Bioconjugate Chemistry

Review

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Versatility of Microbial Transglutaminase

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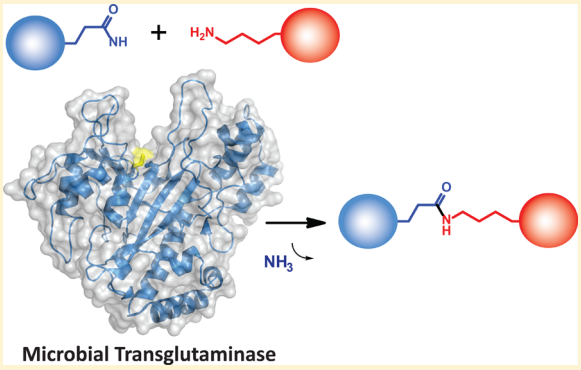
ABSTRACT: Although microbial transglutaminases (mTGs) were initially discovered to offset the cost of producing mammalian transglutaminases for food applications, they have quickly become important tools in research and biotechnology. Today, mTGs are utilized for a large number of applications to conjugate proteins and peptides to small molecules, polymers, surfaces, and DNA, as well as to other proteins. It is important to know how to maximize the advantages of the enzymatic approach and avoid undesired cross-linking. This review focuses on the versatility of transglutaminases in the field of bioconjugation and covers recent developments in utilizing mTG for generating antibody drug conjugates (ADCs) for therapeutic applications.



INTRODUCTION

Microbial transglutaminases (mTGs) are versatile tools in modern research and biotechnology. The availability of large quantities of relatively pure enzymes, ease of use, and lack of regulation by calcium and guanosine-5′-triphosphate (GTP) has propelled mTG to be the main cross-linking enzyme used in both the food industry and biotechnology. Currently, mTGs are used in many applications to attach proteins and peptides to small molecules, polymers, surfaces, DNA, as well as to other proteins.

Although many groups have demonstrated various mTG-based conjugation methods and the potential for therapeutic advantages of site-specific conjugation, few have reported the issues that exist when the target protein contains multiple mTG-reactive glutamine and lysine residues in the wildtype sequence. Several excellent reviews covering applications of microbial transglutaminase have been published previously.[1−4] This article presents a background on transglutaminase structure and function, as well as an overview of the use of mTGs in the food and biotechnology industries. Some of the currently employed applications of mTGs with a focus on the most recent developments are highlighted. The advantages and versatility of mTG-based site-specific conjugation and the importance of understanding its limitations are also discussed.

Transglutaminase Structure and Function. Transglutaminases are protein-glutamine γ-glutamyltransferases (EC 2.3.2.13), which typically catalyze pH-dependent transamidation of glutamine residues to lysine residues (Figure 1a). In multicellular organisms, the resulting side-chain to side-chain isopeptide bonds add strength to tissues and increase their resistance to degradation.[5] In higher organisms, transglutaminases play important roles in diverse biological functions by selectively cross-linking proteins. Among the members are factor XIIIa, which stabilizes fibrin clots; keratinocyte transglutaminase and epidermal transglutaminase, which cross-link proteins on the outer surface of the squamous epithelium;[6] and transglutaminase 2 (TG2), which shapes the extracellular matrix, promotes cell adhesion and motility, and is involved in pathogenesis of celiac disease.[7−11]

Bacterial transglutaminases have been discovered by screening microorganisms to obtain inexpensive and stable sources of transglutaminase for food applications. mTGs catalyze the same reaction as mammalian transglutaminases; however, they do not share sequence or structural homology. Unlike mammalian transglutaminases, mTGs are not regulated by calcium or guanosine-5′-triphosphate (GTP) and have broader substrate specificity and lower deamidation activity.[12,13]

The biological functions of bacterial transglutaminases remain largely unknown. It has been postulated that *Streptomyces mobaraensis* mTG cross-links inhibitory proteins (i.e., *Streptomyces* papain inhibitor, *Streptomyces* subtilisin, transglutaminase activating metalloprotease inhibitor, and Dispase autolysis inducing protein) during the development of aerial hyphae and spores. Thus, one of the potential roles of mTG is to participate in the formation of an antibiotic shield and protect *Streptomyces mobaraensis* against a plethora of host proteases. mTG from *Streptomyces mobaraensis* (formerly classified as *Streptoverticillium mobaraense*) is probably the most well characterized mTG; however, other mTGs have been identified and their production and manufacturing reviewed elsewhere.[14]

Protein Structure and Activity. mTG from *Streptomyces mobaraensis* has no detectable sequence homology, and exhibits a unique structure compared to human TG2. TG2 is the best-characterized member of the human transglutaminase family and will be the focus of the comparison here. Human TG2 consists of four domains: an N-terminal β-sandwich, the catalytic domain containing a Cys-His-Asp catalytic triad, and

Received: March 9, 2014
Revised: March 29, 2014
Published: April 2, 2014

   dx.doi.org/10.1021/bc500099v | *Bioconjugate Chem.* 2014, 25, 855−862

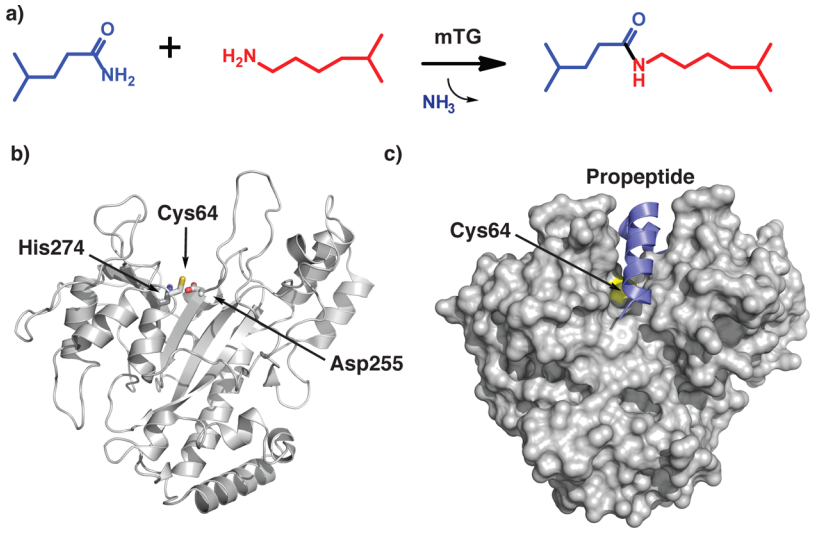


Figure 1. Structure and mechanism of microbial transglutaminase. (a) Mechanism of mTG. Glutamine side chain (acyl donor) reacts with primary amine (acyl acceptor) in the presence of mTG. The reaction proceeds via acyl-enzyme intermediate with one molecule of ammonia released in the course of the reaction resulting in a side-chain to side-chain cross-link. (b) Structure of mTG with the predicted catalytic triad (Cys64, Asp255, His274) shown in cartoon representation.[12] (c) Surface representation of mTG depicting the catalytic Cys64 position at the bottom of the active site cleft. The α-helical propeptide participates/facilitates proper folding of mTG and blocks the active site[19] to keep the enzyme inactive during expression. Upon secretion, the propeptide is cleaved, releasing the active enzyme.

two C-terminal β-barrels.[15,16] The enzymatic activity of TG2 has complex regulation and is modulated by GTP, calcium, and redox potential.[17] Structures in the inactivated and activated forms showed that, upon activation, TG2 undergoes a large conformational change to expose the active site.[15,16] The catalytic Cys (C277) is located in a hydrophobic environment and is occluded by two tryptophan residues, W241 and W332, which reside on distinct loops in the active site.[16,18]

mTG, on the other hand, is a calcium- and GTP-independent transglutaminase containing a central 8-stranded β-sheet surrounded by 11 α-helices.[12] The active site is a 16 Å deep cleft created by two protruding loops, with the catalytic cysteine located at the bottom of the cleft (Figure 1b). The enzyme is produced as a zymogen, where the N-terminus folds into a helical structure that occupies the active site and blocks substrate access.[19] The zymogen is activated by the cleavage and dissociation of the N-terminal helical structure (Figure 1c) by endogenous metalloprotease and tripeptidyl aminopeptidase.[20,21]

Both the human transglutaminases and mTG contain a Cys-His-Asp catalytic triad; however, the structural orientation between the two classes differs. Relative to the active site cysteine, the position of His and Asp are reversed in the two enzymes. The mechanism of human transglutaminases is thought to be similar to a number of cysteine proteases where the first step consists of deprotonation of the active site cysteine thiol by nearby histidine. In mTG, it was proposed that Asp255 plays a similar role as the catalytic triad histidine in TG2. The critical catalytic role of Asp255 in mTG was confirmed by alanine mutagenesis where the activity of D255A mutant was reduced to background levels. The proposed mechanism is further supported by mutagenesis of mTG His274, which reduced the catalytic activity only by 50%, suggesting that His274 does not play a critical role.[12]

Kinetic characterization of mTG with commonly used substrate *N*-α-benzyloxycarbonyl-L-glutaminylglycine revealed an apparent K_m and k_{cat}/K_m values of 52.6 mM and 40.4 mM^{-1} min^{-1}, respectively.[19] Chromogenic hydroxamate assay with 100 mM hydroxylamine as acyl-acceptor substrate was used in these studies. About 20-fold lower K_m (3 mM) was reported for heptapeptide (Ac-WALQRPH-NH2) demonstrating the impact of surrounding residues on K_m.[22] In this assay, the authors utilized continuous enzyme-coupled assay and 10 mM Gly-OMe as acyl-acceptor substrate. mTG was shown to catalyze the reaction over a wide range of pH (pH 4–9) with optimum pH between 5 and 8.[23] The ability of mTG to function at pH 4 shows that the active site cysteine pK_a is significantly shifted. mTG is active at temperatures between approximately 0 and 70 °C with optimum temperature of 55 °C, although it loses activity over time at this temperature.[23]

Substrate Specificity. The sequence effects around the substrate Gln residue were first investigated in context of the heptapeptide substrate (GGGQGGG), where each glycine was individually substituted to several amino acids.[24] The study found that hydrophobic residues at the N-terminus of the Gln accelerated the reaction relative to the GGGQGGG peptide (at the −3, −2, and −1 positions). All tested peptides in the study, however, showed lower reaction velocities when compared to the Z-Gln-Gly model substrate. Another study investigated the preferred substrate sequence of mTG using a phage-displayed peptide library.[25] In this study, a much larger library of linear peptides (10^{11}) was utilized, and most identified clones contained an aromatic amino acid (W, F, or Y) at the −5 to −3 positions, an arginine or hydrophobic residue at the +1 or +2 positions, and hydrophobic residues at the −2 and −1 positions. Few examples of experimentally determined sequences that were used for conjugation with mTG are shown in Table 1.

In addition to the primary sequence specificity surrounding the reactive glutamine residue, it has been suggested that a local secondary structure also plays an important role in determining whether a particular glutamine on the protein surface is a substrate of mTG.[26] Furthermore, it has been postulated that local unfolding and peptide chain flexibility improve reactivity.[27] Taken together, mTG specificity is governed by a combination of primary sequence, secondary structure, as well

dx.doi.org/10.1021/bc500099v | *Bioconjugate Chem.* 2014, 25, 855–862

Table 1. Examples of Protein Sequences Used for Microbial Transglutaminase Conjugations

protein	amino acid sequence
hIgG incorporated tag	LLQG
hIgG1 aglycosylated	PWEEQYNST
Phage panning validated hits	Ac-WALQRPH-NH2
	WALQRPYTLTES
	WALQRPHYSYPD
	WSPIPQMRTVPP
	NPKIYPMQGWFV
	YELQRPYHSELP
Human interlukin-2	VLNLAQSKNFH
Filgrastim	APALQPTQGAM
Human growth hormone	IPKEQKYSF
Myoglobin	MGGSPLAQSHGGS
S-tag	KETAAAKFERQHMDS
α-lactalbumin	TEYGLFQINNDS

as flexibility around the glutamine, and accessibility of the glutamine to mTG. Somewhat broad primary sequence specificity together with secondary structure and steric factors make it challenging to predict whether a given glutamine will be a substrate; therefore, experimental approaches remain the best way to test reactivity.

FOOD APPLICATION SUMMARY

mTGs were discovered in an attempt to find transglutaminases that would be cost-effective for food industry applications. Today, transglutaminases are mainly used in meat, fish, dairy, and baking industries. In the meat and fish industries, the main applications of mTG are to alter mechanical properties of meat and as a bonding agent (Figure 2). Altering mechanical meat properties and meat bonding is used in production of sausages, improving texture, and allowing utilization of lower quality meat. In dairy applications, mTG modulates texture, structure, curd yield, and consistency of yogurts, ice cream, milk, and cheese. In baking, mTG is used for improving flour properties such as elasticity and dough resilience, bread texture and volume, and pasta texture.

Concerns were raised about the usage of mTG for flour modulation due to the role of hTG2 in the deamidation reaction of gluten peptides in the etiology of celiac desease. Deamidated gluten peptides are known to increase immunoreactivity to gluten peptides in celiac patients.[28] Interestingly, recent data suggest that the cross-linked gluten flour has lower immunoreactivity in a rabbit model system, suggesting that the lower deamidation rate of mTG relative to mammalian TGs, together with the cross-linking of gluten peptides, might potentially reduce this risk.[4,29] mTG is used in additional applications such as production of edible protective protein-based films that extend shelf life and freshness. The main substrates that allow mTG to cross-link and change properties of various food products are primarily gluten proteins in wheat flour, myosin and actin in meats, and casein in milk. Further details about use of mTG in the food industry can be found in many excellent reviews.[2,3,14,30,31]

RESEARCH AND BIOTECHNOLOGY SUMMARY

Protein−DNA Conjugates. Numerous strategies for site-specific labeling of recombinant proteins with oligonucleotides utilizing mTG have been reported. In the first example, the use of aminated DNA as an acyl acceptor was attempted with little success. The group went on to react aminated DNA with *N*-carbobenzyloxy glutaminyl glycine (Z-QG) to create Z-QG-DNA. This covalent complex served as an acyl donor and was conjugated to proteins containing reactive lysine residues or tags using mTG.[32] The authors utilized this technique to conjugate alkaline phosphatase and enhanced green fluorescent protein (EGFP) to DNA for use in hybridization or microarray experiments. An alternative approach to chemical conversion of aminated DNA to Z-QG-DNA was developed by utilizing terminal deoxynucleotidyl transferase to incorporate Z-QG-nucleotide at the three prime side (3′-OH) of DNA.[33]

A second approach utilized a synthetic nucleotide analogue, Z-QG-dUTP, that was incorporated into DNA via PCR reaction, resulting in multiple sites of attachment on DNA.[34,35] Z-QG-labeled DNA was conjugated to multiple alkaline phosphatases and used in hybridization experiments, resulting in comparable detection sensitivity to digoxigenin labeled probes.

Protein−Polymer Conjugates. Several groups have published results of mTG-based site-specific PEGylation of pharmaceutical proteins. In most cases, the number of reactive glutamines was typically low in comparison to the total number of surface-exposed glutamines and, in several cases, a single glutamine was identified resulting in site-specific conjugates.[26,36−38]

One of the most well characterized proteins where mTG-based PEGylation was applied is human growth hormone. In the case of human growth hormone, two glutamine residues were identified as major conjugation sites (Q40 and Q141). Mutagenesis of mTG resulted in an mTG variant with increased selectivity toward Q141.[39] In another study, it was found that, for salmon calcitonin and human growth hormone, a change in solvent (presumably leading to a change in the secondary structure surrounding the reactive glutamine) can be used to limit the conjugation to a single glutamine, and therefore increase selectivity.[40,41]

In the case of human interleukin 2 (hIL-2), single reactive glutamine (Gln74) was found and conjugated with 12 kDa PEG or galactose-terminated triantennary glycosides $(Gal)_3$.[42] Both conjugates retained full bioactivity and the PEG12-hIL2 was eliminated more slowly from circulation relative to unmodified hIL2. A monoPEGylated derivative of filgrastim (granulocyte colony stimulating factor) was also prepared using mTG.[36,43] The conjugation yielded an active protein with a single conjugation site (Gln135) that displayed good *in vivo* stability. The PEG conjugate was less active *in vitro* than wild-type protein; however, it was more active than the N-terminally PEGylated and clinically approved protein Neulasta, underlining the relevance of the PEGylation site.[43]

Attachment of hydroxyethyl starch (HES) is also being explored as an alternative to PEGylation of proteins due to its water-soluble and biodegradable properties. HESylation has been also achieved using mTG, where HES worked both as an acyl donor as well as an acyl acceptor when conjugated to test small molecule model compounds;[44,45] however, HES conjugations to proteins have not been demonstrated yet. Oligosaccharides were also conjugated to trypsin in an effort to create a more stable enzyme with decreased autoproteolytic activity.[46,47] Trypsin was conjugated with mTG to mono-6-amino-6-deoxy derivatives of α, β, and γ cyclodextrins and the resulting conjugates showed improved thermostability and resistance to autoproteolysis.

dx.doi.org/10.1021/bc500099v | *Bioconjugate Chem.* 2014, 25, 855−862

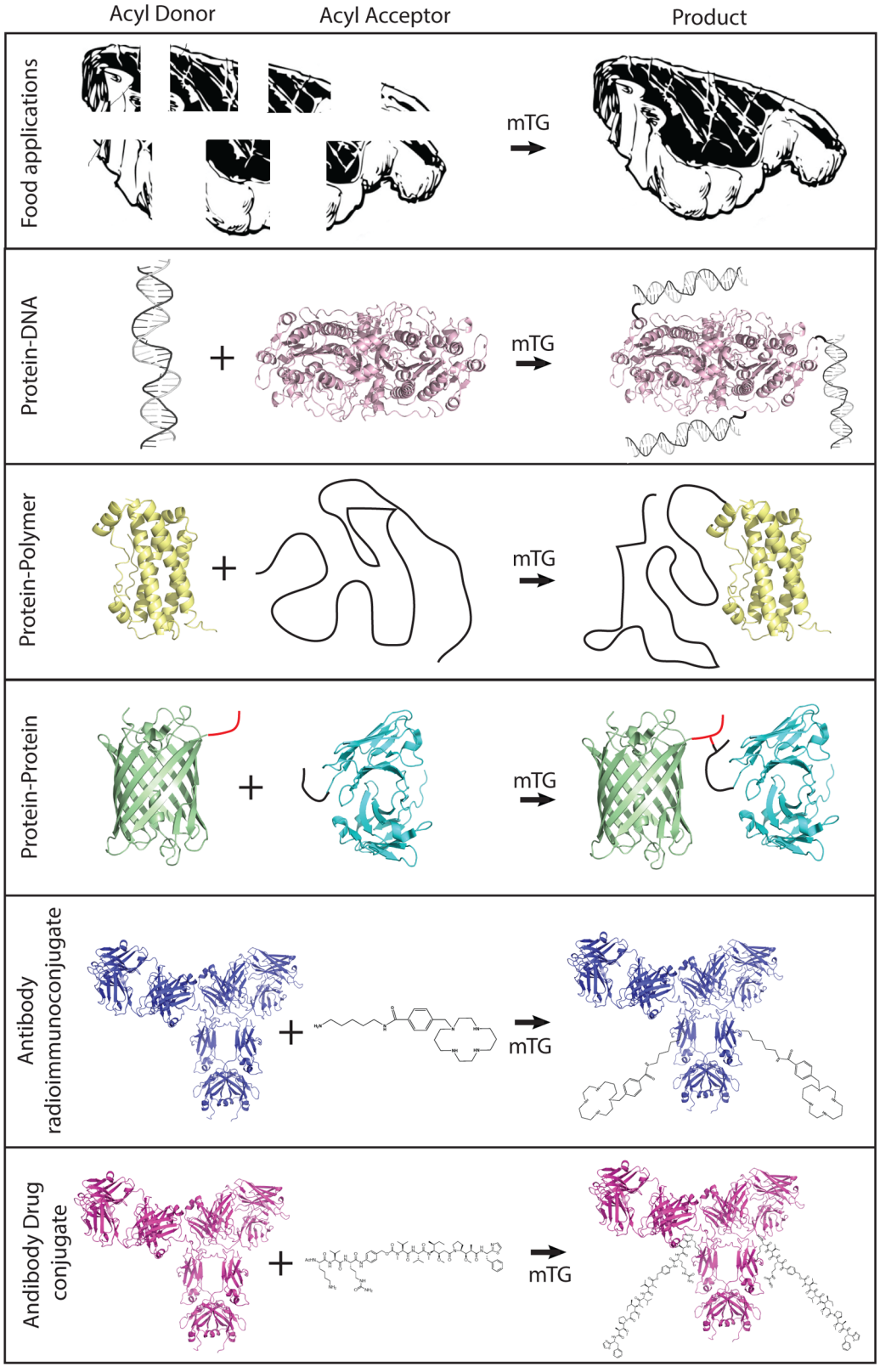


Figure 2. Examples of microbial transglutaminase application versatility.

Protein−Protein Conjugates. As an alternative to chemical coupling of two proteins for immunoassays, several groups have started to use mTG to covalently attach two proteins together. Protein G was successfully coupled to soybean peroxidase with approximately three peroxidases per one protein G molecule.[48] In a separate study, it was shown that wild-type EGFP is not susceptible to self-cross-linking;[49] therefore, adding a specific tag allows for the attachment of EGFP or other GFP variants to different proteins for use in Florescence Resonance Energy Transfer assays.

Incorporation of an mTG tag into proteins that are not substrates of mTG allows for site-specific conjugation and coupling of two proteins. This approach has been successfully applied to coupling of alkaline phosphatase and single chain antibody fragments to generate reagents for immunoassays.[50] In a similar strategy, a single domain antibody from camelidae that binds tumor necrosis factor was multimerized by incorporation of mTG tag and cross-linked by mTG to form dimers and multimers showing improved inhibition *in vitro*.[51] mTG-based immobilization of alkaline phosphatase carrying transglutaminase tag to casein-coated polyacrylic beads was shown to result in higher specific activity and stability relative to chemical conjugation.[52]

Full-Length IgG Conjugation. Protein conjugation or modification using mTG provides the advantages of selectivity, simplified reaction procedures, and mild reaction conditions. One of the requirements, however, is that the protein of interest has to be free of reactive glutamines or have a very low number of them. This can be a challenge especially for larger molecules, such as antibodies, where approximately 60 glutamines exist in a typical immunoglobulin Gamma1 (IgG1). While there are many surface-exposed glutamines on antibodies, one study showed conjugation of only one to two biotins per antibody.[53] In their report, site specificity was not demonstrated and the targeted glutamine residue(s) were not identified. Therefore, it is unknown whether one particular glutamine was being conjugated, or if several glutamines were conjugated at low levels. Because more efficient methods for generating site-specific antibody conjugates were needed, we independently investigated mTG-based conjugation of antibodies. In contrast to the published work,[53] under the utilized conditions, no significantly reactive glutamines were found in a set of multiple glycosylated human IgGs.[54,55] Our data are consistent with the work of Jeger et al. where no conjugation was observed to the chCE7 and Rituxan antibodies.[56] Today, the mTG IgG conjugation methods developed by us[54,55,57] and others[53,56,58−60] are mainly used in the fields of radioimmunoconjugates and antibody drug conjugates.

Radioimmunoconjugates. Aglycosylated chCE7 antibody was conjugated with mTG to deferoxamine for chelation of ^{67}Ga for radiodistribution study and ^{89}Zr for positron emission tomography.[56] This conjugation strategy utilized a difference in conjugation efficiency between glycosylated and aglycosylated antibodies. The difference in conjugation of glycosylated and aglycosylated antibodies was ascribed to the introduction of an additional glutamine or subtle structural changes to the antibody to make glutamine residues more accessible.[60] Later experiments showed that the absence of glycosylation exposes the glutamine at position 295 for conjugation (EU residue numbering). Removal of glycosylation in the Fc region and exposure of glutamine 295 for conjugation can be accomplished by either enzymatic deglycosylation or by mutagenesis of Asn297 (site of glycosylation in IgGs).[54−59] Both the chCE7 ^{67}Ga conjugate and the deglycosylated Rituxan conjugated with 4-(1,4,8,11-tetraazacyclotetradec-1-yl)methyl benzoic acid and radiolabeled with $^{64/67}$Cu were compared *in vivo* to chemically labeled antibodies. In both cases (aglycosylated chCE7 and deglycosylated Rituxan), the mTG-based enzymatic conjugates showed improved selectivity to target tissues relative to the chemically conjugated antibodies.

Antibody Drug Conjugates (ADCs). We investigated the use of mTG in the field of ADCs.[54] The attachment of cytotoxic drugs to antibodies can result in an effective therapy with better safety profile than nontargeted cytotoxics.[61−64] The potential of ADC therapeutics has been recently realized with the approvals of Kadcyla and Adcetris for metastatic breast cancer and Hodgkin lymphoma, respectively.[65]

Kadcyla and Adcetris, as well as most ADCs in clinical development, rely on chemical conjugation methods that yield heterogeneous mixtures of varying number of drugs attached at different positions.[66] The potential benefits of site-specific drug conjugation in terms of stability, manufacturing, and improved therapeutic index led us to develop an enzymatic method for site-specific antibody drug conjugation using mTG.[54,57] Other site-specific approaches, including introduction of cysteine residues,[67] incorporation of unnatural amino acids,[68−70] as well as enzymatic approaches utilizing other enzymes such as sulfatase modification factor,[71−73] have been reported and are reviewed elsewhere.[74−78]

In order to obtain multiple positions on an antibody to conjugate to, we performed a glutamine tag scan in hIgG1 and identified multiple positions with >90% conjugation yields. By optimization of linkers and site selection we were able to obtain both cleavable as well as noncleavable linkers that conjugated with high yields to fluorophores, chelators, and different toxins including microtubulin inhibitors, heat shock protein 90 inhibitors, and DNA damaging agents. Through this approach we can conjugate to multiple sites in the Fab domain (both on the heavy chain and the light chain) as well as in the Fc domain. We have also shown that it is possible to conjugate the native glutamine Q295 and mutant N297Q with cytotoxic drugs.[55] Changing the conjugation position on the antibody, the linker, and the drug allowed us to investigate how the site of conjugation influences stability, toxicity, and efficacy.

We conjugated the same linker-toxin at the C-terminus of heavy chain in one antibody and to the C-terminus of the light chain in a second antibody. We found that the position of the conjugated toxin significantly impacted the total antibody distribution. When tested in rat, the C-terminus of the heavy chain showed markedly faster distribution than the C-terminus of the light chain. In mouse, however, this difference was much smaller. In addition to the total antibody distribution, we also found a significant difference in the cleavage rate of the dipeptide (Val-Cit) linker in circulation. In mouse, the heavy chain conjugate showed faster linker degradation relative to the light chain. This difference, however, was not very prominent in rat. The data suggested that species differences play an important role in ADC pharmacokinetics and caution must be used when interpreting and comparing efficacy and toxicity data, which are typically obtained in mouse and rat, respectively.

Taken together, we showed that the site of attachment has significant impact on ADC stability and pharmacokinetics and that this difference is also species-dependent. These differences can be attributed directly to the position of the conjugation rather than to the chemical instability observed with maleimide based conjugations.[67,79] Recently, conjugation to residues Q295 and Q297 mutant was also reported by another group utilizing Herceptin and cytotoxins monomethyl auristatins E and F.[58,59] In this work, the authors reported using a chemo-enzymatic approach to obtain high reaction yields. Glutamines at position 295 and 297 were first conjugated by mTG to spacers containing click chemistry handles and in a second step were reacted with toxins via click chemistry.

Availability of mTG and good expression levels of the glutamine-containing monoclonal antibodies make these

approaches easily scalable. Our data highlight the utility of mTG-based site-specific ADC conjugation in not only generating homogeneous and reproducible ADCs, but also allowing us to dissect the role of position, linker, and payload in an effort to generate homogeneous molecules, which allow us to optimize the therapeutic index of these molecules.

CONCLUSION

These diverse applications highlight the versatility and specificity of enzymatic mTG based conjugation strategies. mTG conjugations are orthogonal to other chemical and enzymatic approaches and have been utilized across a broad set of applications ranging from food industry to ADCs (Figure 2). The therapeutic potential for ADCs in the treatment of cancer was one motivation to develop an enzymatic method for site-specific antibody drug conjugation using mTG. The combined efficacy and safety results from mTG based ADC against Trop2 are expected to give a favorable therapeutic index and clinical development is currently underway.

When utilizing mTG for site-specific conjugation, care must be taken to ensure that the protein of interest does not already have multiple/excessive mTG-reactive glutamines and lysines. In such cases, reactions typically result in extensive cross-linking and limit the advantage of the enzymatic approach. Such efforts are typically abandoned and not reported in the literature. It is possible that the literature is somewhat biased toward reports where only one or two glutamines have been identified as mTG-reactive and where the utility of mTG-based conjugations is the highest. As additional uses for mTGs are discovered or the frequency of use increases, especially in biotechnology, it will be critical that the limitations are fully understood and that appropriate precautions are taken so that maximum benefit can be achieved.

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Notes

The author declares the following competing financial interest(s): Pavel Strop is a current employee of Rinat-Pfizer, Inc.

ACKNOWLEDGMENTS

I would like to thank Arvind Rajpal and Kirk Hansen for reading of the manuscript and their insightful comments.

ABBREVIATIONS

ADC, antibody drug conjugate; EGFP, enhanced green fluorescent protein; GTP, guanosine-5′-triphosphate; HES, hydroxyethyl starch; IgG, immunoglobulin G; mTG, microbial transglutaminase; TG2, transglutaminase 2; Z-QG, N-carbobenzyloxy glutaminyl glycine

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