performed significantly faster compared to its linear counterpart. It seems plausible that the efficient substrate recognition by MTG is governed by the architecture of the tag. We demonstrated the utility of the MTG-tag by generating bioconjugates of the therapeutic antibody cetuximab, and showed that its binding to EGFR-overexpressing tumor cells was not affected by the bioconjugation.

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