Protein Labeling

DOI: 10.1002/anie.201004243

Site-Specific and Stoichiometric Modification of Antibodies by Bacterial Transglutaminase**

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The therapeutic efficacy of antibodies can be substantially enhanced by conjugation of cytotoxic compounds such as chemotherapeutics and particle-emitting radionuclides.[1] Intuitively one would assume that the therapeutic index would improve as the number of cytotoxic entities conjugated to the antibody increases. However, recent studies on auristatin–antibody conjugates in mice have demonstrated that a drug/antibody molar ratio of 4:1 results in optimal efficacy and in vivo tolerability.[2]

Unfortunately, conventional chemical strategies for protein modification are difficult to control and give rise to heterogeneous populations of immunoconjugates with variable stoichiometries, each of which has its own in vivo characteristics.[3] The introduction of artificial, bio-orthogonal groups for site-specific and stoichiometric protein modification offers a potential solution to this problem.[4–6] Such strategies are en vogue but are often laborious and still risk product heterogeneity.

Transglutaminases (TGs, E.C. 2.3.2.13) catalyze acyltransfer reactions between the γ-carboxamide group of glutamine (a side chain, which is otherwise chemically inert under physiological conditions) and the primary ε-amino group of lysine, to form catabolically stable isopeptide bonds (Figure 1 a).[7] Most TGs are promiscuous with respect to the lysine substrate and accept even simple 5-aminopentyl groups as lysine surrogates. The criteria for a glutamine residue to be recognized by the enzyme, however, are much more stringent: it should be both located in a flexible region of the protein and flanked by specific amino acids.[8] Given this inherent selectivity, we hypothesized that TG would be an alternative for the site-specific and stoichiometric functionalization of antibodies.[9] For this study we used bacterial transglutaminase (BTG) because it is robust, inexpensive, and easy to handle.

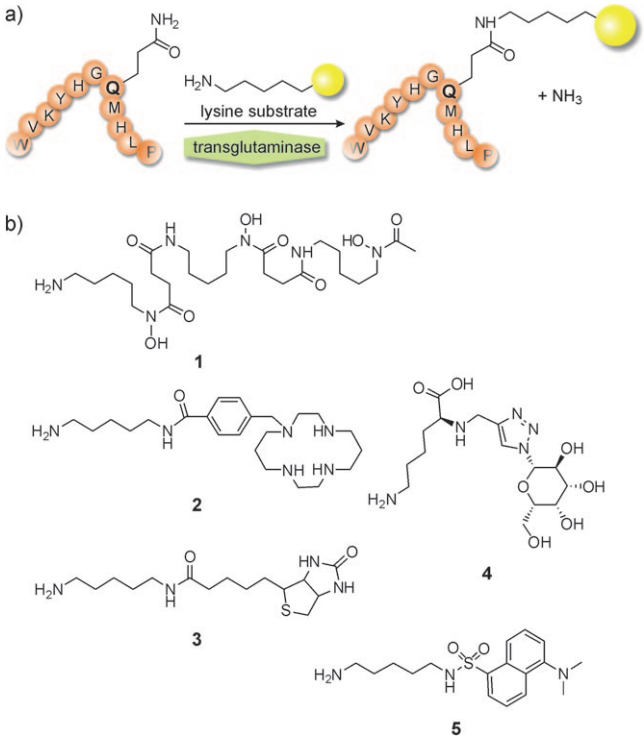

Figure 1. a) TG-mediated modification of Gln (Q) with a substrate containing lysine or a lysine surrogate. b) Substrates used in this study.

Our group is interested in radioimmunoconjugates for diagnostic and therapeutic applications, where low off-target accumulation of radioactivity is crucial. Earlier studies performed with radiolabeled monoclonal antibodies (mAbs) demonstrated that high numbers of metal chelators adversely affect the biological behavior of radioimmunoconjugates.[10,11] Therefore, we tested the features of BTG for the preparation of immunoconjugates that are functionalized with different metal chelators and radiolabeled with different diagnostic and therapeutic radionuclides. Deferoxamine (DF, **1**), an antidote for metal poisoning, has recently been identified as a suitable chelator for radionuclides such as ^{67}Ga and ^{89}Zr.[12] During the course of our studies we recognized that without further derivatization deferoxamine is already a potent BTG substrate. Furthermore, the metal chelating system 4-(1,4,8,11-tetraazacyclotetradec-1-yl)methyl benzoic acid (CPTA, **2**) was derivatized with a 1,5-diaminopentane (cadaverine) spacer (Figure 1 b; see the Supporting Information for details on the synthesis). To probe the scope of the new strategy we investigated other (model) substrates, which are of potential

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[**] This work was supported by the Swiss National Science Foundation (No. 112437).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201004243.

interest for mAb modification such as the biotin–cadaverine conjugate **3**, the galactopyranose–lysine derivative **4**, and the fluorescent dansyl–cadaverine derivative **5**. The anti-L1-CAM mAb chCE7 and the commercial anti-CD20 antibody rituximab (RTX) were functionalized with these substrates in the presence of BTG. The immunoconjugates were characterized by LC–MS after reduction with dithiothreitol (for experimental details see the Supporting Information).

To our surprise, no modification of native chCE7 or RTX was observed with any of the substrates in the presence of BTG, despite the fact that sequence analysis revealed abundant Q residues (see the Supporting Information). In parallel to these experiments we also tested the aglycosylated mutant chCE7agl, in which asparagine at position 297 has been replaced by glutamine to abort N-glycosylation.[13] This mutation was originally engineered to reduce the serum half-life of the radiolabeled immunoconjugate.[13] In the context of the present study, the N297Q mutation has two interesting consequences: 1) the antibody has an additional glutamine residue, and 2) removal of the Fc glycans leads to an increased mobility of the C/E loop (Q295–T299) as reported in the literature.[14–16] The deconvoluted mass spectra of the chCE7agl conjugates revealed no modification of the LC but exactly two modifications of the HC with all of the substrates tested (see the Supporting Information). Thus, completely homogeneous immunoconjugates with a defined substrate/mAb stoichiometry of 4:1 (the optimal drug/mAb ratio found for auristatin immunoconjugates[2]) were formed (Figure 2b,d). SDS PAGE and western blot analyses of immunoconjugates enzymatically functionalized with the biotin and the fluorescent substrates confirmed the exclusive functionalization of the HC (see the Supporting Information). For comparison, mass spectra of chCE7 and RTX chemically functionalized at lysine with isothiocyanate or hydroxysuccinimide analogues of substrates **1**–**3** showed varying numbers of molecules attached to both the HC and LC (see the Supporting Information). Chemical conjugation therefore yields a population of conjugates with two types of molecular heterogeneity: first, species with different numbers of molecules per mAb and second, isostoichiometic conjugates, in which the substrates are linked to different sites of the mAb. MALDI-TOF MS analyses unambiguously proved that Q295 and Q297 in chCE7agl are indeed the sites of BTG-mediated conjugation. The tryptic fragment containing Q295 and Q297 ($[EEQ_{295}YQ_{297}STYR+H]^+$, calcd 1203.52, found 1203.59) of the unconjugated chCE7agl was invariably modified with two substrates. For example, the spectrum of the digested fragment from the reaction with substrate **1** corresponds to $[EEQ_{295}(\mathbf{1})YQ_{297}(\mathbf{1})STYR+H]^+$ (calcd 2290.17, found 2290.41; Figure 2d).

Comparative B-factor analysis of the glycosylated Fc fragment of RTX (DOI:10.2210/pdb1L6x/pdb) with that of a deglycosylated Fc fragment of a human IgG1 (DOI:10.2210/pdb3dnk/pdb) revealed significant shifts of the C_α positions in the C/E loop towards a more open conformation (e.g. approximately 5 Å was observed for positions Q295 and N/D297 in the deglycosylated variant; see the Supporting Information). Based on these findings we concluded that the differences between native and aglycosylated mAbs with respect to enzymatic modification must be predominantly related to the greater flexibility of the C/E loop. To prove this hypothesis, we removed the glycans from chCE7 and RTX

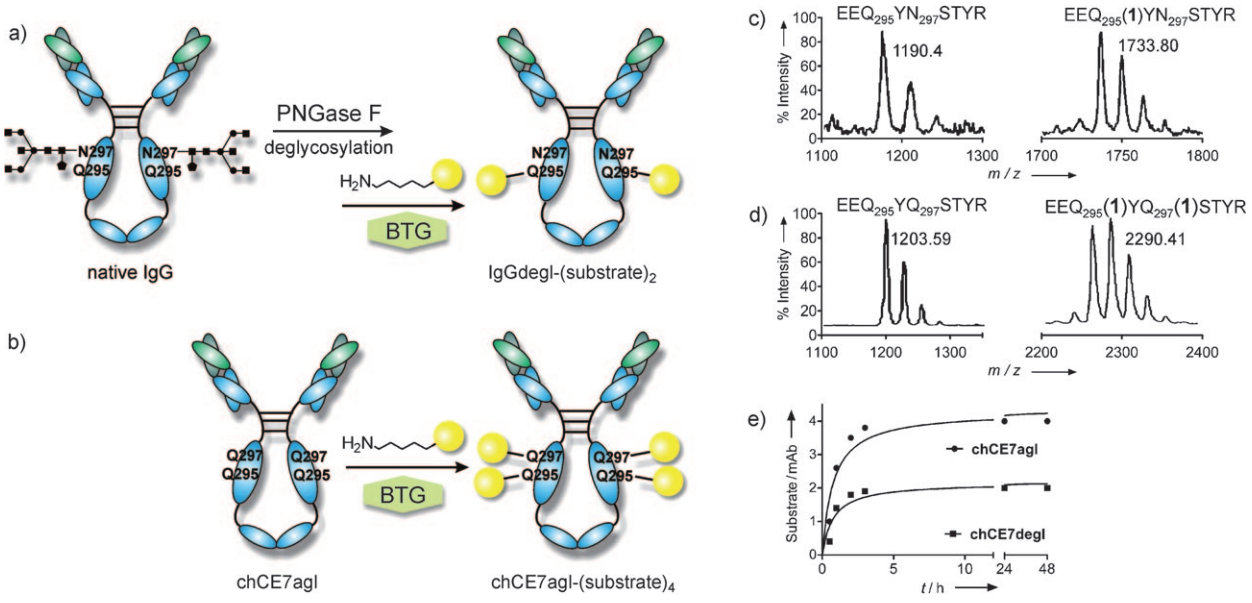

Figure 2. a) BTG recognizes exclusively Q295 located in the Fc region of deglycosylated IgGs (IgGdegl) as a site for modification with a suitable substrate. This leads to an exact substrate/mAb ratio of 2:1. Deglycosylation: mAb (1 mg mL^{-1}), PNGase F (100 U), PBS (pH 7.4), 37 °C, overnight. Conjugation: mAB (1 mg mL^{-1}), BTG (1 U mL^{-1}), **1**–**5** (400 μM), PBS (pH 8), 37 °C. b) In the aglycosylated mutant chCE7agl (N297Q) Q297 is recognized as a second site for modification resulting in a exact substrate/mAb ratio of 4:1 under the same reaction conditions. c,d) MALDI-TOF mass spectra of the tryptic fragments containing Q295 or Q295 and Q297 (left) and the corresponding immunoconjugates (right) proved the exact location and stoichiometry of the modification. e) Time-dependent BTG-mediated modification of chCE7degl (■) and chCE7agl (●) with **1**. The substrate/mAb ratio reaches a plateau after ca. 4 h.

with N-glycosidase F (PNGase F). The deglycosylated antibodies (chCE7degl and RTXdegl) were incubated with BTG and substrates **1** and **2**. MS analysis of the reaction products of both deglycosylated antibodies showed products with exactly one substrate per HC while the LC remained unmodified. This gave rise to homogeneous immunoconjugates with a substrate/antibody stoichiometry of exactly 2:1. MALDI-TOF MS confirmed Q295 to be the site of modification (Figure 2c).

To assess the effect of the moderate substrate/mAb ratio and the homogeneity of the immunoconjugates on the in vivo behavior, chCE7agl-(**1**)$_4$ was radiolabeled with ^{67}Ga and injected into female nude mice bearing SKOV3ip human ovarian carcinoma xenografts. Maximal mean uptake in the tumor was 58.7% ID g^{-1} at 72 hours after injection (p.i.; Figure 3a and Table S11 in the Supporting Information).

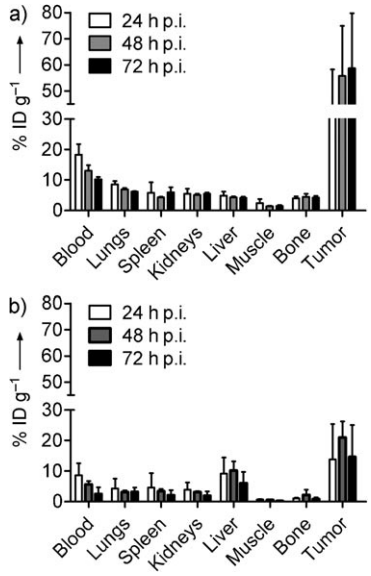

Figure 3. Time-dependent organ distribution of a) enzymatically and b) chemically conjugated ^{67}Ga-chCE7agl-(**1**)$_4$ in tumor-bearing mice.

Nonspecific uptake in all of the off-target organs determined was lower than 9% ID g^{-1} at all time points (with the exception of blood). This gave rise to high tumor/liver and tumor/kidney ratios of 14.0 and 10.7, respectively (72 h p.i.). For comparison, the chemically prepared analogue (with a calculated average of four ligands per mAb) had a maximum tumor uptake of 21.0% ID g^{-1} at 48 hours p.i. and high levels of radioactivity in the liver (between 6.0–10.2% ID g^{-1}) (Figure 3b and Table S11 in the Supporting Information). Positron emission tomography of mice injected with ^{89}Zr-labeled chCE7agl-(**1**)$_4$ correlated well with the biodistribution data of the ^{67}Ga-labeled analogue (Figure S5 in the Supporting Information). A comparative biodistribution study of RTXdegl, enzymatically and chemically modified with CTPA and radiolabeled with $^{64/67}$Cu, in mice bearing CD-20 positive Ramos xenografts also revealed improved target-to-nontarget ratios for the enzymatically prepared conjugate (Table S12 in the Supporting Information).

In summary, we have shown that enzymatic modification of mAbs using BTG is site-specific and versatile (with the potential to be readily scaled up), and leads to homogenous immunoconjugates with defined stoichiometries. The in vivo characteristics of such immunoconjugates are superior to those prepared using chemical coupling methods. Since position 295 is located in the constant Fc region, the enzymatic conjugation approach is applicable not only to other human IgG1s, but also to mAbs belonging to subtypes IgG2, IgG3, and IgG4, all of which conserve the Q295 residue.[17] Thus, the method is broadly applicable and permits the systematic assessment and improvement of immunoconjugates.

Received: July 12, 2010
Revised: September 13, 2010
Published online: November 25, 2010

Keywords: bacterial transglutaminase · enzymes · protein labeling · radioimmunoconjugates

[1] P. Carter, *Nat. Rev. Cancer* **2001**, *1*, 118–129.
[2] K. J. Hamblett, P. D. Senter, D. F. Chace, M. M. C. Sun, J. Lenox, C. G. Cerveny, K. M. Kissler, S. X. Bernhardt, A. K. Kopcha, R. F. Zabinski, D. L. Meyer, J. A. Francisco, *Clin. Cancer Res.* **2004**, *10*, 7063–7070.
[3] T. U. Gerngross, *Nat. Biotechnol.* **2004**, *22*, 1409–1414.
[4] J. R. Junutula, H. Raab, S. Clark, S. Bhakta, D. D. Leipold, S. Weir, Y. Chen, M. Simpson, S. P. Tsai, M. S. Dennis, Y. Lu, Y. G. Meng, C. Ng, J. Yang, C. C. Lee, E. Duenas, J. Gorrell, V. Katta, A. Kim, K. McDorman, K. Flagella, R. Venook, S. Ross, S. D. Spencer, W. Lee Wong, H. B. Lowman, R. Vandlen, M. X. Sliwkowski, R. H. Scheller, P. Polakis, W. Mallet, *Nat. Biotechnol.* **2008**, *26*, 925–932.
[5] P. Wu, W. Q. Shui, B. L. Carlson, N. Hu, D. Rabuka, J. Lee, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 3000–3005.
[6] W. Peters, S. Willnow, M. Duisken, H. Kleine, T. Macherey, K. E. Duncan, D. W. Litchfield, B. Lüscher, E. Weinhold, *Angew. Chem.* **2010**, *122*, 5296–5299; *Angew. Chem. Int. Ed.* **2010**, *49*, 5170–5173.
[7] L. Lorand, R. M. Graham, *Nat. Rev. Mol. Cell Biol.* **2003**, *4*, 140–156.
[8] A. Fontana, B. Spolaore, A. Mero, F. M. Veronese, *Adv. Drug Delivery Rev.* **2008**, *60*, 13–28.
[9] M. Bartholomä, J. Valliant, K. P. Maresca, J. Babich, J. Zubieta, *Chem. Commun.* **2009**, 493–512.
[10] K. Knogler, J. Grünberg, I. Novak-Hofer, K. Zimmermann, P. A. Schubiger, *Nucl. Med. Biol.* **2006**, *33*, 883–889.
[11] D. L. Kukis, G. L. DeNardo, S. J. DeNardo, G. R. Mirick, L. A. Miers, D. P. Greiner, C. F. Meares, *Cancer Res.* **1995**, *55*, 878–884.
[12] E. C. Dijkers, J. G. Kosterink, A. P. Rademaker, L. R. Perk, G. A. van Dongen, J. Bart, J. R. de Jong, E. G. de Vries, M. N. Lub-de Hooge, *J. Nucl. Med.* **2009**, *50*, 974–981.
[13] K. Knogler, J. Grünberg, K. Zimmermann, S. Cohrs, M. Honer, S. Ametamey, P. Altevogt, M. Fogel, P. A. Schubiger, I. Novak-Hofer, *Clin. Cancer Res.* **2007**, *13*, 603–611.
[14] S. Matsumiya, Y. Yamaguchi, J. Saito, M. Nagano, H. Sasakawa, S. Otaki, M. Satoh, K. Shitara, K. Kato, *J. Mol. Biol.* **2007**, *368*, 767–779.
[15] M. J. Feige, S. Nath, S. R. Catharino, D. Weinfurtner, S. Steinbacher, J. Buchner, *J. Mol. Biol.* **2009**, *391*, 599–608.
[16] S. Krapp, Y. Mimura, R. Jefferis, R. Huber, P. Sondermann, *J. Mol. Biol.* **2003**, *325*, 979–989.
[17] P. Sondermann, R. Huber, V. Oosthuizen, U. Jacob, *Nature* **2000**, *406*, 267–273.

