# Dual, Site-Specific Modification of Antibodies by Using Solid-Phase Immobilized Microbial Transglutaminase



**Author(s):**
Spycher, Philipp R.; Amann, Christian A.; Wehrmüller, Jöri E.; Hurwitz, David R.; Kreis, Olivier; Messmer, Daniel; Ritler, Andreas; Küchler, Andreas; Blanc, Alain; Behe, Martin; Walde, Peter; Schibli, Roger

**Publication date:**
2017-10-05

**Permanent link:**
https://doi.org/10.3929/ethz-b-000280185



**Originally published in:**
ChemBioChem 18(19), https://doi.org/10.1002/cbic.201700188

A EUROPEAN JOURNAL OF CHEMICAL BIOLOGY

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# Accepted Article

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**To be cited as:** *ChemBioChem* 10.1002/cbic.201700188

**Link to VoR:** http://dx.doi.org/10.1002/cbic.201700188

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10.1002/cbic.201700188

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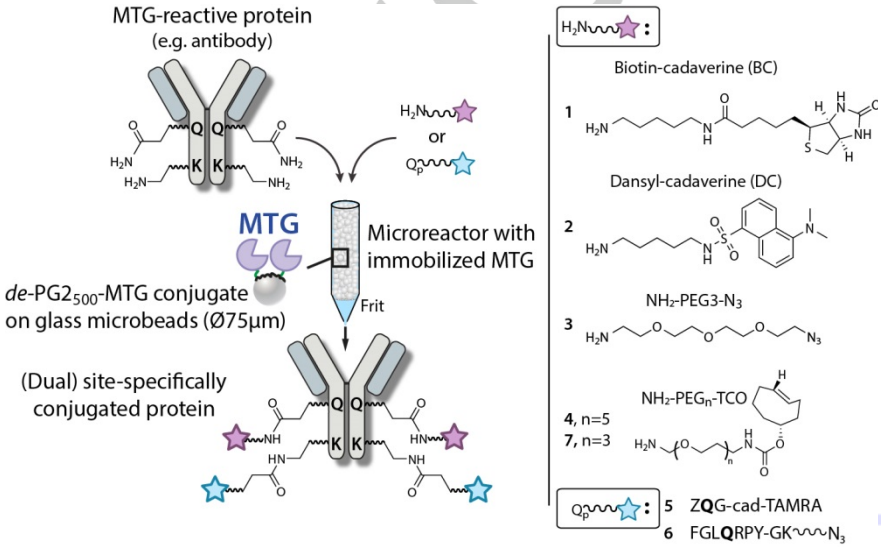
COMMUNICATION

# Dual site-specifically modified antibodies using solid-phase immobilized microbial transglutaminase

Philipp R. Spycher, Christian A. Amann, Jöri E. Wehrmüller, David R. Hurwitz, Olivier Kreis, Daniel Messmer, Andreas Ritler, Andreas Küchler, Alain Blanc, Martin Béhé, Peter Walde, Roger Schibli*

**Abstract:** Microbial transglutaminase (MTG) has been stably solid-phase immobilized on glass microbeads using a second generation dendronized polymer. Immobilized MTG enabled the efficient generation of site-specifically conjugated proteins including scFvs and Fab-fragments as well as whole antibodies via distinct glutamines and unprecedented, also via lysines with various bifunctional substrates with defined stoichiometry. With this method we generated dual site-specifically modified antibodies comprising a fluorescent probe and a metal chelator for radiolabeling – a strategy anticipated to design antibodies for imaging and therapy. Furthermore, we provide evidence that immobilized MTG features higher site-selectivity than soluble MTG.

The efficient modification and conversion of desired substrates by enzymes have found widespread application in a variety of fields including protein bioconjugation.[1] Enzymes usually operate under physiological conditions and possess a high specificity and selectivity towards their substrates, making them ideal candidates for protein conjugation, typically in a site-specific manner. Despite these benefits, the enzyme subsequently has to be removed from the mixture which can be avoided through solid-phase enzyme immobilization.[2] Policarpo et al. recently reported on the non-covalent immobilization of a Sortase A-variant onto microbeads, assembled into a microreactor.[3] Non-covalent immobilization of the endoglycosidase EndoS has furthermore been used for deglycosylation of antibodies.[4] Moreover, immobilization of enzymes has been reported to often enhance enzyme stability and can also lead to an increased activity or selectivity.[5]




**Scheme 1.** Assembly of the microreactor containing microbead-immobilized MTG that catalyzes the formation of isopeptidic bonds between reactive glutamine and lysine residues or other lysine surrogates. The dendronized polymer-enzyme conjugate *de*-PG2$_{500}$-MTG is adsorbed onto glass microbeads and assembled into a microreactor device. Proteins containing a MTG-reactive glutamine (Gln, Q) and/or lysine (Lys, K) residue are subjected to the microreactor, using a suitable primary amine substrate for Gln-conjugation or a glutamine containing peptide ($Q_p$) for Lys-conjugation. The blue and purple stars represent functional groups, e.g. dye or moieties suitable for click-chemistry.

We describe the stable solid-phase immobilization of microbial transglutaminase (MTG, ~38kDa) from *Streptomyces mobaraensis* onto glass microbeads for the subsequent generation of site-specifically modified proteins (Scheme 1). MTG has attracted major interest for the generation of homogenous, stoichiometrically and site-specifically modified antibody-drug-conjugates (ADCs) with improved pharmacokinetic properties.[6] So far, quantitative functionalization of antibodies using MTG via formation of an isopeptidic bond between primary amines and glutamines has been reported in solution.

We challenged the conjugation capability of the

[*] Dr. P. R. Spycher, C. A. Amann, J. E. Wehrmüller, D. R. Hurwitz, O. Kreis, A. Ritler, A. Blanc, Dr. M. Béhé, Prof. Dr. R. Schibli
Center for Radiopharmaceutical Sciences ETH-PSI-USZ
Paul Scherrer Institute
5232 Villigen PSI (Switzerland)
roger.schibli@psi.ch

D. Messmer, Dr. A. Küchler, Prof. Dr. P. Walde
Laboratory of Polymer Chemistry
Department of Materials
ETH Zurich
Vladimir-Prelog-Weg 5, 8093 Zurich (Switzerland)

A. Ritler
Laboratory of Organic Chemistry
Department of Chemistry and Applied Biosciences
ETH Zurich
Vladimir-Prelog-Weg 4, 8093 Zurich (Switzerland)

Prof. Dr. R. Schibli
Institute of Pharmaceutical Sciences
Department of Chemistry and Applied Biosciences
ETH Zurich
Vladimir-Prelog-Weg 4, 8093 Zurich (Switzerland)

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bead-immobilized MTG in three ways: i) We modified therapeutically relevant protein structures such as antibodies or scFvs and Fab-fragments via incorporation of glutamine residues. The latter formats are of particular interest since they have a similar molecular weight as the enzyme and are thus difficult to separate from the enzyme by conventional chromatographic methods; ii) we site-specifically modify proteins via lysine residues using immobilized MTG thereby generating single- or dual-modified, biologically active antibodies; iii) we site-selectively modified proteins via immobilized MTG.

The polymer-MTG conjugate was generated by first coupling the bifunctional linker *N*-succinimidyl-4-formylbenzoate (excess of 1.5eq) to MTG, which resulted in >90%, single-linked MTG. *N*-succinimidyl-6-hydrazinonicotinate was conjugated to the dendronized polymer *de*-PG2$_{500}$ (Figure S1-S4). The reaction of the modified MTG and modified polymer was monitored by UV-VIS spectroscopy (absorption at 354nm; Figure S4).[7] The polymer-MTG conjugate was then adsorbed onto glass microbeads by exploiting the strong affinity of the denpol's ammonium cations to the negatively charged glass surface.[8]

We tested the immobilized MTG towards small and high molecular weight proteins including scFvs, Fab-fragments and antibodies for the site-specific and quantitative incorporation of various functional small molecular weight compounds like biotin or fluorophores. We also investigated molecules suitable for bio-orthogonal click-reactions which are of particular interest, since they allow for the attachment of virtually any moiety of interest.[6d, 9]

In a first endeavor, we investigated the conjugation capability of immobilized MTG using a c-myc-tagged scFv (26kDa) bearing an accessible glutamine moiety, previously shown to be efficiently conjugated in solution[6a] (Figure 1A). We subjected the scFv to various functional substrates including biotin-cadaverine (BC) **1**, dansylcadaverine (DC) **2** (a fluorescent amine donor for MTG) and $NH_2$-PEG3-$N_3$ **3** suitable for SPAAC (strain-promoted alkyne-azide cycloaddition) at a molar ratio of 1:80 for **1** and **3** and 1:8 for **2**. The solutions were incubated in the microreactor for 30-90min and we found a conjugation rate of ≥90-95% as determined by LC-MS (Figure 1B and S5). Similarly, a 52kDa c-myc-tagged Fab-fragment was site-specifically modified to ≥95% within 30min with **1** and **2** at 80 and 8 molar excess, respectively (Figure S5). Enzyme-leaking could be excluded using slot-blot assay and an antibody against MTG (Figure S6).

Encouraged by these findings, we next explored whether full length antibodies could also be conjugated by microbead-immobilized MTG. We previously identified glutamine 295 (Q295) of de- or aglycosylated antibodies, located on the flexible C'E loop of the Fc-domain, as the sole site of modification.[6b] We employed the aglycosylated cAC10 antibody with a N297S point mutation[10], which has been used as an ADC in our previous studies using CD30-positive tumor bearing mice.[6e] The antibody was subjected to the microreactor with substrate **1** at a 1:80 molar ratio. We found ≥95% conversion overnight as determined by LC-MS (Figure 1C and Figure S5). For the cAC10-N297Q mutant, offering two modification sites per heavy chain,[6b, 11] we likewise found ≥95% product conversion with substrate **3** and **4** at 1:80

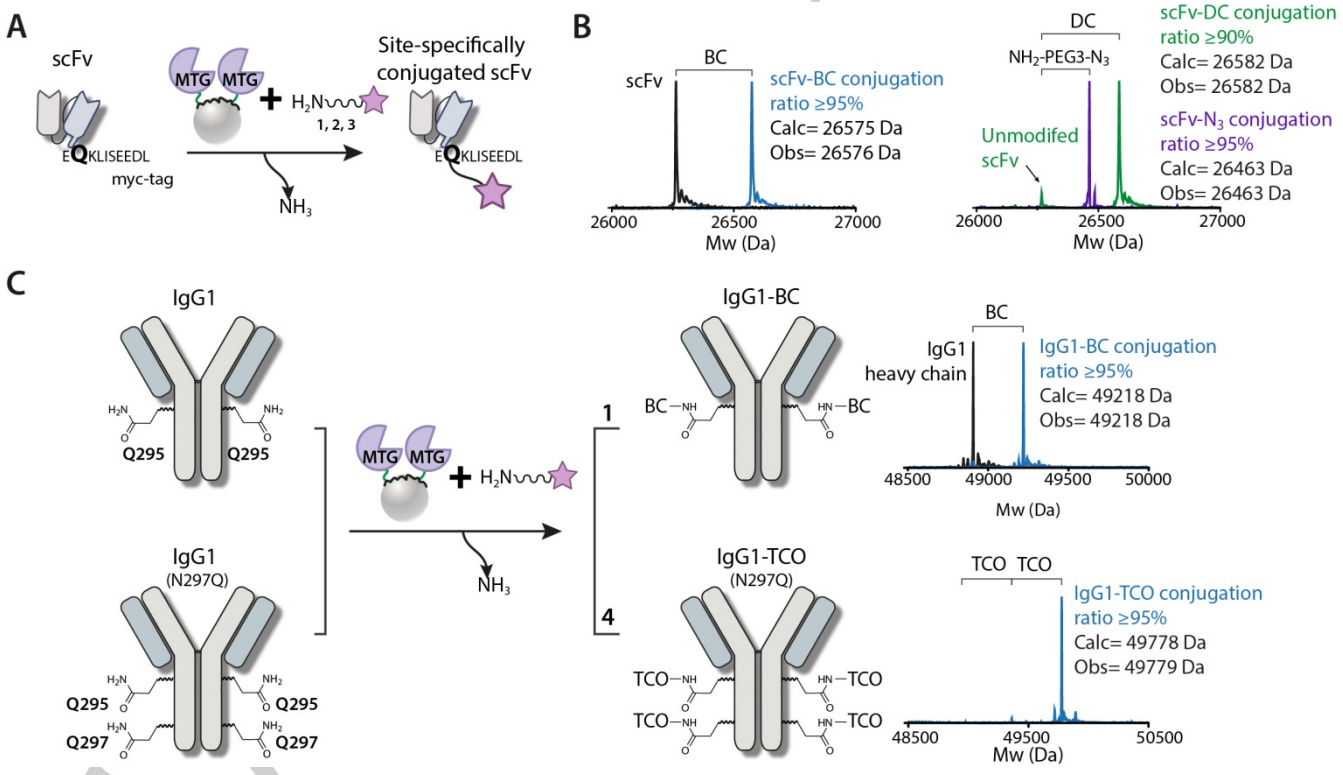


**Figure 1.** (A) Schematic representation of the conjugation process of primary amine substrates to c-myc-tagged scFv (the bold **Q** is targeted by MTG). (B) Deconvoluted LC-MS spectra of substrates **1**-**3** conjugated to c-myc-tagged scFv yielded conversion of ≥90-95% to the desired conjugates. (C) More than 95% conjugation was achieved for aglycosylated cAC10-IgG1 antibodies (N297S and N297Q mutant, resp.) with substrates **1** and **4** after 16h of incubation (see also SI, Figure S5). Antibodies were reduced for LC-MS analysis, deconvoluted spectra are shown.

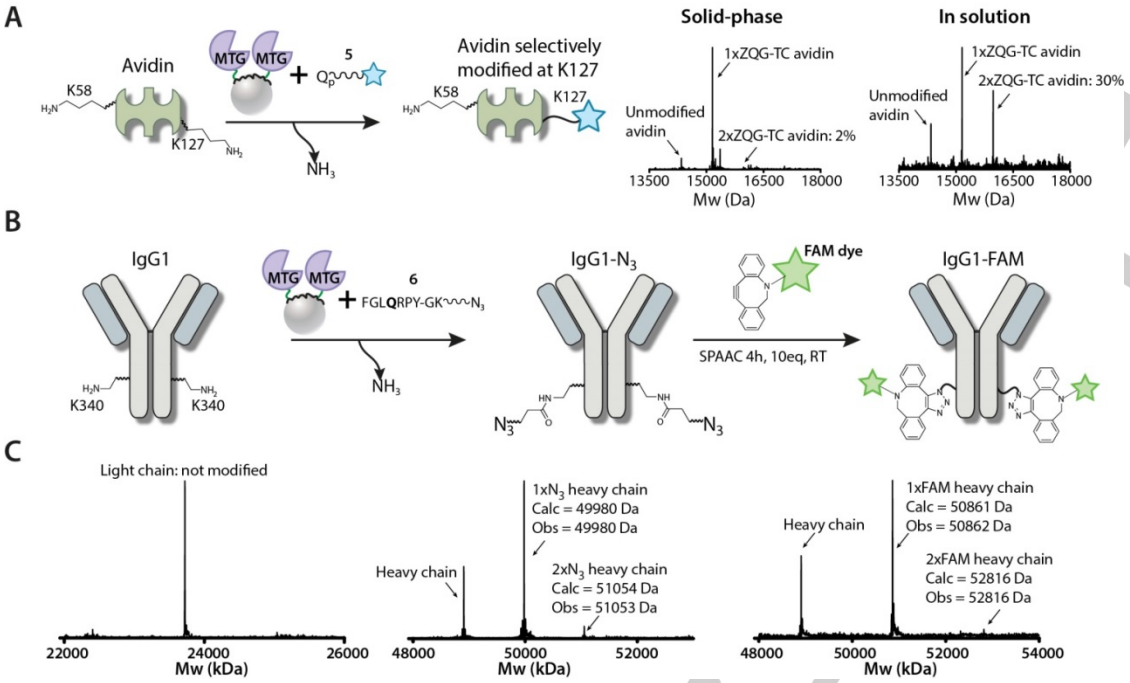


**Figure 2.** (A) Site-specific lysine conjugation of avidin using immobilized MTG and **5** (ZQG-TC, ZQG-cad-TAMRA dye) as the glutamine containing substrate $Q_p$. Solid-phase conjugation showed higher selectivity for K127 compared to conjugation in solution where both lysines were strongly modified. (B) Lysine conjugation of aglycosylated cAC10-IgG1 N297S antibody with glutamine peptide **6** and subsequent SPAAC; the MTG-reactive Q is marked in bold. (C) The conjugation yield was determined to ~71% with one major peak on the heavy chain while on the light chain, no modification could be detected in LC-MS (deconvoluted spectra are shown). Clicking with DBCO-PEG4-5/6-FAM dye showed clean conversion to the fluorescently labelled conjugate.

molar ratio per conjugation site (Figure 1C and Figure S5). Our experiments show that even when the reactive glutamines are in close proximity the aglycosylated antibody can be site-specifically conjugated with immobilized MTG. In contrary, we did not observe any modification of native, glycosylated antibodies presumably due to steric reasons, which is in agreement with our previous observations.[6b]

While site-specificity and stoichiometric modification of proteins via glutamine side chains with primary amine substrates has been reported, the reverse approach, i.e. conjugation to MTG-reactive lysine residues on native proteins with Gln-substrates is scarcely described.[12] Fontana et al. recently described MTG-mediated lysine modifications of interferon α subtypes[12a] and avidin[12b] at multiple sites and non-stoichiometrically using glutamine containing ZQG-derivatives (ZQG, *N*-benzyloxycarbonyl-L-glutaminylglycine). Since immobilization of enzymes can increase site-selectivity[5] as e.g. reported for Lipase[13] we speculated whether this may also apply to immobilized MTG. Based on Fontana's work, we focused on avidin that is reported to possess two MTG-reactive lysines (Lys58 and Lys127).[12b] Indeed, whereas the reaction with soluble MTG gave rise to a mixture of single and double modified avidin when reacted with substrate **5** (up to 30% dual modification; Figure 2A), we found that with immobilized MTG almost exclusively modified Lys127 with only negligible conjugation of Lys58 (Figure 2A and S7). We attribute this to a decreased rotational flexibility of immobilized MTG that makes substrate binding and conjugation to less-well exposed residues such as Lys58 located in a loop structure more challenging. Lys127, however, is located in the highly disordered region of the C-terminus making binding and thus conjugation likely more efficient.[12b] From these data we can conclude that immobilization of MTG can lead to higher site-selectivity which to the best of our knowledge has not been reported yet for enzymes modifying macromolecules.

Whereas modification of avidin with substrate **5** was almost quantitative functionalization of antibodies via lysine residues was unsatisfactory with the ZQG-peptide.[14] Therefore, we speculated whether other glutamine-containing peptide sequences would be better substrates for MTG From a small library of glutamine containing peptides we could identify peptide FGLQRPY to be twice as reactive as ZQG (Figure S8-S11). For the FGLQRPY azide-derivative **6** a conjugation yield of >70% was achieved to aglycosylated cAC10 with immobilized MTG (Figure 2B, C and Figure S8-S12). Peptide mapping confirmed two modification sites at Lys288/290 and Lys340 on the heavy chain (confirmed with LC-MS and SDS-PAGE; Figure 2C, 3B and S13), which due to its amino acid sequence presumably is the preferred modification site for MTG (Figure S13). Bioorthogonal reaction of the **6**-modified antibody with 10eq the dye DBCO-PEG4-5/6-FAM for 4h yielded quantitatively the fluorescently labeled antibody (Figure 2C and S7).

Having established glutamine and lysine conjugation with immobilized MTG, we tested whether site-specific dual antibody modification would be feasible. This is an attractive strategy for diagnostic or therapeutic applications or a combination thereof.[15] We focused on the generation of antibodies with two imaging probes suitable for e.g. non- invasive and intra/post-operative imaging.[16] Q295 was first conjugated with **7** (≥95%) followed by modification of K340 with **6** resulting in a slightly lower yield of 38% as compared to sole conjugation with **6** (Figure 3A). Nevertheless, simultaneous dual-clicking of (Tz)-PEG4-DOTAGA and DBCO-PEG4-5/6-FAM (see Figure S7) could be elegantly performed in a single step yielding >85% IgG1 exclusively labelled on the heavy chain as confirmed by SDS-PAGE (Figure 3B). Biological activity and binding specificity of the dual-modified antibodies was proven by flow- cytometric analysis using

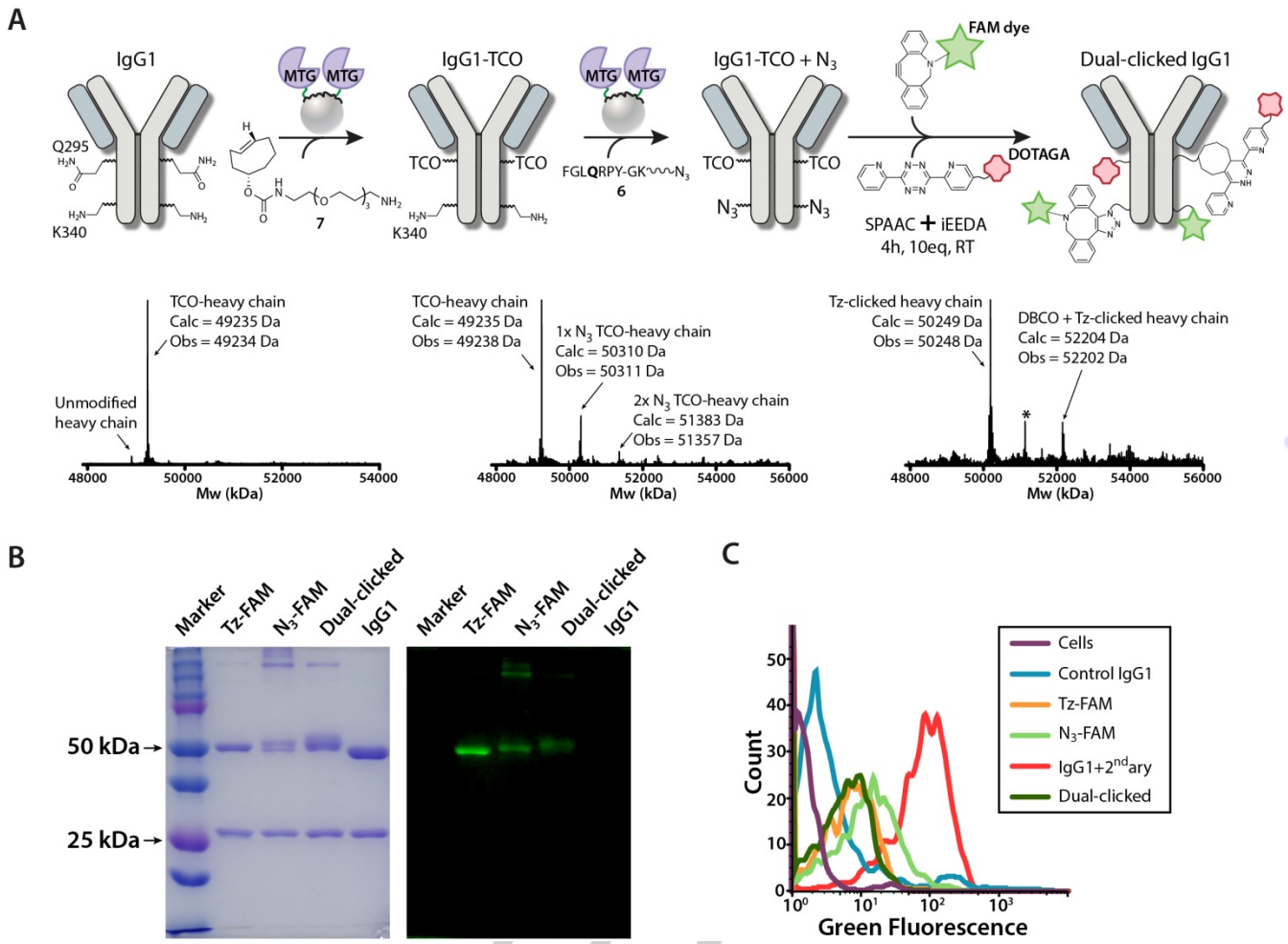


**Figure 3.** (A) Preparation of the dual site-specifically modified cAC10-IgG1 N297S and the subsequent simultaneous bioorthogonal reaction with DBCO-PEG4-5/6-FAM dye (SPAAC) and tetrazine(Tz)-PEG4-DOTAGA metal chelator (inverse electron demand Diels-Alder, iEEDA). IgG1 N297S was first conjugated with **7** (yield ≥95%) and subsequently with **6** (~38% conjugation). The mass peak indicated by an asterisk (51190 Da) corresponds to DBCO-clicked heavy chain with no Tz-clicking. (B) SDS-PAGE with coomassie staining and fluorescence detection of the antibodies showed exclusive modification on the heavy chain. (C) Flow-cytometry of conjugated antibodies using CD30 expressing Karpas299 cells demonstrated specific binding. IgG1 corresponds to unmodified cAC10-N297S antibody for which a secondary antibody was used (see SI).

Karpas299 cells expressing the CD30 receptor.[10, 17] antibodies generated by immobilized MTG showed binding to CD30 (Figure 3C).

In conclusion, we have demonstrated that with MTG, which was immobilized by adsorbing a water soluble dendronized polymer-enzyme conjugate (*de*-$PG_{500}$-MTG) onto glass microbeads, various types and sizes of proteins can be site-specifically and in most cases quantitatively modified with different substrates. Immobilization of MTG also increased the enzymes site-selectivity in certain cases. Finally, the method allowed dual site-specific modification of aglycosylated IgG1s which is particularly interesting for the design of dual labeled antibodies for imaging purposes or highly potent antibody-drug-conjugates. However, further optimization to improve incorporation efficiency is required for application to ADCs.

## Acknowledgements

This work was supported by the European Union Seventh Framework Programme FP7/2007-2013 under Grant 602820. Dr. Tim Hungerland is gratefully acknowledged for the synthesis of polymer precursors.

**Keywords:** antibodies • dual site-specific modification • transglutaminase • immobilization • bioconjugation

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10.1002/cbic.201700188

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**Entry for the Table of Contents**

# COMMUNICATION

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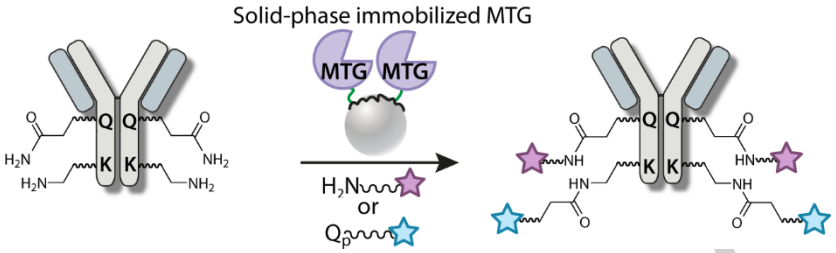
**Dual site-specifically modified antibodies using solid-phase immobilized microbial transglutaminase**

**Dual site-specific conjugation:** Solid-phase immobilized microbial transglutaminase (MTG) enabled the site-specific modification of glutamine and/or lysine residues of various proteins with probes suitable for bio-orthogonal click-reactions. Dual site-specific modification of antibodies is demonstrated with chemical entities suitable for dual-imaging applications without affecting antibody antigen binding.



Supporting Information

69451 Weinheim, Germany

**DOI: 10.1002/anie.2016XXXXX**

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## 1. Experimental Procedures

If not mentioned otherwise, ultra-pure water was used for all experiments and if not stated otherwise, reactions were carried out at room temperature (22°C).

### 1.1 Buffers

MES buffer was prepared as follows: 100mM 2-(*N*-Morpholino)ethanesulfonic acid 1-hydrate (AppliChem GmbH, Darmstadt, Germany) and 150mM NaCl were dissolved in ultra-pure MilliQ water and adjusted to pH 4.7. MOPS buffer was prepared by

dissolving 100mM 3-(*N*-Morpholino)propanesulfonic acid (AppliChem GmbH, Darmstadt, Germany) and 150mM NaCl in ultra-pure MilliQ water and was adjusted to pH 7.6. PBS7.2 was prepared as follows: 150mM sodium phosphate dibasic dihydrate (Sigma-Aldrich, Buchs, Switzerland) and 150mM NaCl were dissolved in ultra-pure and adjusted to pH 7.2. TrisHCl buffer contained 50mM TrisHCl (Sigma-Aldrich, Buchs, Switzerland) and was adjusted to pH 7.6. Metal-free (chelexed) PBS was prepared as follows: PBS Dulbecco Instamed was dissolved in ultrapure water at 9.55g/L (w/o $Ca^{2+}$) (Merck-Millipore, Switzerland) and incubated with 5g/L Chelex100 resin (Bio-Rad, Switzerland) for 2h at room temperature while gentle shaking was applied. The resin was removed by 0.22µm filtering and the pH was adjusted to 7.4 with metal-free 5M HCl (Sigma-Aldrich, Switzerland).
For the antibody lysine screening the following buffers were used: BisTris-buffer for pH 6, sodium phosphate buffer for 7.4 and Trizma base-buffer for pH 8.5 (all from Sigma-Aldrich, Switzerland). The BisTris-buffer was prepared by dissolving Bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methan in ultrapure water to a concentration of 50mM, followed by pH adjustment to pH 6 using 6 M NaOH. The sodium phosphate buffer was prepared by dissolving sodium phosphate to a concentration of 50mM in ultra-pure water and subsequent pH adjustment to pH 7.4 using 6M NaOH. The Trizma base-buffer contained Trizma base dissolved to a concentration of 50mM in ultra-pure water at pH 8.5.

### 1.2 Assembly of the *de*-PG2$_{500}$-MTG conjugate

The principle how MTG is attached to dendronized *de*-PG2$_{500}$ polymer is schematically shown in Figure S1 and will be outlined in detail below. MTG and polymer are first modified with N-succinimidyl-4-formylbenzoate (S-4FB) and N-succinimidyl-6-hydrazinonicotinate (S-HyNic), respectively (Figure S1). Afterwards, both conjugates are mixed which leads to the formation of a bis-aryl-hydrazone (BAH) bond quantifiable at 354nm by UV-VIS spectrometry and to the formation of the *de*-PG2$_{500}$-BAH-MTG conjugate, respectively. The polymer-enzyme conjugate is then adsorbed onto glass microbeads and assembled into a reactor (Scheme 1).

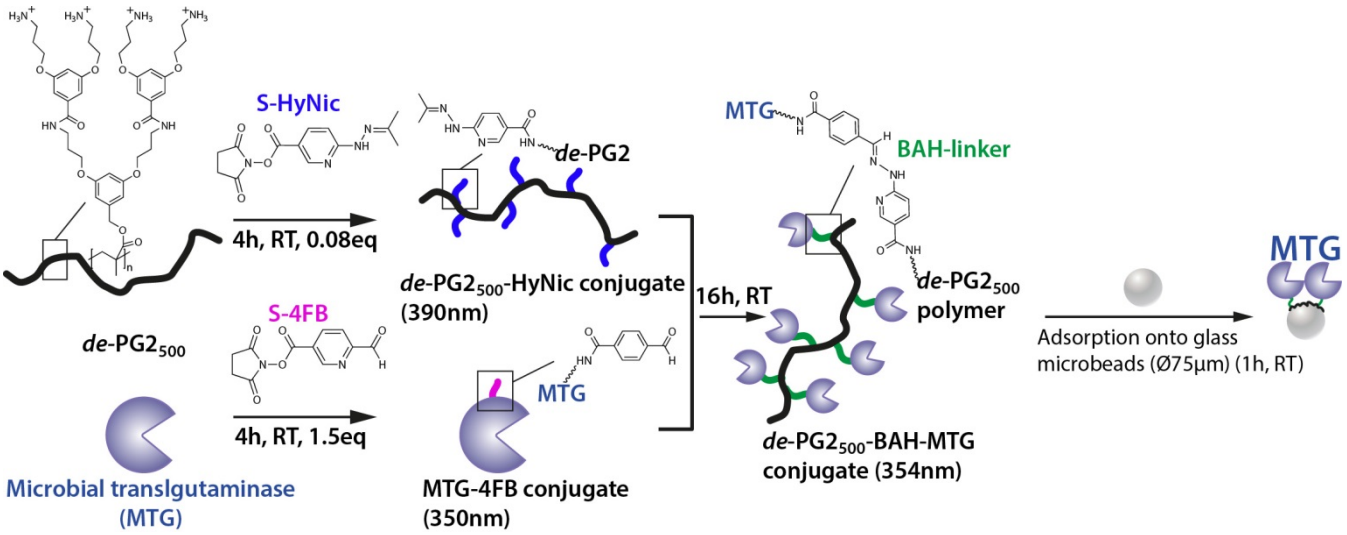



**Figure S1.** Assembly of *de*-PG2$_{500}$-MTG conjugate using the bifunctional linkers S-HyNic and S-4FB. In a first step, denpol *de*-PG2$_{500}$ and microbial transglutaminase (MTG) are modified with the linkers S-HyNic and S-4FB, resp. Both linkers can be quantified with UV-VIS spectrometry after conjugation (corresponding wavelength indicated) using specific detection reagents (Figure S3). Afterwards, both conjugates are mixed, which leads to the formation of a bis-aryl-hydrazone bond (BAH) that can be quantified by UV-VIS spectrometry at 354nm. The polymer-MTG conjugate is subsequently adsorbed onto glass microbeads and assembled into a flow-device (Scheme 1).

### 1.3 Synthesis of dendronized polymer *de*-PG2$_{500}$

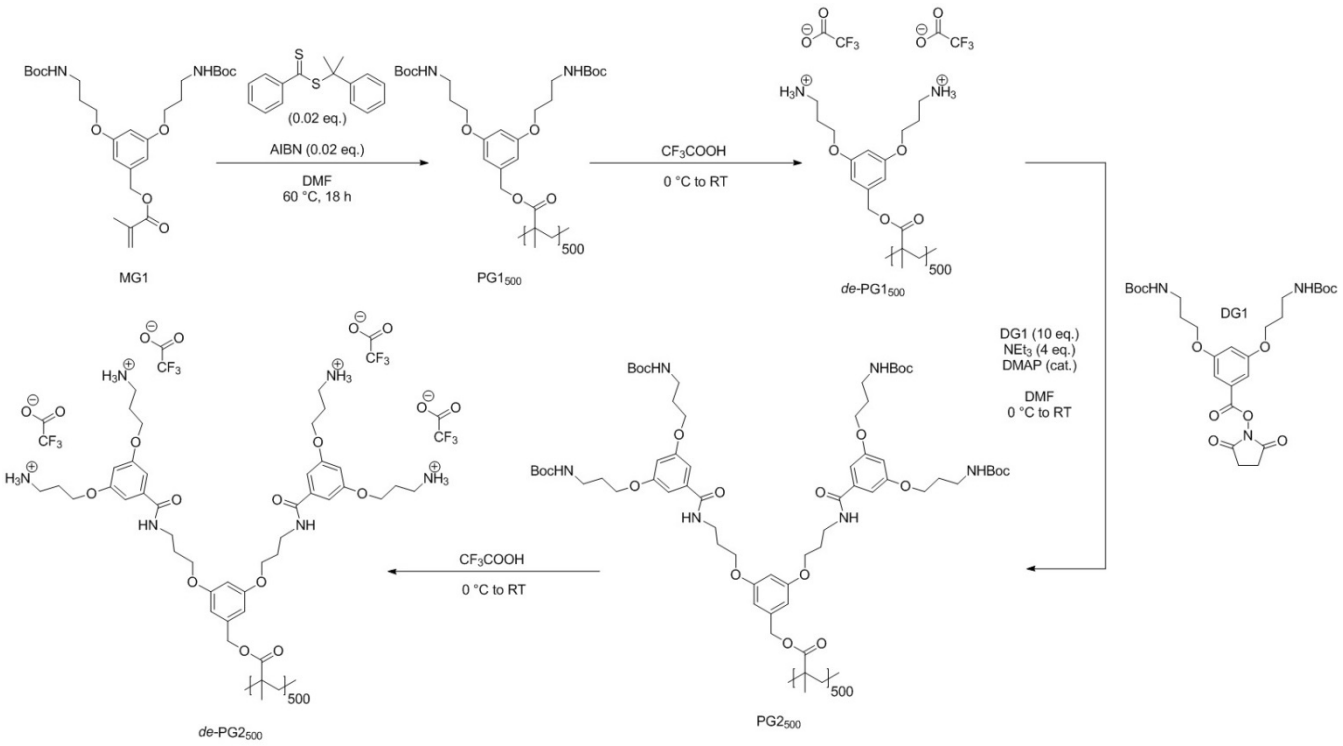



**Figure S2.** Synthesis of the de-protected, poly(methacrylate)-based second generation dendronized polymer PG2 (*de*-PG2$_{500}$) consisting of approx. 500 repeating units. PG2=Polymer of generation 2.

PG2$_{500}$ was synthesized according to established methods (Figure S2);[1] briefly, monomer MG1 was polymerized using a RAFT protocol with cumyl dithiobenzoate as transfer agent and AIBN as radical initiator. The resulting polymer PG1$_{500}$ was characterized by GPC (DMF containing 0.1% LiBr as eluent) and had a $P_n \approx 500$ at PDI = 1.41 (evaluated using universal calibration from narrowly dispersed PMMA standards). Boc protecting groups were cleaved using neat trifluoroacetic acid, affording *de*-PG1$_{500}$, which was reacted with active ester DG1 under basic conditions, affording PG2$_{500}$. Quantification of unreacted amines by the established Sanger labeling method[2] verified >99.5% conversion of amines. The final deprotection step is outlined in detail below:
A 15mL vial containing PG2$_{500}$ (112.7mg, 92µmol repeating units) and a magnetic stir bar was cooled in an ice bath. Ice-cold trifluororacetic acid (4mL, 52mmol, 560eq. per repeating unit) was added slowly and the mixture was stirred at 0°C until all polymer chunks were visibly broken up into a milky-white dispersion. Methanol was added in single droplets until the mixture turned clear; the reaction was then stirred at room temperature overnight. The mixture was quenched by slow addition of methanol (5mL) while stirring in an ice bath, then concentrated by rotary evaporation. Addition of methanol (2 x 5mL) and concentration of the mixture was repeated twice, then solvents were evaporated to dryness. The resulting glassy solid was dissolved in deionized water (ca. 1.5mL) and lyophilized to quantitatively afford *de*-PG2$_{500}$ as an off-white foam. The polymer was aliquoted to 2mg units and stored at -20°C. For conjugation reactions the polymer was dissolved in MOPS buffer and the repeating unit concentration was determined with UV-VIS spectrometry at 285nm ($\varepsilon_{285}$=5'000M$^{-1}$cm$^{-1}$).[3]

### 1.4 Conjugation of S-4FB to MTG and S-HyNic to *de*-PG2$_{500}$ polymer

The bifunctional linkers S-4FB and S-HyNic[4] (Figure S1) were dissolved in DMF (Sigma-Aldrich, Switzerland) to a stock concentration of 20mM, aliquoted and stored at -20°C. A 1.5eq molar excess of S-4FB was reacted with 80µM MTG for an incubation time of 4 hours at room temperature. For that matter the MTG concentration was determined experimentally with UV-VIS spectrometry at 280nm using the ExPASy[5] computed molecular weight of 38334Da for MTG and a molar extinction coefficient of $\varepsilon_{280nm}$=71'850M$^{-1}$cm$^{-1}$ derived from the MTG sequence (personal communication, Dr. Martin Hils, Zedira, Germany). S-HyNic was conjugated to *de*-PG2$_{500}$ polymer at 1.45mM repeating unit concentration using 0.08 molar equivalent of S-HyNic for 4h at room

temperature. Both conjugates were purified in MES buffer by repeated ultracentrifugation using Amicon Ultra-4 filter tubes with a 10kDa MWCO (Merck-Millipore, Switzerland).

### 1.5 Determination of S-4FB and S-HyNic linker concentration

*Principle of linker quantification*

The concentration of the conjugated S-4FB and S-HyNic-linkers, resp. can be quantified using corresponding UV-traceable detection reagents that will form a bis-aryl-hydrazone bond with the respective linkers.[4a] The S-4FB linker concentration can be quantified with 2-HPDC (2-hydrazinopyridine-dihydrochloride) at 350nm ($\varepsilon_{350nm}$=24'500$M^{-1}cm^{-1}$) and the S-HyNic linker using 4-NBA (4-nitrobenzaldehyde) at 390nm ($\varepsilon_{390}$=24'000$M^{-1}cm^{-1}$) (Figure S3). The corresponding absorption increase at 350nm and 390nm, resp. was measured immediately after mixing (t=0) and after 1h of incubation (t=1h).

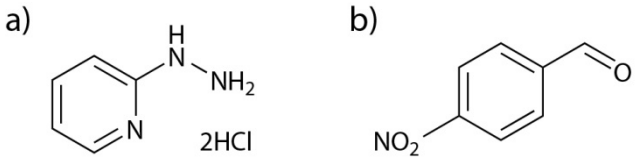



**Figure S3.** a) The dectection reagent 2-hydrazinopyridine-dihydrochloride (2-HPDC) was used to determine the concentration of 4FB conjugated to MTG. The resulting bis-aryl-hydrazone bond can then be detected at 350nm and the linker concentration be determined using $\varepsilon_{350nm}$=24'500$M^{-1}cm^{-1}$. (b) 4-nitrobenzaldehyde (4-NBA) was used to determine the amount of HyNic-functionalization of the *de*-$PG2_{500}$ polymer by measuring the absorbance of the bis-aryl-hydrazone bond at 390nm and $\varepsilon_{390nm}$=24'000$M^{-1}cm^{-1}$.

*UV-VIS spectrophotometric linker quantification of MTG-4FB & de-$PG2_{500}$-HyNic*

The linker concentration of S-4FB was determined as follows: 2µl of the MTG-4FB solution and 2-HPDC (50mM in MES) were added to 196µl MES buffer and mixed well. The mixture was swiftly transferred to a quartz cuvette (10mm) and the absorption was measured at t=0 and t=1h at 350nm. The corresponding difference in absorption was used to determine the concentration of bound linker via Lambert-Beer's law with $\varepsilon_{350nm}$=24'500$M^{-1}cm^{-1}$. The concentration of *de*-$PG2_{500}$-bound HyNic was determined analogously with 4-NBA (50mM in DMF) measuring the absorption at 390nm ($\varepsilon_{390nm}$=24'000$M^{-1}cm^{-1}$).

### 1.6 Formation of the *de*-$PG2_{500}$-MTG conjugate

The MTG-4FB was mixed with dendronized polymer *de*-$PG2_{500}$-HyNic using 75µM 4FB-linker and 50µM HyNic-concentration, resp. in MES buffer. The reaction was incubated at room temperature for 16h during which a UV-VIS quantifiable bis-aryl-hydrazone (BAH) bond was formed at 354nm. The conjugation reaction was performed using a Lambda 35 UV/VIS spectrophotometer (Perkin Elmer, Switzerland) and an Eppendorf UVette (Vaudaux-Eppendorf, Switzerland) at a path length of 2mm to monitor the formation of the UV-traceable BAH-bond at 354nm. Subsequently, the polymer-enzyme conjugate was purified by repeated ultracentrifugation using an Amicon Ultra-4 filter tube with a 100kDa MWCO in TrisHCl buffer.

### 1.7 Adsorption of polymer-enzyme conjugate on glass beads

Glass beads with a mean diameter of 75µm (Sigma-Aldrich, Switzerland) were first purified by 20min ultrasonication in toluene followed by 20min ultrasonication in isopropanol. Excess solvent was decanted and the glass beads were dried at 80°C for 6h to remove residual solvent.

For the immobilization process, the purified polymer-enzyme conjugate solution was set to 5µM BAH concentration in TrisHCl pH 7.6. The desired amount of beads (typically 1-1.5g) was placed in a suitable tube and the conjugate was added. The bead-slurry was suspended well to allow for a homogeneous polymer-enzyme distribution and was then transferred to a column with a 20µm pore size frit at the bottom (Isolute, Biotage AB, Sweden). The solution was allowed to pass through the column using gravity flow, was collected and repeatedly transferred back into the column for 1h after which no conjugate adsorption could be detected anymore using UV-VIS spectroscopy at 354nm (10mm UVette). After saturating the bead surface with polymer-enzyme conjugate, the column was rinsed with at least 10ml of TrisHCl buffer to wash out excess conjugate.

### 1.8 Proteins and antibodies

Microbial transglutaminase (MTG) (Zedira, Germany) was dissolved in PBS7.2 for immobilization experiments or in ultrapure water for experiments performed in solution. PNGase F (Hoffmann-La Roche AG, Switzerland) was dissolved in ultrapure water. Herceptin IgG1 antibody (kindly provided by Alexander Meisel, Waidspital Zürich, Switzerland) was deglycosylated applying PNGase F at a concentration of 6 U/mg antibody for 16h at 37°C. Afterwards, the deglycosylated antibody was buffer exchanged by PD-10 size exclusion chromatography (GE Healthcare, Switzerland) to TrisHCl pH 7.6 and concentrated to >8mg/ml by ultracentrifugation using a Vivaspin 50kDa MWCO filter (Sartorius, Germany). The anti-CD30 IgG1 antibody cAC10 which has the same polypeptide backbone as Adcetris[6] was used in its aglycosylated form, i.e. either containing a serine (N297S) or glutamine (N297Q) point mutation at its amino acid position N297 (antibodies kindly provided by Innate Pharma, France). A glutamine N297Q point mutation generates aglycosylated antibodies offering two modification sites per heavy chain.[7]
Myc-tagged Fab-fragment and scFv were generously provided by Dr. Patrick Dennler and Dr. Maria Mitsi, (Paul Scherrer Institute, Switzerland). Avidin was obtained from Sigma-Aldrich (Switzerland). All proteins were kept at 4°C.

### 1.9 Conjugation of proteins with immobilized MTG and click-reactions

In order to allow for a controllable sample flow, a custom-built tubing set-up (tubing from ISMATEC, Germany) was attached to the outlet of the microreactor and connected to a peristaltic pump. Such a set-up allowed for a controllable flow sample application and sample flow (~25µl/min) through the microreactor. After thorough washing with TrisHCl pH7.6 under gravity flow (see above) the microreactor was mounted into the tubing set-up. Before sample application, a pre-push solution was applied containing the substrate to be conjugated (either the primary amine containing substrate or the glutamine containing peptide). Once the pre-push had completely entered the packed bed of the microreactor the corresponding protein and the substrate were loaded. Afterwards, a second push-solution was mounted and allowed to enter the packed bed completely before the flow was stopped. The reactor column was then removed, the outlet sealed and incubated at room temperature for 16h under gentle shaking. After incubation the microreactor was mounted again into the pumping device and elution of the antibody mixture was performed by rinsing the reactor with substrate solution at constant flow. If not mentioned otherwise, an 80eq molar excess of the substrate was used compared to the protein to be conjugated. For avidin a molar excess of 160eq was used and for IgG1cAC10-N297Q, 80eq molar excess per conjugation site.
80µM myc-tagged scFv and Fab-fragment were applied for **(1)** (for numbering see main text**)** biotin-cadaverine (Zedira, Germany) and 40µM for **(2)** dansyl-cadaverine (Zedira) at an 8eq excess. 25µM scFv was used for conjugation to **(3)** $NH_2$-PEG3-$N_3$ (Jena Bioscience, Germany). Avidin was conjugated at 15µM with ZQG-cad-TAMRA (or ZQG-TC) (Zedira, Germany) (see Figure S7). Antibodies (IgG1cAC10-N297S and N297Q) were conjugated between 1-2.5mg/ml (6.6-16.7µM); substrate **(4)** TCO-PEG5-$NH_2$ was purchased from Conju-Probe, USA. The dual-modified IgG1cAC10-N297S was first conjugated to **(7)** TCO-PEG3-$NH_2$ (Conju-Probe, USA) and after incubation purified with an Amicon Ultra-4 filter tube with a 30kDa MWCO in TrisHCl buffer. Purified IgG1-TCO antibody was then conjugated to FGLQRPY-GK*($N_3$) and purified in chelexed PBS with Amicon Ultra-500 filter tube with a 30kDa MWCO. Simultaneous dual-clicking was then performed with 10eq molar excess of the metal chelator tetrazine-PEG4-DOTAGA (Chematech, France) and DBCO-PEG4-5/6-FAM fluorescent dye (Jena Bioscience, Germany) (see Figure S7 for structures) versus the antibody for 4h at room temperature and subsequently purified with an Amicon Ultra-0.5mL filter tube 30kDa MWCO in chelexed PBS. For SDS-PAGE analysis purified IgG1-TCO was clicked with DBCO-PEG4-5/6-FAM at 10eq, for 4h and purified as above. Conjugation reactions were done in 50mM TrisHCl, pH7.6. Peptide mapping was done at Functional Genomics Center Zurich (FGCZ, Zurich, Switzerland).

### 1.10 Probing polymer-enzyme desorption by slot blot

Since robust enzyme immobilization is critical for downstream applications, in particular for prospective therapeutic proteins, we checked for enzyme-leaking using slot-blot assay and an antibody against MTG (Zedira, Germany).
Slot blots were performed using a vacuum-driven Bio-Dot Apparatus (Bio-Rad Laboratories, USA). First, an Immobilon® Transfer membrane (Merck-Millipore, Switzerland) was activated in methanol for 5 minutes, then equilibrated in 50mM TrisHCl pH 7.6 buffer for 5 minutes and subsequently washed with 50mM TrisHCl pH 7.6 buffer for 3 x 5min. Then, the slot blot apparatus was assembled according to the manufacturer, and droplet fractions were pipetted into the slots and transferred onto the membrane by applying a vacuum. The membrane was then blocked with 2% BSA in TBST (Tris buffered saline containing 0.1% Tween-20; 50mM Tris, 150mM NaCl in $H_2O$ pH 7.6 with 0.1% Tween-20) solution for 1 hour before adding the rabbit polyclonal anti-MTG antibody (Zedira, Darmstadt) using a 1:1500 fold dilution in 2% BSA solution in TBST. Afterwards, the secondary anti-rabbit HRP-linked antibody was applied for 1 hour in a 1:1500 dilution (Cell Signalling Technologies, USA). After each step, gentle shaking was performed, followed by a washing step consisting of 3 x 5 minutes washing with TBST. Supersignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific, UK) was used for detection according to manufacturer's instruction.

### 1.11 Conjugation of avidin in solution with MTG

Avidin was conjugated at 15µM using 1:160eq excess of ZQG-cad-TAMRA dye with 6U/ml MTG in TrisHCl pH 7.6, 16h at room temperature.

### 1.12 Glutamine containing peptides for lysine screening

If not further specified, the glutamine containing peptides used for antibody lysine screening were purchased form LifeTein (USA) and used as obtained. To dissolve the peptides, the following method was applied (according to LifeTein, USA).[8] First, the overall charge of the peptide was determined by assigning a value of +1 to each basic residue (R, K, H and the N-terminal -$NH_2$) and a value of -1 to the acidic residues (D, E and C-terminal -COOH). If the overall charge of the peptide was negative, it was dissolved in 0.1M ammonium bicarbonate, while positively charged peptides were dissolved in ultrapure water. After dissolving the peptides, the concentration was determined by UV-VIS using the calculated extinction coefficient from ExPASy[5] or calculated directly based on the amount of peptide dissolved in case of peptides not exhibiting UV absorption. The concentration was adjusted to 25mM or at least 10mM if dissolving proved difficult. The peptides were then aliquoted and stored at -20°C. Z-glutaminyl-glycine (ZQG or *N*-Benzyloxycarbonyl-L-Glutaminylglycine) and Ac-WALQRPHYSYPD-COOH (Zedira, Germany) were dissolved in DMSO (Sigma-Aldrich, Switzerland) at 35mM and 50mM, resp., aliquoted and stored at -20°C.

### 1.13 Synthesis of Peptide FGLQRPY and FGLQRPY-GK*($N_3$)

The peptides $NH_2$-FGLQRPY-COOH and $NH_2$-FGLQRPY-GK*($N_3$)-COOH (see Figure S8 for structures) were synthesized on trityl chloride resin from Novabiochem (Merck-Millipore, Switzerland) following standard Fmoc-based solid state peptide synthesis protocols, F-moc-protected amino acids were purchased from Bachem (Switzerland) and Fmoc-Lys($N_3$)-OH was obtained from Anaspec (USA). 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) was used as coupling reagent together with Hünig's base. After cleavage from the resin with 95/2.5/2.5 (v/v/v) trifluoroacetic acid (TFA)/$H_2O$/triisopropylsilane the peptides were purified by preparative reversed phase HPLC on a Ultimate 3000 semi preparative HPLC from Dionex (USA) equipped with a ReproSil Gold 120 C18 (10µm, 30x16mm) column at 50°C with a gradient of 10-40% buffer B in buffer A over 15 minutes with a flow rate of 6 mL/min (buffer A: 0.1% TFA in acetonitrile/$H_2O$ 1:100 v/v, buffer B: pure acetonitrile).
The peptide structures were confirmed by high resolution mass spectrometry on a Bruker UltraFlex II spectrometer (Bruker, USA) and the purity was determined by reversed-phase UHPLC on a Ultimate 3000 from Dionex (USA) equipped with a ReproSil Gold 120 C18 (5µm, 150x4mm) column (Dr. Maisch, Germany) at 50°C with a gradient of 10-50% buffer B in buffer A over 20 minutes with a flow rate of 1 mL/min (buffer A: 0.1% TFA in acetonitrile/$H_2O$ 1:100 v/v, buffer B: pure acetonitrile) by monitoring UV-absorption at 214nm, 254nm and 280nm.

### 1.14 Screening for potent glutamine containing peptides

The screening experiments for antibody lysine conjugation were carried out in solution using 6U/ml of microbial transglutaminase, 1 mM glutamine containing peptide and 1mg/ml deglycosylated IgG1 (Herceptin) which was incubated for 16h at room temperature (22°C) in the corresponding buffer. After incubation, MTG was blocked with MTG-blocker (Zedira, Germany) using at least 1 mM for 30min at room temperature.

### 1.15 LC-MS analysis of protein conjugates

Conjugated protein samples were analysed as follows. Liquid chromatography (LC) was performed with an Aeris WIDEPORE XB-C18 column (3.6µm, 100mm x 2.1mm; Phenomenex, USA) at a column temperature of 80°C, applying the LC-gradient shown in Table S1. Conjugated antibody samples were reduced by the addition of 50mM DTT (Sigma-Aldrich, Switzerland) and incubation at 37°C for at least 20min prior to LC-MS ESI analysis (Waters LCT Premier Instrument United States). Since LC-MS analysis of conjugated avidin proved difficult deglycosylation was necessary. The solution was incubated for 5min at 95°C in 0.1 v/v-% *Rapi*Gest SF (Waters, USA) and allowed to cool down for 5min followed by the addition of PNGase F (1U) and incubation for 4h at 50°C. For the LC-MS analysis of the MTG-4FB conjugate a different gradient was used (described in Table S2).

**Table S1.** HPLC gradient. Percentage of different solvents over time.

| Time [min] | Water [%] | Acetonitrile [%] | 2-Propanol [%] | Curve |
|---|---|---|---|---|
| 0 | 75 | 20 | 5 | immediate |
| 3 | 73 | 22 | 5 | linear |
| 18 | 40 | 55 | 5 | linear |
| 20 | 10 | 85 | 5 | linear |

**Table S2.** HPLC gradient. Percentage of different solvents over time.

| Time [min] | Water [%] | Acetonitrile [%] | 2-Propanol [%] | Curve |
|---|---|---|---|---|
| 0 | 90 | 10 | 5 | immediate |
| 3 | 70 | 25 | 5 | linear |
| 15 | 58 | 37 | 5 | linear |
| 20 | 5 | 90 | 5 | immediate |

The obtained spectra were analysed using MassLynx V4.1 and deconvoluted using the MaxEnt1 algorithm. The conjugation ratio $R_c$ was calculated as follows:

$$R_c = I_c / (I_c + I_{nc}) \times 100\%$$

With $I_c$ corresponding to the intensity of the conjugated and $I_{nc}$ of the unconjugated protein, resp.

### 1.16 SDS-PAGE and flow-cytometric analysis of conjugated antibodies

10µg of the various conjugated antibodies was incubated in Laemmli buffer at 95°C for 5min. A 12.5% polyacrylamide-gel, 0.75mm was applied for 1.5h at 120V. The gel (coomassie stained and unstained) was imaged with an Amersham Imager 600 (GE Healthcare, Switzerland).
CD30-expressing Karpas299 cells (ECACC 06072604, UK) were cultivated in RPMI1640 containing 10%FCS, 2mM L-Glutamine and 1%PSF (all from Bioconcept, Switzerland) and kept at a density between 0.5 – 2*$10^6$ cells/ml in 5% $CO_2$, 37°C. For flow-cytometric analysis, cells were pelleted at 1000xg for 10min, washed with PBS and again centrifuged. The cell pellet was re-suspended in PBS containing 1%BSA (1%BSA-PBS). 100µl of the cell suspension containing $10^6$ cells were then transferred into the wells of a 96-well plate. For the following steps, the well plate was always stored on ice. First, 5µg of either unlabelled cAC10 IgG1 (N297S) or isotype humanized IgG1 (LifeTechnologies, Switzerland) were applied to the control wells and incubated for 15min, resuspended and again incubated for 15min with continuous, gentle shaking. Afterwards the cells were washed twice with 1%BSA-PBS buffer and resuspended in 100µL of the same buffer. Then, 100µl of the cell suspension containing $10^6$ cells were transferred to the other wells and 5µg of conjugated antibodies were applied and preceded as above. For the control wells a secondary goat anti-human IgG-FITC (1:75 dilution, Santa Cruz Biotechnology, USA) was applied. Flow-cytometry was done with Guava easyCyte Flow Cytometer (Merck-Millipore, Switzerland) and data were analysed with FlowJo software (TreeStar Inc, USA).

## 2. Results and Discussion

### 2.1 Linker quantification of MTG-4FB & *de*-PG2$_{500}$-HyNic and formation of the *de*-PG2$_{500}$-MTG conjugate

Different linker excesses of S-4FB to MTG were investigated with LC-MS and spectrophotometrically by UV-VIS, finding both methods to correlate well (Figure S4a). We chose to use 0.08eq molar equivalent of S-HyNic versus the repeating units of the polymer and 1.5eq 4FB-linker excess versus MTG to ensure MTG integrity which resulted in >90% pure, single-linked MTG (Figure S4b). Higher linked polymer-enzyme conjugates could not be purified, probably due to precipitation of over-crosslinked MTG-polymer conjugates. The MTG-4FB was mixed with dendronized polymer *de*-PG2$_{500}$-HyNic using 75µM 4FB-linker and 50µM HyNic-concentration, resp. in MES buffer. Upon mixing and incubation of the 4FB-MTG and HyNic-polymer components for 16h a characteristic absorption increase and peak formation at 354nm in UV-VIS spectroscopy could be detected confirming the successful bis-aryl-hydrazone (BAH) bond formation of the polymer-enzyme conjugate (Figure S4c).[9]

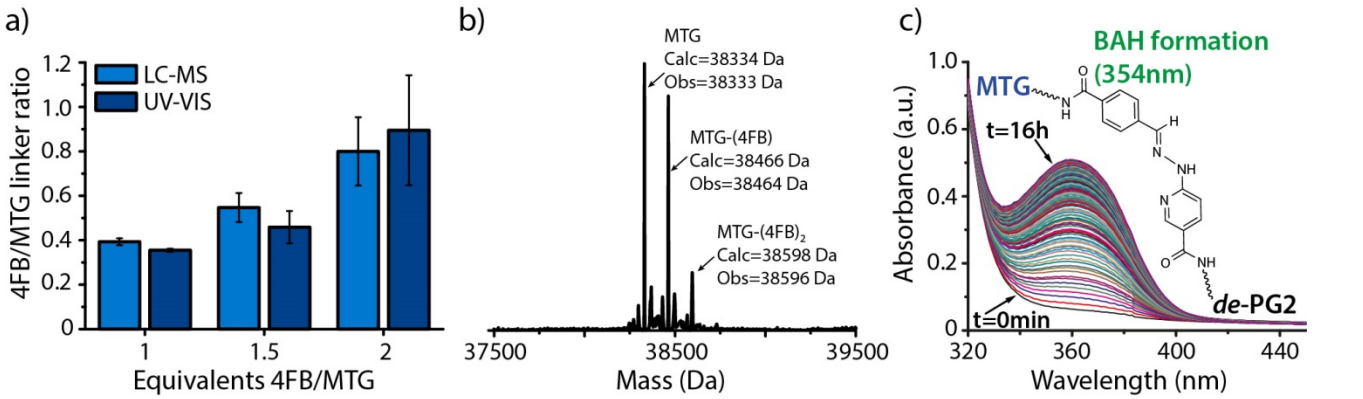



**Figure S4.** (a) Quantitative analysis of 4FB linker-to-MTG ratio investigated with LC-MS and UV-VIS showed good correlation. (b) Deconvoluted LC-MS spectra of 1.5eq of 4FB to MTG showing unconjugated MTG and MTG carrying one or two 4FB-linkers. Since unconjugated MTG is removed during purification >90% single linked, pure MTG is obtained. (c) Absorption increase at 354nm (d=2mm) followed by UV-VIS spectrometry indicated the successful bis-aryl-hydrazone (BAH)-bond formation and MTG immobilization on the denpol-polymer (*de*-PG2$_{500}$), resp.

### 2.2 Estimating the amount of adsorbed polymer-enzyme conjugate on glass microbeads

The UV-VIS quantifiable bis-aryl-hydrazone bond measured at 354nm (29'000 $M^{-1}cm^{-1}$)[4a] allowed us to estimate the amount of MTG immobilized on the beads. Since the MTG is mostly single cross-linked to the polymer, we estimated an adsorbed mass of ~400ng/cm$^2$. These values correspond to previously published results using proteinase K[10] or horseradish-peroxidase[11] immobilized on denpol-polymers and other enzymes, covalently immobilized on silica-polymer-surfaces.[12]

### 2.3 Conjugation of different substrates to various protein formats with immobilized MTG

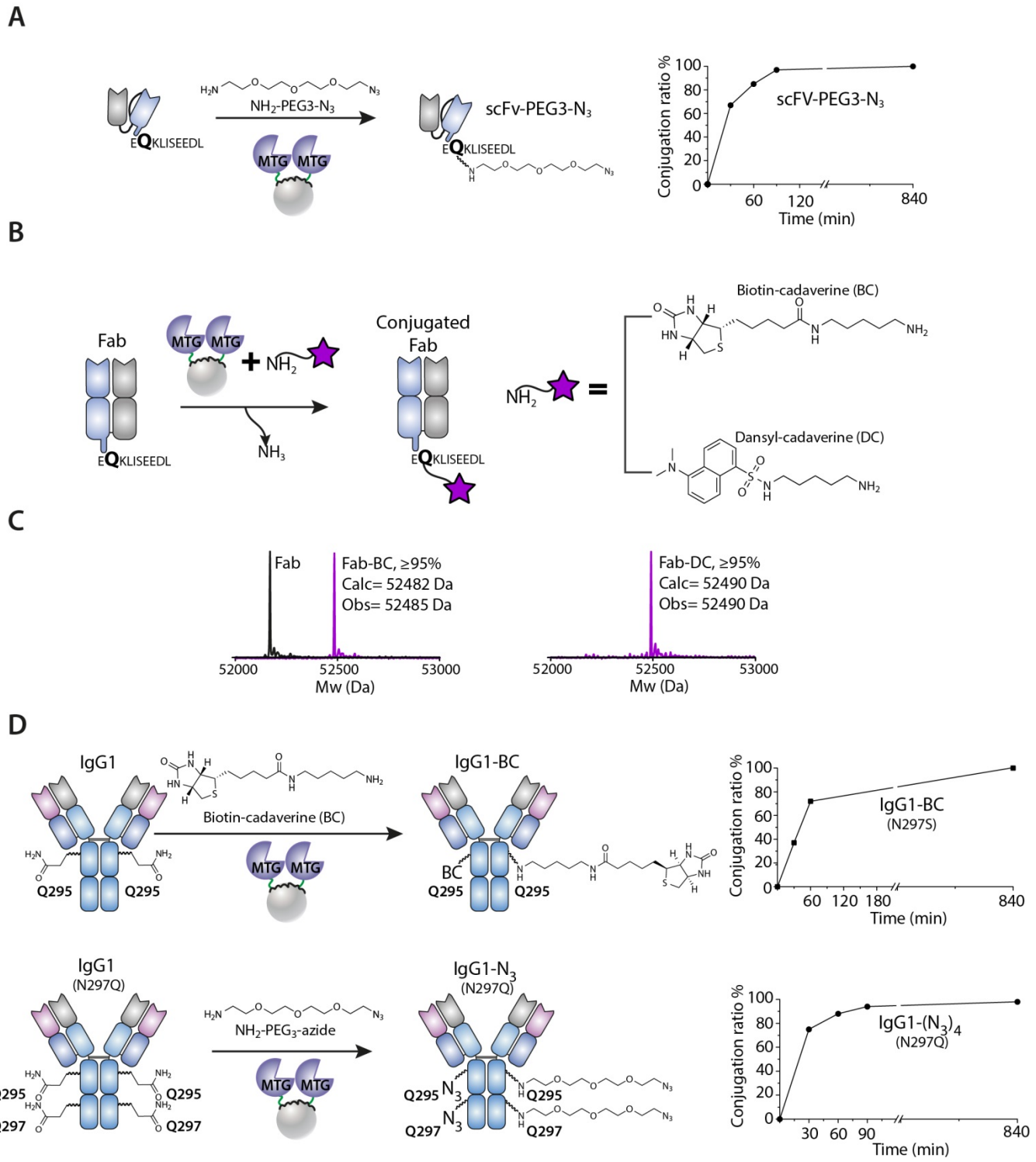



**Figure S5.** (A) Conjugation of $NH_2$-PEG3-$N_3$ to scFv using prolonged incubation time. (B, C) Conjugation of c-myc-tagged Fab-fragment was successfully achieved (≥95%) with amine-donor substrates biotin-cadaverine and the fluorescent dansylcadaverine after 30min of incubation time (1:80 and 1:8 molar substrate ratios, resp.). (D) Conjugation of biotin-cadaverine to aglycosylated antibody N297S and of $NH_2$-PEG3-$N_3$ to aglycosylated antibody N297Q overtime with immobilized MTG. Molar substrate ratio 1:80 (N297S) and 1:80 per conjugation site (N297Q).

### 2.4 Estimating polymer-enzyme desorption by slot blot

Both positive controls, polymer-MTG conjugate (700ng) as well as native MTG (20ng) (Figure S6, i and ii, resp.) showed a strong signal as expected. To thoroughly test our platform, we applied a sample mixture (200µL) incubated for 14h with microbead immobilized MTG (Figure S6, iii). Assuming the 20ng as the lower limit of detection, we can estimate that <0.1% of the immobilized MTG (~150µg, 1mL sample volume) has desorbed from the microbead surface. This indicates that MTG-attachment to the polymer and polymer-enzyme conjugate adsorption on the microbeads are solid with no free or leaking enzyme detectable.

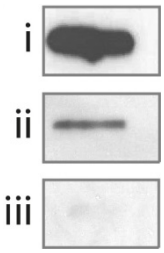



**Figure S6.** Slot blot assay using antibody against MTG revealed no enzyme leaking from the microreactor. Lane i: Polymer-MTG conjugate (700ng), ii: MTG (20ng), iii: conjugated protein sample incubated for 14h on immobilized MTG.

### 2.5 Structures of used compounds: Tetrazine-PEG4-DOTAGA, DBCO-PEG4-5/6-FAM for click-chemistry and ZQG-cad-TAMRA

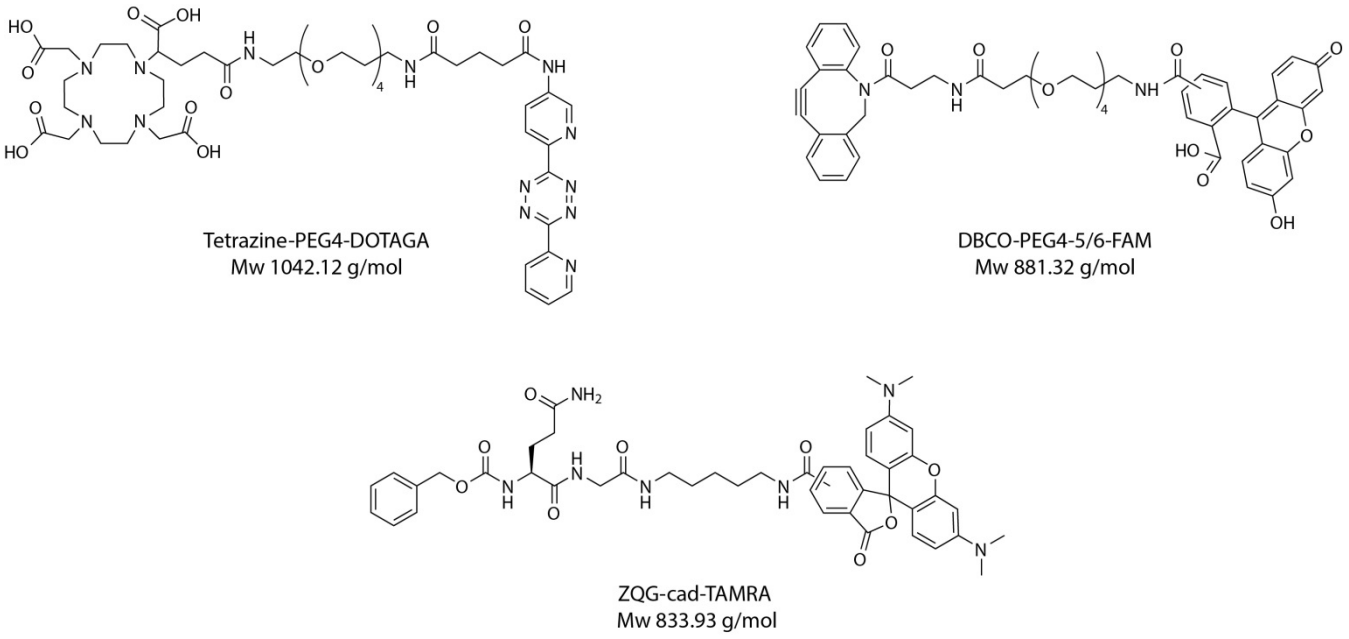
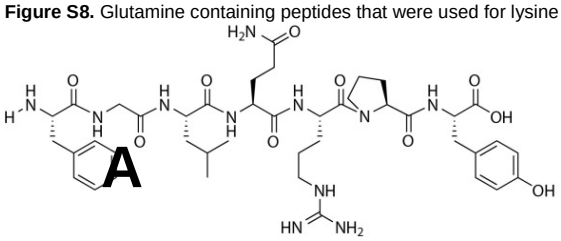



**Figure S7.** Linkers used for SPAAC (strain-promoted alkyne-azide cycloaddition) and iEEDA (inverse electron demand Diels-Alder) click-chemistry that were used throughout this study as well as substrates for lysine conjugation with immobilized MTG.

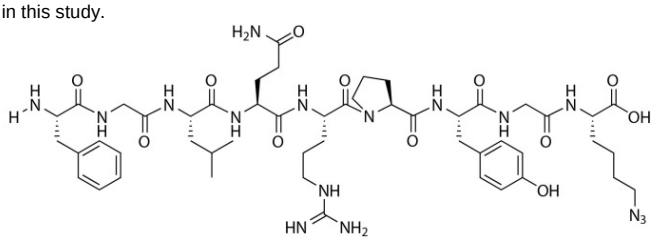
### 2.6 Results of synthesis of peptides FGLQRPY and FGLQRPY-GK*($N_3$)

Both products were analyzed by HPLC and HR-MS and their identity was confirmed (Figure S9 and S10).

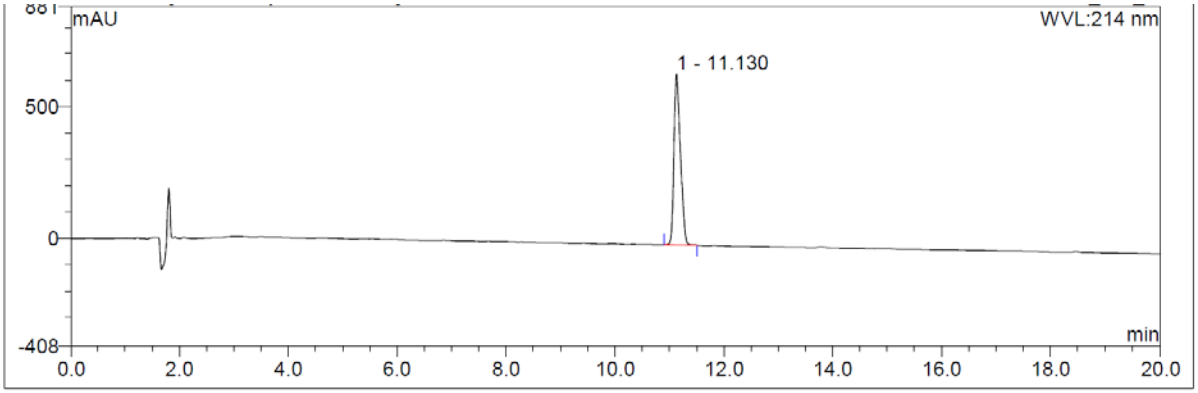
**Figure S8.** Glutamine containing peptides that were used for lysine conjugation in this study.



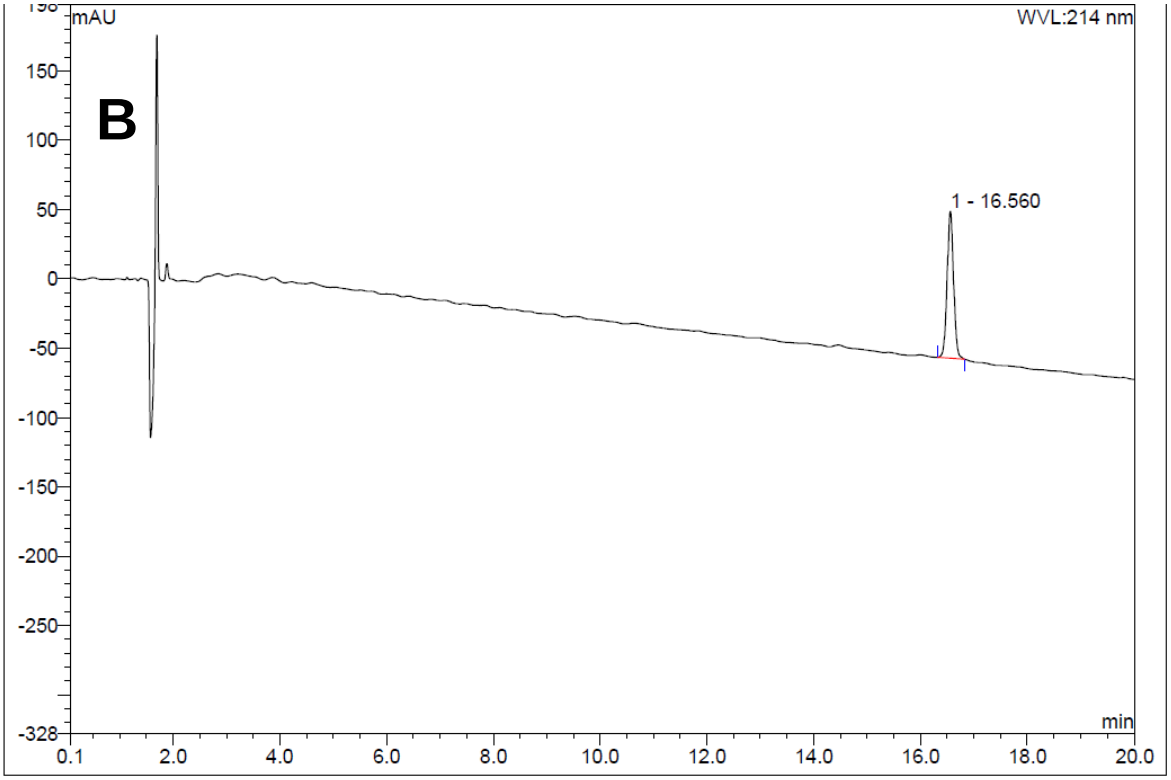
FGL**Q**RPY
Mw 880.02 g/mol



FGL**Q**RPY-GK*($N_3$)
Mw 1091.24 g/mol

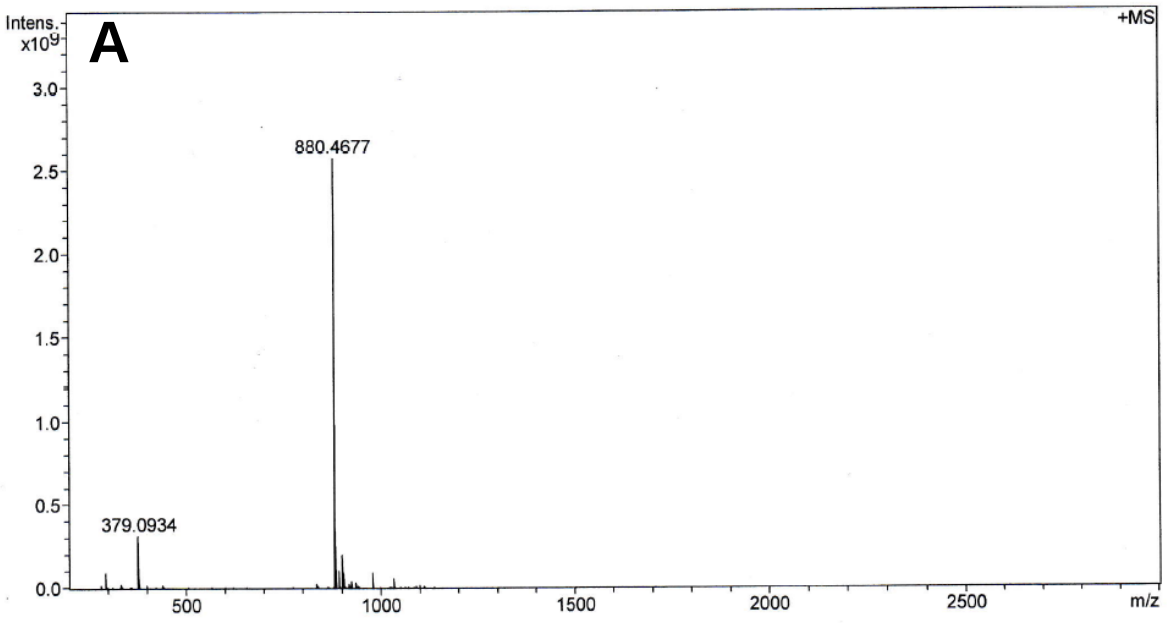



| No. | Ret.Time min | Peak Name | Height mAU | Area mAU*min | Rel.Area % | Amount | Type |
|---|---|---|---|---|---|---|---|
| 1 | 11.13 | n.a. | 648.533 | 88.519 | 100.00 | n.a. | BMB* |
| **Total:** | | | 648.533 | 88.519 | 100.00 | 0.000 | |




| No. | Ret.Time min | Peak Name | Height mAU | Area mAU*min | Rel.Area % | Amount | Type |
|---|---|---|---|---|---|---|---|
| 1 | 16.56 | n.a. | 105.9017 | 14.8842 | 100 | n.a. | BMB* |

**Figure S9.** (A) HPLC analysis of peptide FGLQRPY and (B) of peptide FGLQRPY-GK*($N_3$) showed in both cases 100% pure products.



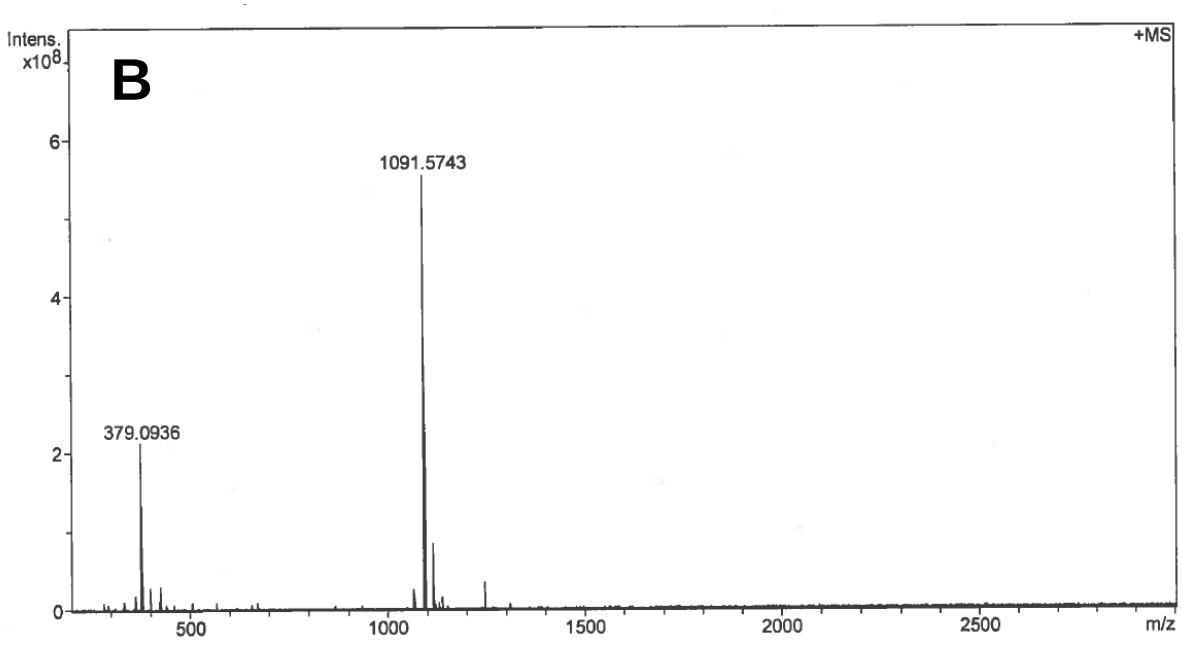
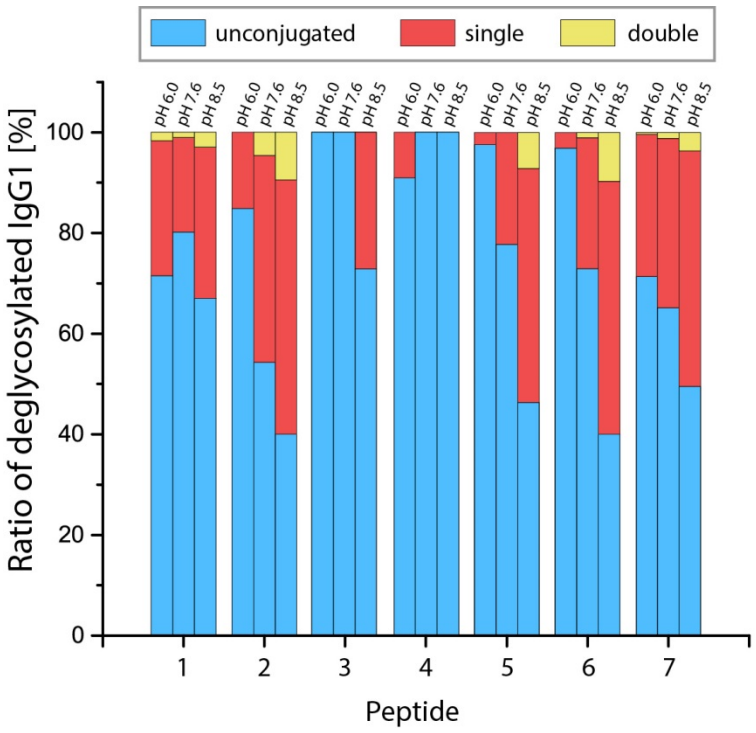



**Figure S10.** (A) HRMS of peptide FGLQRPY, *m/z* calculated $C_{42}H_{62}N_{11}O_{10}^{+}$ [M+H]$^{+}$: 880.4676; found: 880.4677. (B) HRMS *m/z* calculated for $C_{50}H_{75}N_{16}O_{12}^{+}$ [M+H]$^{+}$: 1091.5745; found: 1091.5743. Both analysis confirmed peptide identities.

### 2.7 Screening for potent glutamine containing peptides




| Peptide | Sequence |
|---|---|
| 1 | ZQG |
| 2 | $H_2N$-FGL**Q**RPY-COOH |
| 3 | Ac-WAL**Q**RPHYSYPD-COOH |
| 4 | $H_2N$-TV**Q**QEL-COOH |
| 5 | $H_2N$-WAL**Q**RPH-COOH |
| 6 | $H_2N$-SLL**Q**G-COOH |
| 7 | $H_2N$-FL**Q**RP-COOH |

**Figure S11.** Screening of glutamine containing peptides (sequence given in table on the right) for antibody lysine conjugation to deglycosylated IgG1 (Herceptin) at different pH conditions in solution. Although pH 8.5 showed increased conjugation ratios this was not applicable to solid-phase immobilized MTG as at pH >8.2 the primary amines of the *de*-$PG2_{500}$ polymer will get deprotonated leading to desorption of the polymer from the glass microbeads. Since peptide 2 showed very high conjugation ratio at pH 7.6 further experiments were performed using this peptide. Peptide 1, ZQG, in comparison showed a lower conjugation ratio. Interestingly, peptide 2 was previously reported not to be very active[13] while peptide 3, corresponding to the "Hitomi peptide M42", was reported to be a very reactive MTG substrate[14] which we were not able to confirm. A shorter peptide sequence (Peptide 5) seems to be more reactive similar to previous findings where it was used

as a tag in proteins.[15] The reactions were performed at a peptide concentration of 1mM, an incubation time of 16h, an antibody concentration of 1mg/ml (deglycosylated Herceptin) and 6U/ml MTG at room temperature (22°C).

## 2.8 Conjugation of glutamine containing peptides FGLQRPY and FGLQRPY-azide to aglycosylated IgG1 with immobilized MTG

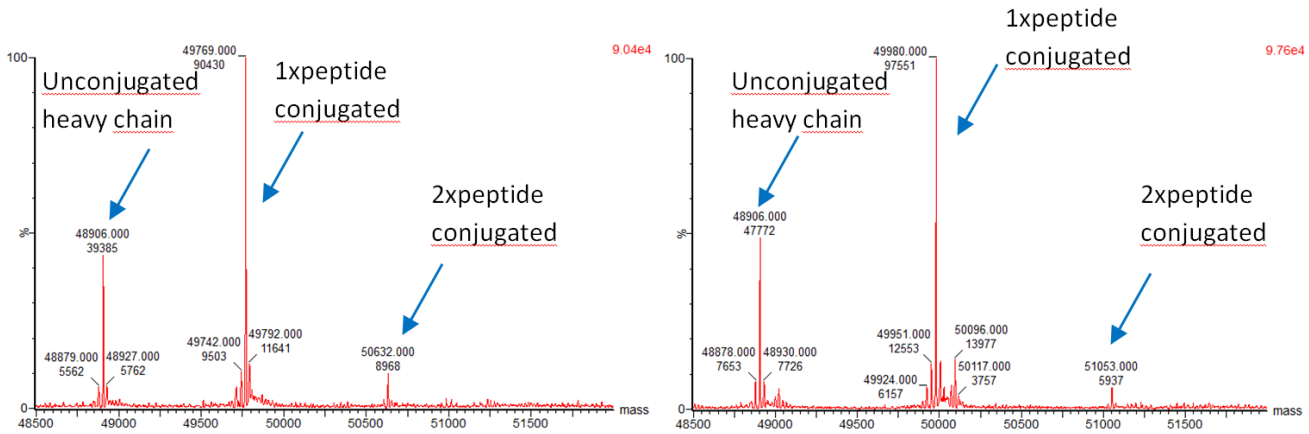



**Figure S12.** Left: Conjugation of FGLQRPY to IgG1 N297S with immobilized MTG (78%). Right: conjugation of FGLQRPY-GK*($N_3$) to IgG1 N297S with immobilized MTG (72%). Both gave comparable conjugation efficiencies showing that the $N_3$-group did not alter the conjugation behavior of MTG.

## 2.9 Structural analysis of lysine conjugated IgG1 antibody

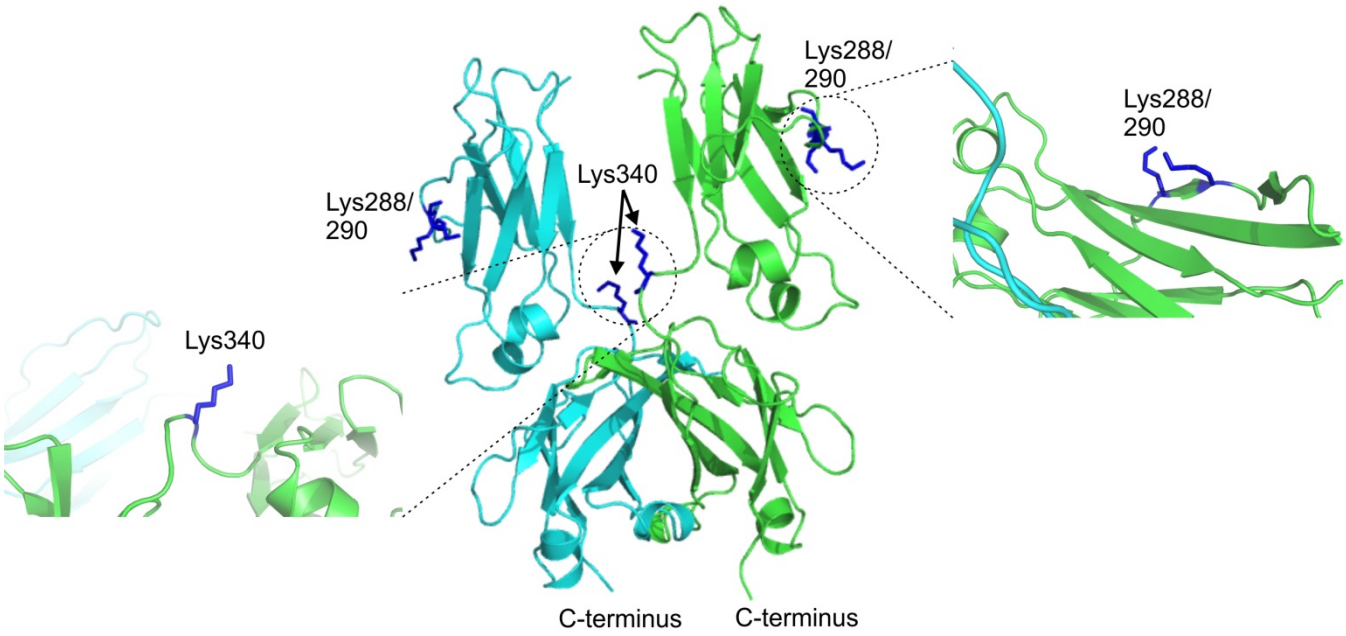



**Figure S13.** Antibody IgG1 Fc-region (Herceptin) with lysine residues prone to MTG processing indicated in blue (Lys288/290 and Lys 340). The lysines, modified by MTG, could be identified with peptide mapping to be Lys288/290 and Lys340. We were not able to discriminate by peptide mapping which lysine residue is preferentially conjugated by MTG and whether lysine 288 or lysine 290 has been modified. All lysines are located on the CH2 domain on flexible loops, a type of structure reported to be preferentially targeted by MTG.[7a, 16] Given the observation of Fiebig et al.,[16b] who reported residues that are located on flexible loops and embedded by small/polar/hydrophobic and uncharged residues to be preferentially targeted, we speculate Lys340 (Sequence: …ISKA**K**GQPR…) to be the major target of MTG. In contrast, Lys288/290 seem to be located on a small β-sheet and are surrounded by charged residues (Sequence: …EVHNA**KT**K**K**PREEQ…); both factors are reported to result in decreased MTG targeting and thus to minor conjugation.[16b] PDB entry used: 3D6G.[17]

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## Author Contributions

P.R.S.: Designed and performed research, evaluated data, wrote the manuscript, degree: lead

C.A.A., J.E.W., D.R.H., O.K.: Performed research, evaluated data, degree: supporting

D.M., A.R.: Performed research, degree: supporting

A.K., A.B., M.B., P.W.: Evaluated data, degree: supporting

R.S.: Designed research, evaluated data, wrote the manuscript, degree: lead