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Peptide-Directed Attachment of Hydroxylamines to Specific Lysines of IgG Antibodies for Bioconjugations with Acylboronates

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Abstract

The role of monoclonal antibodies as vehicles to deliver payloads has evolved as a powerful tool in cancer therapy in recent years. The clinical development of therapeutic antibody-conjugates with precise payloads holds great promise for targeted therapeutic interventions. The use of affinity-peptide mediated functionalization of native off-the-shelf antibodies offers an effective approach to selectively modify IgG antibodies with a drug antibody ratio (DAR) of 2. Here, we report the traceless, peptide-directed attachment of two hydroxylamines to native IgGs followed by chemoselective potassium acyltrifluoroborate (KAT) ligation with quinolinium acyltrifluoroborates (QATs), which provide enhanced ligation rates with hydroxylamines under physiological conditions. By applying KAT ligation to the modified antibodies, conjugation of small molecules, proteins, and oligonucleotides to off-the-shelf IgGs proceeds efficiently, in good yields, and with simultaneous cleavage of the affinity peptide-directing moiety.

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Introduction

The site-specific modification of monoclonal antibodies excites significant interest owing to the value of antibody conjugates in targeted therapy, particularly in cancer treatment.[1-4] While first generation antibody-conjugates primarily relied on stochastic conjugations to lysine and cysteine residues, the heterogeneity of the resulting constructs resulted in poor pharmacokinetics.[5] A second generation offered enhanced selectivity, stability and clinical efficacy, yet product heterogeneity continued to be a limiting factor in their applications.[5-6] The need to further reduce heterogeneity in order to improve the therapeutic index of antibody-conjugates led to the development of site-specific conjugation strategies, which now dominate the field. Although these largely bio-engineered and chemoenzymatic strategies address the challenge of controlling the sites of modification and drug-antibody ratio (DAR),[7-18] the attachment of larger payloads including proteins and nucleic acids remains a challenge.

A particularly valuable chemical approach for site-specific modifications of off-the-shelf antibodies relies on Fc-affinity tags for the directed modification of specific lysine residues in the heavy chain. In 2019, Ito and co-workers identified a 17-residue affinity-tag (IgG-BP) that binds to the Fc site of human IgG1, IgG2, and IgG4 between the CH2 and CH3 domain interface. This affinity tag shows a similar binding mode as the Fc-III peptide discovered by Wells and co-workers (PDB: 1DN2 and 6IQG).[19-20] By introducing an NHS ester into a mutated lysine (R8K), the affinity tag was successfully linked covalently at lysine 248 on trastuzumab. Although the conjugated Fc affinity tags could not be removed in the early reports, this method established the potential for the site-specific modification of IgGs.[21] Yamada and Mendelsohn used a similar 17 amino acid residue affinity tag (RGNCAYHRGQLVWCTYH), discovered through biopanning against human IgG1 on a T7 phage display, that showed a high affinity to human IgG Fc (K_d = 9 nM). The incorporation of a disulfide bond into the affinity tag and a subsequent reduction with TCEP led to the advent of AJICAP technology, in which the affinity tag is cleaved and the IgGs can be attached to thiol-reactive payloads leading to ADCs with DAR2 (Figure 1A). To obliviate the need for TCEP throughout the conjugation step, the Ajinomoto group enhanced their conjugation approach by linking a thioester to the affinity tag. The thioester can be subsequently cleaved with hydroxylamine hydrochloride, leading to free thiols in a streamlined one pot fashion, referred to as AJICAP second generation.[22]
They further showed that their conjugation method did not affect the antigen binding in the case of trastuzumab.[23] Additional approaches using engineered peptide tags have enabled

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recent development in the traceless modifications of off-the-shelf antibodies allowing for the convenient synthesis of homogeneous ADCs without compromising their structural integrity or requiring de novo expression or engineering.[24-26]

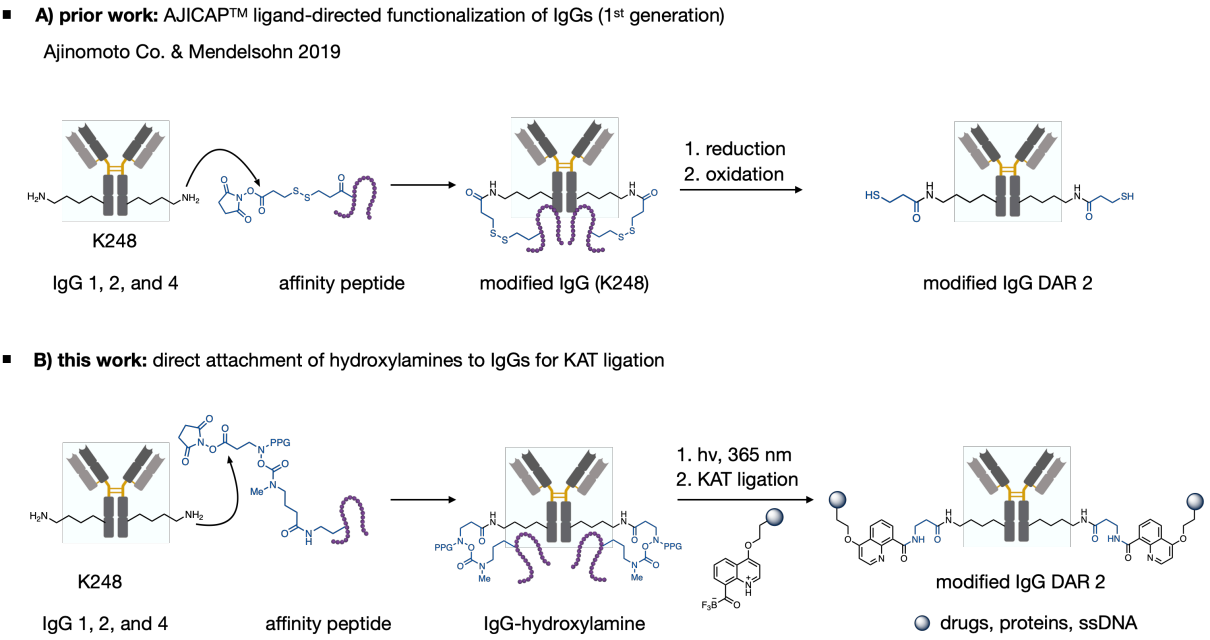

Figure 1. **A.** Affinity peptide mediated functionalization of IgGs (AJICAP). The peptide sequence Ac-RGNCAYHKGQLVWCTYH-NH_2 was modified with Lomant's reagent to introduce a 3-mercaptopropionate group at lysine residues K248 (EU numbering) at the heavy chain of IgGs after reduction with tris(2-carboxyethyl)phosphine (TCEP) followed by a reoxidation step using dehydroascorbic acid. **B.** Affinity peptide directed introduction of a photocaged hydroxylamine to IgG antibodies for KAT ligation at physiological conditions. The ligation leads to a concomitant loss of the peptide tag, resulting in antibody-conjugates with DAR 2.

In this report, we document the direct attachment of hydroxylamines to specific lysine residues in IgGs antibodies using a variant of the lysine-modified affinity peptide Ac-RGNCAYH**K**GQLVWCTY-NH_2 (**1**) developed by Yamada and Mendelsohn.[23] The resulting stable, photoprotected peptide-antibody conjugates undergo traceless amide formation with potassium acyltrifluoroborates (KATs) and their quinolinium counterparts, which participate in rapid ligations at pH 7 (k = 4.5 M^{-1} s^{-1}, Figure 2A).[27] The affinity peptide, which is linked to the antibody by an amide bond and an *O*-carbamoyl hydroxylamine, is removed concomitantly during the KAT ligation. This facile, two-step process operates directly on commercial IgG antibodies under mild, aqueous conditions to give DAR 2 modified antibodies and enables the attachment of small molecules, peptides, proteins, and ssDNA.

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Results and Discussion

At the outset of our studies, it was unclear if a modified affinity peptide with a linker containing a hydroxylamine would be suitable for site-specific lysine labeling (Figure 2B). We began our efforts by preparing the known affinity peptide **1** by standard Fmoc-SPPS protocols followed by formation of the cyclic disulfide bond with hydrogen peroxide.[23] Photoprotected hydroxylamine **7** was prepared in five steps and attached via its carboxylic acid to the lysine of **1,** using HATU as coupling reagent. *tert*-Butyl deprotection and NHS ester formation led to the desired construct **10** (Figure 2C), which was purified by RP-HPLC and stored as a stock solution in DMSO. To ensure stability and shelf-life through the synthesis and HPLC purification of the hydroxylamine-containing affinity peptide **10**, we elected to protect it as an *ortho*-nitrobenzyl derived carbamate, which can be readily cleaved in minutes upon irradiation at 300 – 360 nm.[28-29] Based on prior work from Yamada and Mendelsohn, the NHS ester was expected to react with lysine K248 (EU numbering) to form an amide bond on both sides of the heavy chain, leading to an antibody : peptide ratio = 2.[30]

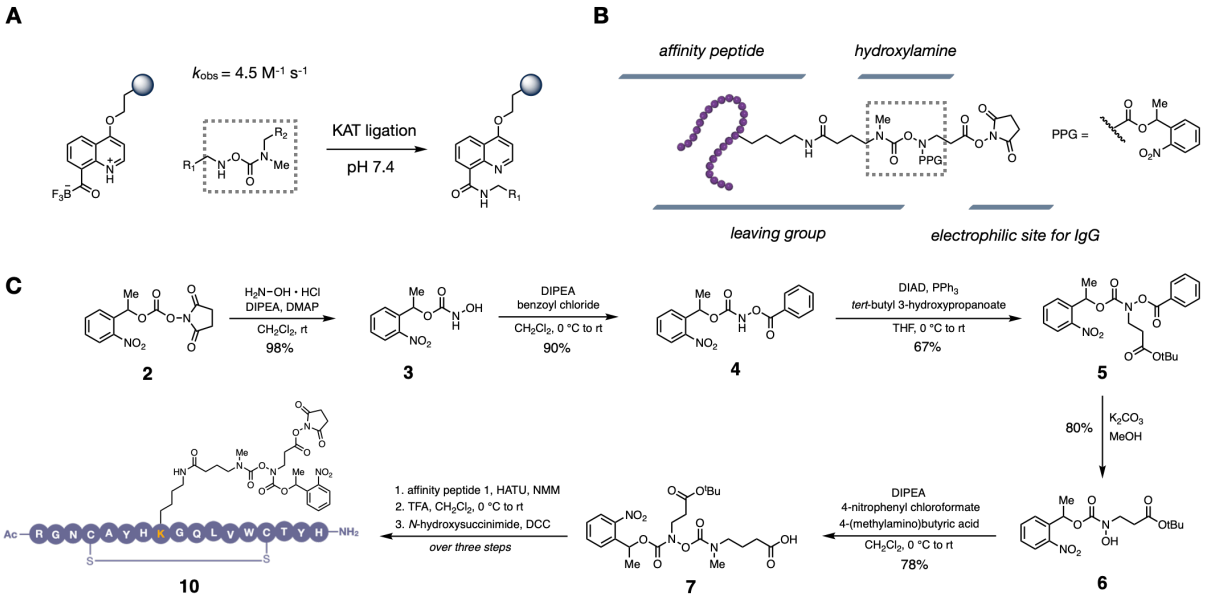

Figure 2. A. Rapid KAT ligation between hydroxylamines and quinolinium acyltrifluoroborates at physiological pH. **B.** Design of modified affinity peptide **10** for IgG1, IgG2, and IgG4 with built-in hydroxylamine. The affinity peptide is incorporated into the leaving group of the hydroxylamine, resulting in its intended loss after KAT ligation with a quinolinium acyltrifluoroborate. **C.** Synthesis of **10**: detailed protocols included in *Supporting Information*.

We tested the ability of hydroxylamine-containing affinity peptide **10** to selectively modify the Fc-region on the IgG1 antibody trastuzumab (**29**). For these initial studies, trastuzumab (**29**) was deglycosylated (indicated with an asterisk *) by PNGase F, and treated with 10 equiv of

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10 (100 µM stock solution in DMSO) in sodium acetate buffer at pH 5.5 (Figure 3A). A slightly acidic pH range was chosen to prevent NHS ester hydrolysis and to suppress unspecific lysine modification. After 30 min at room temperature, mass analysis of the intact antibody revealed clean conjugation of **10** to trastuzumab, leading to **30*** (**29*** + 2 x 2480 Da) with a precise ratio of two hydroxylamines per antibody (DAR 2). Interestingly, the excess of **10** did not lead to unspecific conjugation and could be fully removed from the mixture by spin filtration. To demonstrate the applicability of our approach, **10** was further tested on pembrolizumab and bevacizumab (IgG4 and IgG1, respectively). In all cases, a total mass difference of 4960 Da was observed, indicating full conversion of the native antibodies to the corresponding IgG-hydroxylamines with a DAR of 2 in under 30 minutes (Figure 3B).

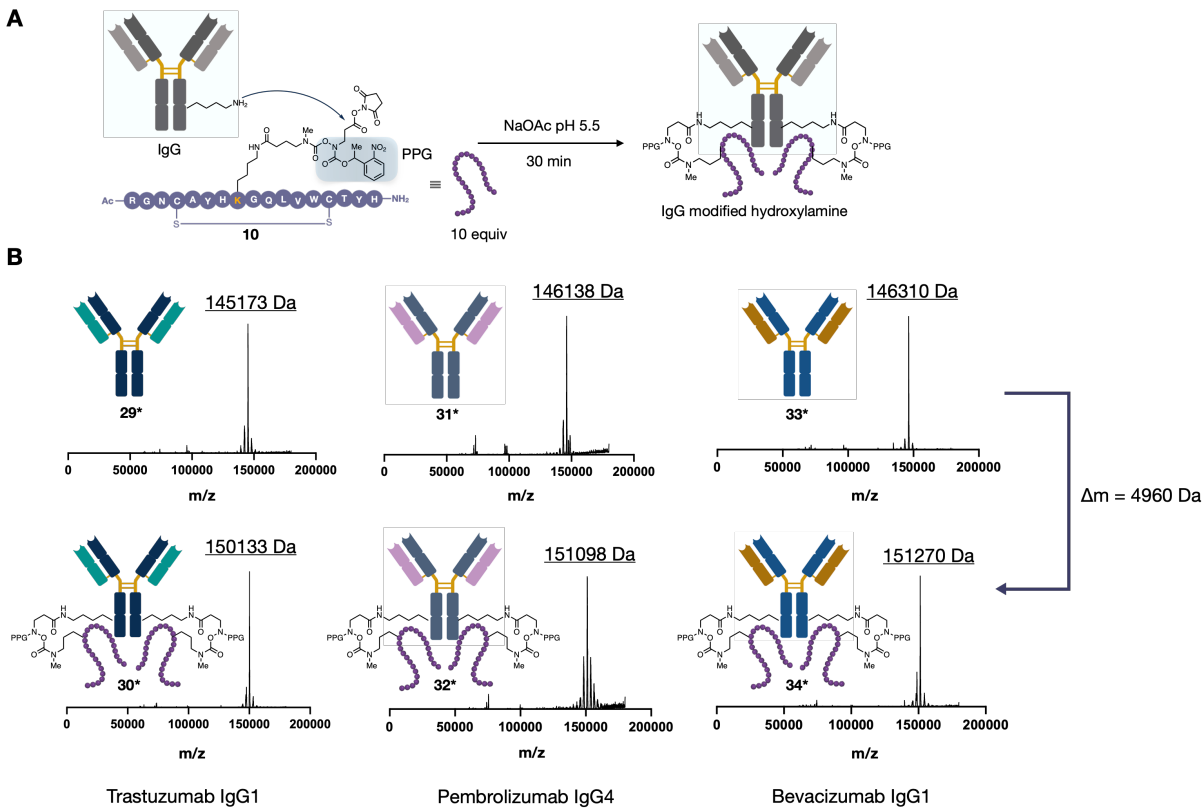

Figure 3. A. Reaction of commercial IgGs and affinity peptide **10** in sodium acetate buffer at pH 5.5. The concentration of native antibodies was set to 1 µM and 10 equiv of **10** in DMSO were added. The mixture was agitated for 30 min at rt and subjected to mass analysis. **B.** Mass spectra of intact trastuzumab, pembrolizumab, and bevacizumab (**29***, **31***, **33***) and their respective hydroxylamine-modified analogues (**30***, **32***, **34***). The asterisk refers to antibodies deglycosylated with PNGase F; detailed protocol in *Supporting Information*.

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To test the proposed concomitant amide ligation / affinity peptide removal, the trastuzumab-hydroxylamine conjugate was, after removal of excess **10** and buffer exchange to 50 mM potassium phosphate buffer (pH 7.4), irradiated at 365 nm for 10 min to remove the photoprotecting group and release the deprotected trastuzumab-hydroxylamine **35***. Unprotected **35*** was stable for several days in the reaction buffer at room temperature. Treatment of **35*** (1 µM) with QAT **22** (1 mM, 20 equiv) for 15 min at room temperature resulted in full conversion of both hydroxylamines, as confirmed by LCMS (Figure 4A). To facilitate mass analysis by QTOF MS, the reaction mixture was reduced with tris(2-carboxyethyl)phosphine (TCEP) prior to mass analysis, promoting the disassembly into heavy chain (HC) and light chain (LC). Analysis of the heavy chain indicated full conversion from **35*** (51437 Da) to **36*** (49628 Da), with simultaneous cleavage of the affinity tag (Figure 4B). In-gel tryptic digestion of the heavy chain (**36***) followed by tandem MS/MS analysis showed a selective modification of either lysine residue K246 or K248 (EU numbering), in agreement with the findings of Yamada and co-workers on similar systems, who showed that the conjugation proceeds exclusively at K248.[23, 30] The mass difference of fragment *y6* and *y12*$^{++}$ (Figure 4C) corresponds to PPKPK^{248}D with one modified lysine using QAT **22** for the modification (details in *Supporting Information*).

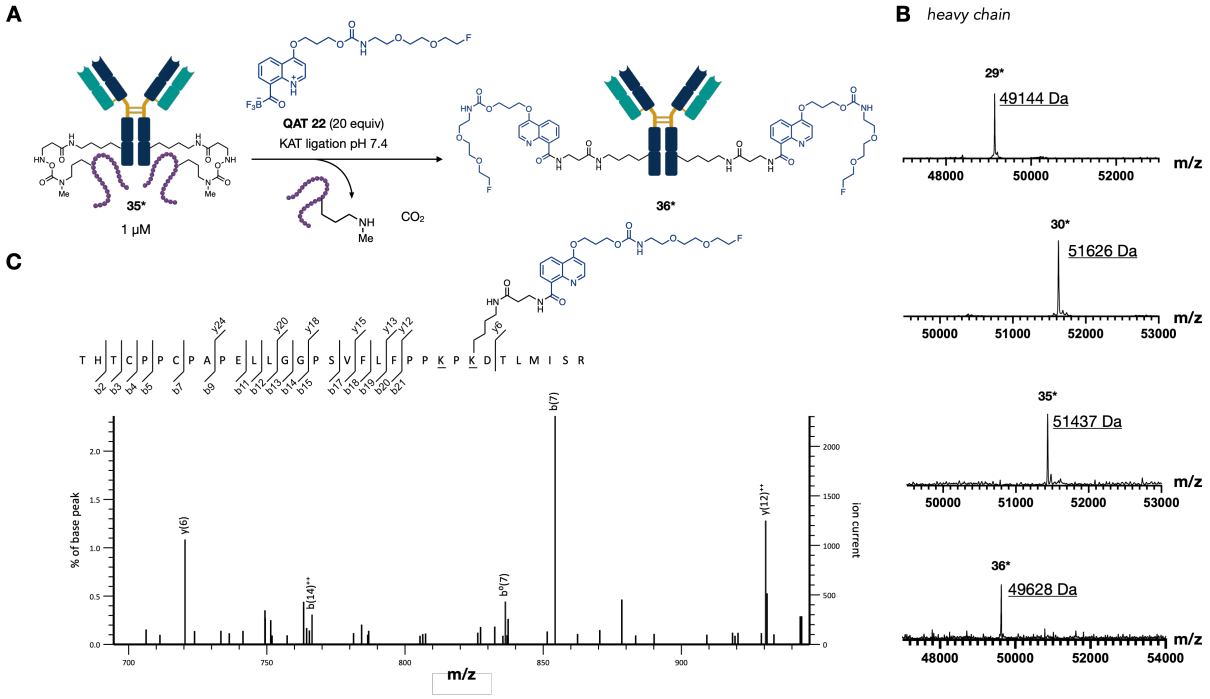

Figure 4. A. KAT ligation of **35*** (1 µM) with 20 equiv of **22** under aqueous conditions at pH 7.4 . The reaction was agitated for 15 min at rt. **B.** Heavy chain mass spectra of **29*** (49144 Da), **30*** (51626 Da), **35*** (51437 Da), and **36*** (49628 Da) after reduction with TCEP, indicating full conversion of KAT ligation. **C.** Tandem MS/MS spectrum of the heavy chain of **36*** shows a selective modification of either K246 or K248 (EU numbering). Parent ion represents THTCPPCPAPELLGGPSVFLFPPK^{246}PK^{248}DTLMISR (1035.2660 m/z, 4+ including 2

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carbamidomethylation and one modified lysine). The mass difference of fragment *y6* (720.4072 m/z) and fragment *y12*$^{++}$ (920.4902 m/z) corresponds to PPK^{246}PK^{248}D with one modified lysine.

To showcase the applicability of our method, we synthesized QATs bearing functional handles, including QAT-azide **14** and QAT-maleimide **16**. These molecules were readily prepared from **11** through S_NAr reactions (*Supporting Information*). We attached commercial mertansine (DM1) to QAT-maleimide **16** (Figure 5, entry 3), to form the analogous construct of the FDA approved ADC trastuzumab-emtansine (Kadcyla® or T-DM1), used for the treatment of HER2-positive metastatic breast cancer.[31-33] For these ligations, 20 of equiv QAT were added to glycosylated trastuzumab-hydroxylamine **35** in pH 7.4 potassium phosphate buffer and stirred for 15 min at room temperature. The solutions were reduced by TCEP and subjected to mass analysis to observe the modification of the heavy chain. In all examples, we observed full conversion by KAT ligation to the trastuzumab conjugates **37**, **38**, and **39** (Figure 5). In the case of the trastuzumab-maleimide conjugate (**38**), the heavy-chain masses observed (51222 Da and 51384 Da) correspond the maleimide-TCEP adduct as TCEP has been reported to form phosphorus-based 4-ylenes with maleimides irreversibly.[34]

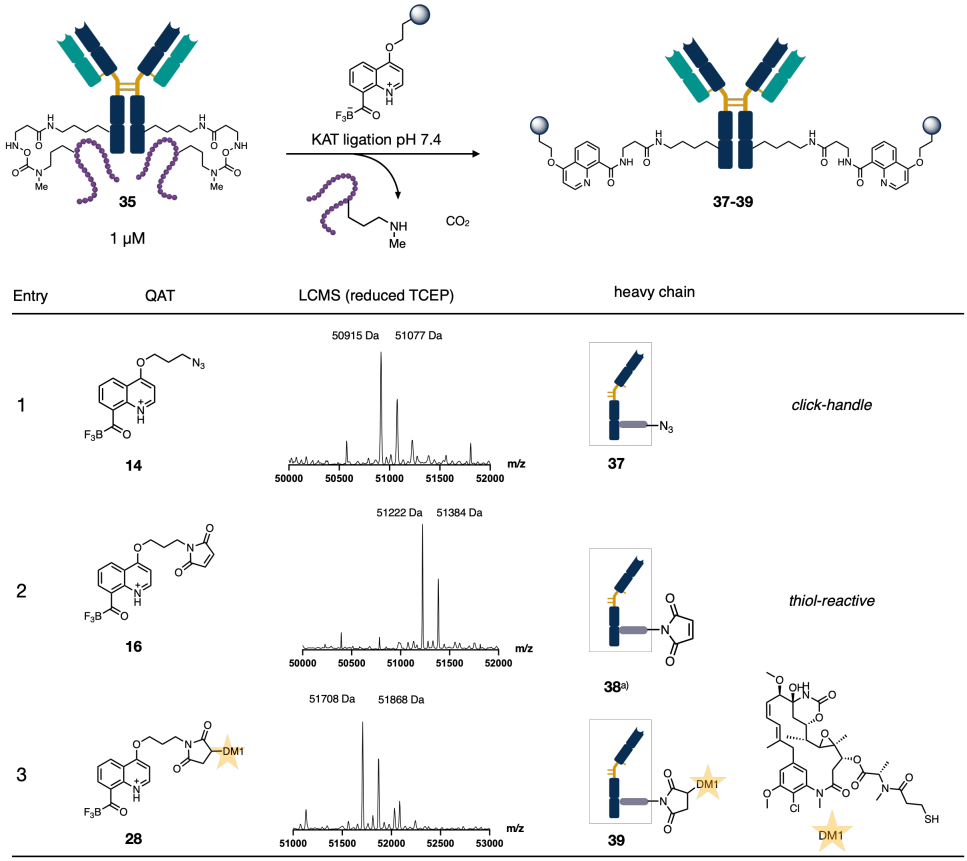

Figure 5. KAT ligation of glycosylated trastuzumab **35** with QAT-azide **14**, QAT-maleimide **16** and QAT-DM1 **28**. The concentration of **35** was set to 1 μM and 20 equiv of each QAT were added and the resulting solution mixed

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for 15 min at room temperature. After reduction with TCEP, the solutions were evaluated by mass analysis (Xevo G2-XS QTOF). The masses observed correspond to the ligated products. a) The heavy chain mass of **38** (51222 Da) corresponds to the maleimide-TCEP 4-ylene adduct after reduction.

The expected modifications were observed on the heavy chain only; no unspecific conjugation of light chains was detected. To further confirm this observation, we prepared coumarin-labeled QAT **19** for analysis of the KAT ligation‡ by SDS-PAGE on a 8-16% discontinuous gradient gel after denaturing (Figure 6). Both starting material **35** and the trastuzumab-coumarin conjugate **40** were loaded in both their intact and reduced form. The intact antibodies (Figure 6: lane 2, 3, and 5, *left gel*) run as multiple bands due to glycosylation of asparagine N297. When comparing the reduced bands in lane 6, the heavy chain emits a fluorescent signal whereas the light chain remains unmodified indicating that only the heavy has been labeled by our construct **10**. Further, the coumarin-labeled QAT **19** did not show unspecific imine-formation with other lysines on the light chain, despite the fact that 20 equiv of **19** was employed.

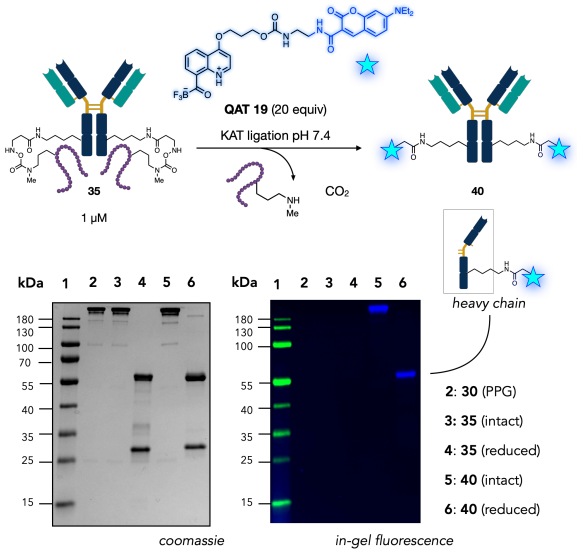

Figure 6: Reaction of trastuzumab-hydroxylamine conjugate **35** and coumarin-labeled QAT **19** in 50 mM potassium phosphate buffer pH 7.4 for SDS-PAGE analysis. The fluorescence (*right gel*) was measured on a Bio-Rad ChemiDoc MP Imaging System before staining with coomassie (*left gel*). Lane 1: MW protein ladder, lane 2: intact trastuzumab-hydroxylamine with photo-protecting group (PPG), lane 3: trastuzumab-hydroxylamine after photo-deprotection at 365 nm for 10 min, lane 4: **35** after reduction with TCEP, lane 5: reaction mixture of **35** with coumarin-labeled QAT **19** (intact), lane 6: reaction mixture after reduction with TCEP. No fluorescent light chain band was observed by SDS-PAGE.

‡ Despite the absence of a potassium counterion in QATs, the term 'KAT ligation' is retained to avoid introducing a new acronym.

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The ability of KAT ligation with quinolinium acyltrifluoroborates to proceed rapidly (k = 4.5 M^{-1} s^{-1}) at pH 7 makes it an ideal candidate for the conjugation of biological macromolecules, including proteins and nucleic acids, to antibodies. To test its utility for protein–antibody conjugation, we prepared UbK63C-QAT **42** by expressing ubiquitin K63C (UbK63C) **41** and treating it with bifunctional linker **27** bearing a thiophilic moiety[35] and a quinolinium acyltrifluoroborate (Figure 7A). Ubiquitin-antibody fusion proteins have recently been prepared and studied as a means of using Ub as handle for generating bispecific antibodies.[36] Trastuzumab-hydroxylamine conjugate **35** was coupled with 10 equiv of **42** at rt for 22 h. A small fraction was reduced using TCEP and denatured for SDS-PAGE analysis (Figure 7C) where the ubiquitin-labeled heavy chain was detected with an estimated conversion of 64% as judged by gel densitometry. Mass analysis of the reaction mixture after reduction by Q-TOF LC/MS (Figure 7D) confirmed the formation of a ubiquitin-labeled heavy chain. The two prominent peaks 59924 Da and 60085 Da stem from the N-linked oligosaccharides G0F and G1F on the intact monoclonal antibody.

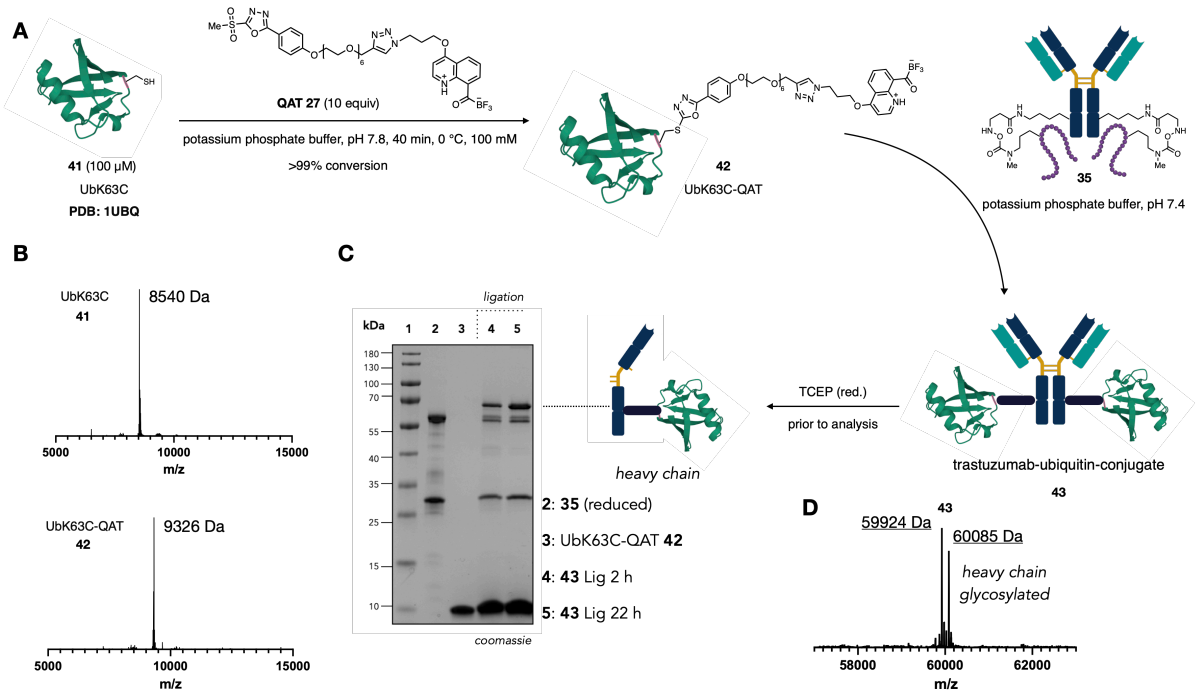

Figure 7. A: Reaction of a UbK63C mutant **41** (100 µM) and 10 equiv of bifunctional linker **27** in 50 mM potassium phosphate buffer at pH 7.8. The reaction was incubated at 0 °C for 40 min, before purifying using a desalting column. An LCMS was measured to ensure that residual **27** was fully removed. 10 equiv of **42** were added to the trastuzumab-hydroxylamine conjugate **35** at a final antibody concentration of 1 µM in potassium phosphate buffer at pH 7.4. Small aliquots were taken at 2 h and 22 h and reduced with TCEP for analysis. **B:** Deconvoluted mass spectra of UbK63C **41** and UbK63C-QAT **42** after purification, indicating full conversion. **C:** SDS-PAGE: lane 1: MW protein ladder, lane 2: trastuzumab-hydroxylamine **35** (reduced), lane 3: UbK63C-QAT **42**, lane 4: reaction monitoring of **35** and **42** after 2 h (reduced), lane 5: reaction monitoring after 22 h (reduced). Gel densitometry

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analysis estimated 64% of ubiquitin-labeled heavy chain after 22 h. **D:** Deconvoluted Xevo G2-XS QTOF mass spectrum of ubiquitin-labeled heavy chain derived from **43** after reduction with TCEP. Two prominent peaks appear due to glycosylation (G0F, G1F).

Oligonucleotide-conjugated antibodies represent a family of chimeric constructs that have gained attention owing to their use in protein diagnostics and as therapeutic drugs.[37-39] While the high specificity of antibodies towards antigens can be used as a delivery tool, oligonucleotides exert their functions as therapeutic drugs (antisense nucleotides) or as sensitive detection moieties e.g. through immuno-PCR.[40-42] Methods to successfully prepare antibody-oligonucleotide conjugates (AOCs) have been previously described, including both non-covalent and covalent approaches.[43-49] The majority of the covalent approaches are not amenable to the direct, off-the-shelf use of antibodies and often rely on the modification of lysine and cysteine residues that can lead to heterogenous outcomes.

We hypothesized that ssDNA derived QATs could provide an efficient route towards AOCs. A commercially available ssDNA 5'-SH-ss29-mer **44** with a 5'-thiol C6 modifier was obtained and transformed into the respective QAT using **27** (Figure 8A). A 250 µM stock solution of **44** in PBS was fully converted to the 5'-QAT labeled ssDNA **45** in 30 min by adding 4.0 equiv of **27** and excess **27** was removed by spin filtration. Mass spectra of **44** and **45** showed the full conversion and signals were detected as $[M]^{4-}$ charge state (Figure 8C). Subsequently, trastuzumab-hydroxylamine conjugate **35** in PBS was mixed with 10 equiv of **45** at a final antibody concentration of 1 µM and the reaction was stirred for 14 h at room temperature (Figure 8B). A small fraction of **46** was reduced using TCEP and denatured for SDS-PAGE analysis (Figure 8D). SDS-PAGE indicated a nearly full conversion to the ssDNA-labeled heavy chain of trastuzumab.

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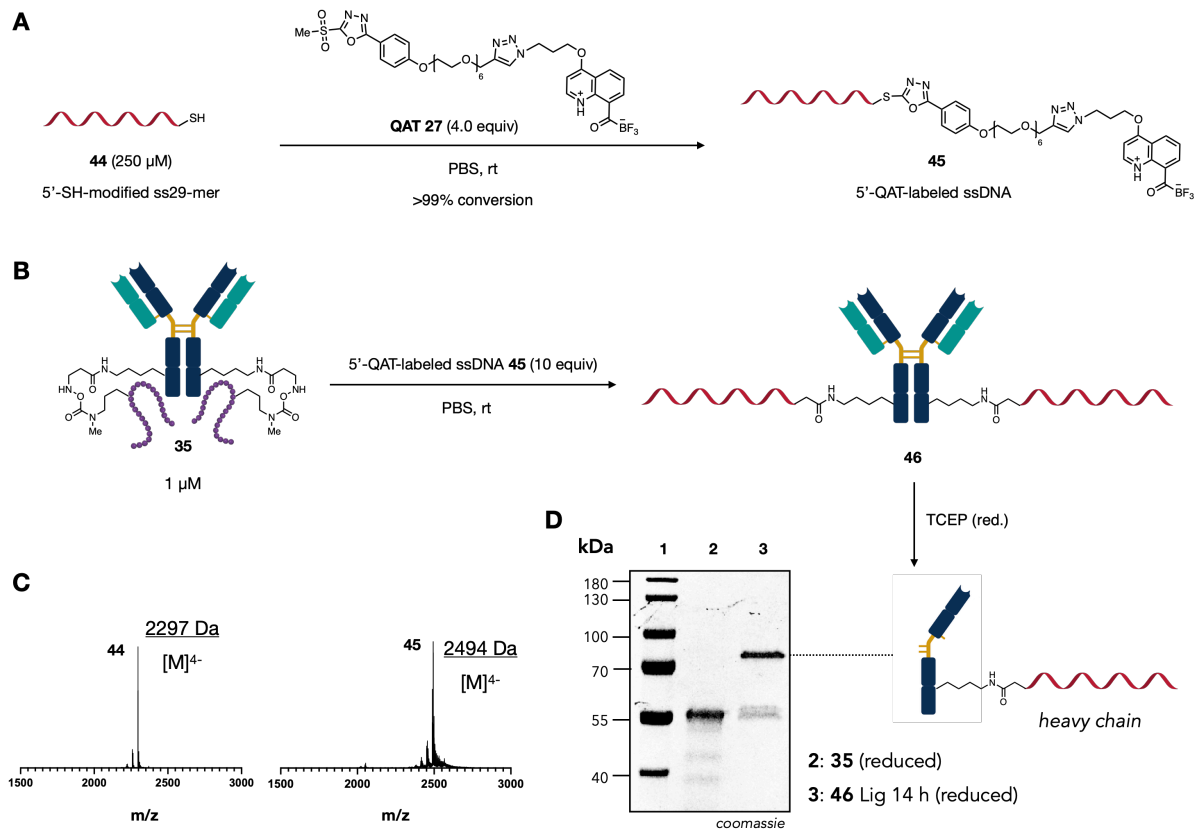

Figure 8. A: Modification of 5'-thio-ssDNA GGAGACCCTATAGTGAGTCGTATTAAGGT (5' to 3') with 4.0 equiv of **27** in PBS buffer at 250 µM. **B:** Reaction of trastuzumab-hydroxylamine-conjugate **35** and 10.0 equiv of **45** at a final antibody concentration of 1 µM. The solution was stirred for 14 h at rt in PBS. **C:** Mass spectra of **44** and **45** indicating full conversion to the 5'-QAT-labeled ssDNA. Masses were detected as $[M]^{4-}$ charge state. **D:** SDS-PAGE: lane 1: MW protein ladder, lane 2: trastuzumab-hydroxylamine **35** (reduced), lane 3: reaction monitoring of **35** and **45** after 14 h (reduced), indicating the formation of **46**.

The site-specific modification of K248 has been shown to maintain the antibodies affinities in precedented studies.[23-24] To confirm that modification site of the IgG antibodies does not compromise antigen binding, SKOV-3 (HER2 positive) ovarian cancer cells and MCF-7 (HER2 negative) breast cancer cells were grown (*Supporting Information*) and treated with a trastuzumab-biotin conjugate **47** prepared from the biotin-QAT **29** and the trastuzumab-hydroxylamine conjugate **35**, following the general procedure (Figure 9A). Upon treatment with Atto 488-streptavidin in a second step, the cells could be imaged via confocal microscopy. Detection of green fluorescence was observed within the HER2 positive SKOV-3 cells, whereas the MCF-7 cells did not exhibit any observable fluorescent signal. A control experiment involving the incubation of both cell lines with biotin-QAT **29**, followed by Atto 488-streptavidin revealed no background fluorescence, confirming that SKOV-3 cells were labeled by the trastuzumab-biotin conjugate **47**. In a subsequent control experiment, treatment of both cell lines with stochastically conjugated Atto 565-trastuzumab (via NHS ester), resulted in red

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fluorescence specifically in SKOV-3 cells, with no detectable fluorescence in MCF-7 cells (Supporting information 9.2).

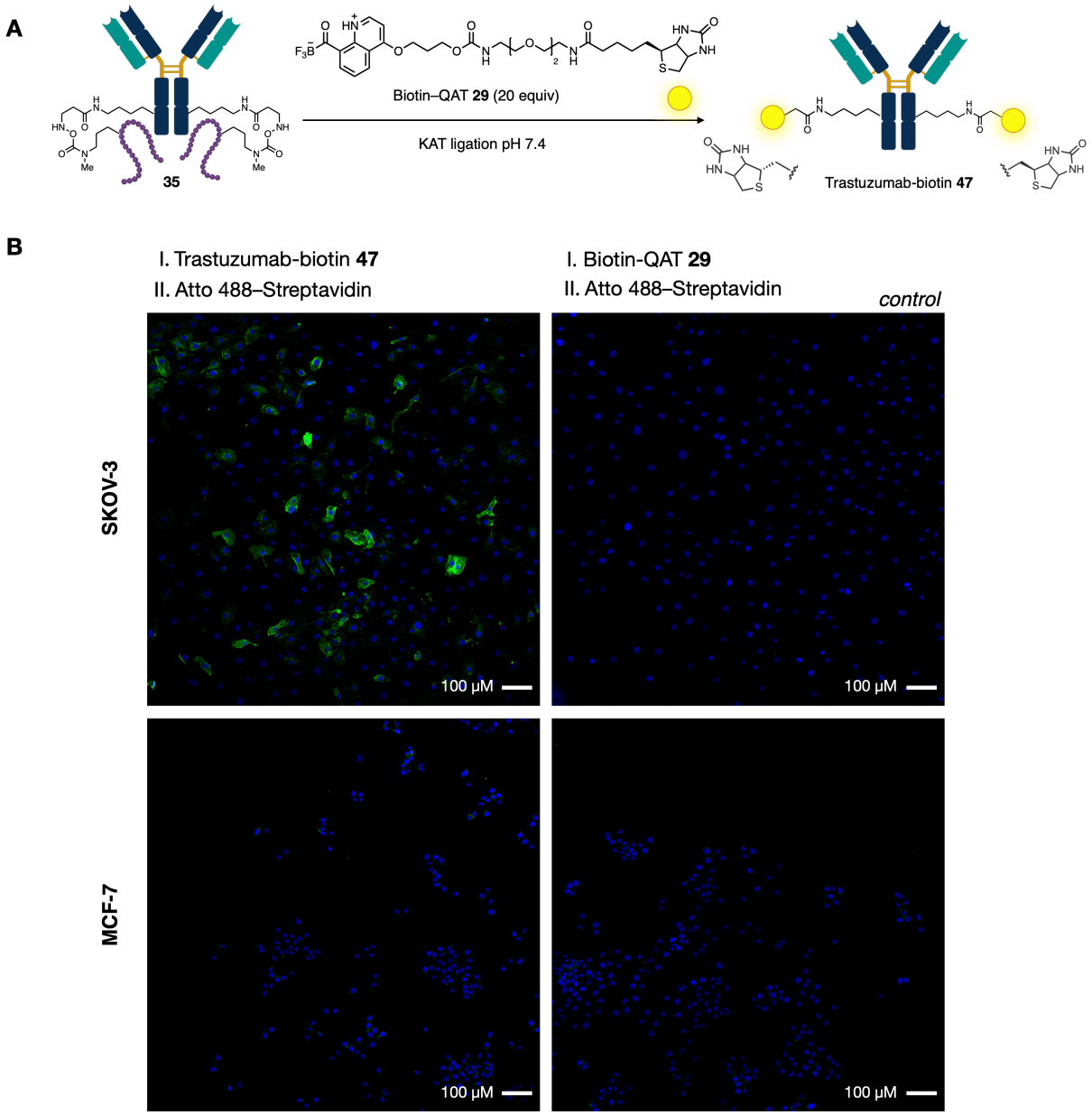

Figure 9. A. Preparation of DAR 2 modified trastuzumab-biotin conjugate **47** from trastuzumab-hydroxylamine conjugate **35** and biotin-QAT **29**. **B.** Confocal microscopy of HER2-positive SKOV-3 cells (*top*) and HER2-negative MCF-7 cells (*bottom*) treated with trastuzumab-biotin conjugate **47** and detected using Atto 488 labeled streptavidin. Green fluorescence could be detected for SKOV-3 cells when treated with the trastuzumab-biotin conjugate **47**, followed by Atto 488-streptavidine, whereas MCF-7 cells remained unlabeled (*left*). Control experiments where both cell lines were subjected to only biotin-QAT **29**, followed by Atto 488-streptavidine showed no fluorescent signal (*right*). Hoechst (blue) was used for nuclei counterstaining.

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Conclusion

In summary, we have adapted a site-specific lysine modification of antibodies for the facile introduction of two hydroxylamines into commercial IgGs using affinity peptide **10**. The resulting IgG-hydroxylamine conjugates (DAR = 2) undergo clean KAT ligation with simultaneous removal of the affinity peptide, leaving a simple, lysine-linked amide at the specific site of heavy chain modification. Various functional QATs could be prepared and efficiently ligated to hydroxylamine-containing antibodies under physiological conditions, allowing the attachment of small molecules, anti-cancer drugs, proteins, and ssDNA. Even when excess amounts of the affinity peptide **10** and QATs were used, unspecific labeling of lysine residues was not detected. By combining Yamada's elegant protocol for site-specific antibody modification with KAT ligation, discrete antibody-(bio)conjugates with defined payloads can be readily prepared from off-the-shelf therapeutic antibodies.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: Antibodies • Affinity peptide • Conjugation • Antibody-drug conjugate • KAT Ligation

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References

[1] Adams, G. P.; Weiner, L. M., *Nat. Biotechnol.* **2005,** *23*, 1147-1157.

[2] Polakis, P., *Curr. Opin. Pharmacol.* **2005,** *5*, 382-7.

[3] Sievers, E. L.; Senter, P. D., *Annu. Rev. Med.* **2013,** *64*, 15-29.

[4] Agarwal, P.; Bertozzi, C. R., *Bioconjug. Chem.* **2015,** *26*, 176-192.

[5] Fu, Z.; Li, S.; Han, S.; Shi, C.; Zhang, Y., *Signal Transduct. and Target. Ther.* **2022,** *7*, 93.

[6] You, J.; Zhang, J.; Wang, J.; Jin, M., *Bioconjug. Chem.* **2021,** *32*, 1525-1534.

[7] Junutula, J. R.; Raab, H.; Clark, S.; Bhakta, S.; Leipold, D. D.; Weir, S.; Chen, Y.; Simpson, M.; Tsai, S. P.; Dennis, M. S.; Lu, Y.; Meng, Y. G.; Ng, C.; Yang, J.; Lee, C. C.; Duenas, E.; Gorrell, J.; Katta, V.; Kim, A.; McDorman, K.; Flagella, K.; Venook, R.; Ross, S.; Spencer, S. D.; Lee Wong, W.; Lowman, H. B.; Vandlen, R.; Sliwkowski, M. X.; Scheller, R. H.; Polakis, P.; Mallet, W., *Nat. Biotechnol.* **2008,** *26*, 925-932.

[8] Coumans, R. G. E.; Ariaans, G. J. A.; Spijker, H. J.; Renart Verkerk, P.; Beusker, P. H.; Kokke, B. P. A.; Schouten, J.; Blomenröhr, M.; van der Lee, M. M. C.; Groothuis, P. G.; Ubink, R.; Dokter, W. H. A.; Timmers, C. M., *Bioconjug. Chem.* **2020,** *31*, 2136-2146.

[9] Zeglis, B. M.; Davis, C. B.; Aggeler, R.; Kang, H. C.; Chen, A.; Agnew, B. J.; Lewis, J. S., *Bioconjug. Chem.* **2013,** *24*, 1057-1067.

[10] Jeger, S.; Zimmermann, K.; Blanc, A.; Grünberg, J.; Honer, M.; Hunziker, P.; Struthers, H.; Schibli, R., *Angew. Chem. Int. Ed.* **2010,** *49*, 9995-9997.

[11] Lorand, L.; Graham, R. M., *Nat. Rev. Mol. Cell Bio.* **2003,** *4*, 140-156.

[12] Zuberbühler, K.; Casi, G.; Bernardes, G. J. L.; Neri, D., *Chem. Commun.* **2012,** *48*, 7100-7102.

[13] Li, X.; Yang, J.; Rader, C., *Methods* **2014,** *65*, 133-138.

[14] Vinogradova, E. V.; Zhang, C.; Spokoyny, A. M.; Pentelute, B. L.; Buchwald, S. L., *Nature* **2015,** *526*, 687-691.

[15] Ferreira, V. F. C.; Oliveira, B. L.; D'Onofrio, A.; Farinha, C. M.; Gano, L.; Paulo, A.; Bernardes, G. J. L.; Mendes, F., *Bioconjug. Chem.* **2021,** *32*, 121-132.

[16] Bernardim, B.; Matos, M. J.; Ferhati, X.; Compañón, I.; Guerreiro, A.; Akkapeddi, P.; Burtoloso, A. C. B.; Jiménez-Osés, G.; Corzana, F.; Bernardes, G. J. L., *Nat. Protoc.* **2019,** *14*, 86-99.

[17] Walsh, S. J.; Bargh, J. D.; Dannheim, F. M.; Hanby, A. R.; Seki, H.; Counsell, A. J.; Ou, X.; Fowler, E.; Ashman, N.; Takada, Y.; Isidro-Llobet, A.; Parker, J. S.; Carroll, J. S.; Spring, D. R., *Chem. Soc. Rev.* **2021,** *50*, 1305-1353.

[18] Harmand, T. J.; Bousbaine, D.; Chan, A.; Zhang, X.; Liu, D. R.; Tam, J. P.; Ploegh, H. L., *Bioconjug. Chem.* **2018,** *29*, 3245-3249.

[19] DeLano, W. L.; Ultsch, M. H.; de, A. M.; Vos; Wells, J. A., *Science* **2000,** *287*, 1279-1283.

[20] Kishimoto, S.; Nakashimada, Y.; Yokota, R.; Hatanaka, T.; Adachi, M.; Ito, Y., *Bioconjug. Chem.* **2019,** *30*, 698-702.

[21] Vance, N.; Zacharias, N.; Ultsch, M.; Li, G.; Fourie, A.; Liu, P.; LaFrance-Vanasse, J.; Ernst, J. A.; Sandoval, W.; Kozak, K. R.; Phillips, G.; Wang, W.; Sadowsky, J., *Bioconjug. Chem.* **2019,** *30*, 148-160.

[22] Fujii, T.; Matsuda, Y.; Seki, T.; Shikida, N.; Iwai, Y.; Ooba, Y.; Takahashi, K.; Isokawa, M.; Kawaguchi, S.; Hatada, N.; Watanabe, T.; Takasugi, R.; Nakayama, A.; Shimbo, K.; Mendelsohn, B. A.; Okuzumi, T.; Yamada, K., *Bioconjug. Chem.* **2023,** *34*, 728-738.

[23] Yamada, K.; Shikida, N.; Shimbo, K.; Ito, Y.; Khedri, Z.; Matsuda, Y.; Mendelsohn, B. A., *Angew. Chem. Int. Ed.* **2019,** *58*, 5592-5597.

[24] Zeng, Y.; Shi, W.; Dong, Q.; Li, W.; Zhang, J.; Ren, X.; Tang, C.; Liu, B.; Song, Y.; Wu, Y.; Diao, X.; Zhou, H.; Huang, H.; Tang, F.; Huang, W., *Angew. Chem. Int. Ed.* **2022,** *61*, e202204132.

[25] Postupalenko, V.; Marx, L.; Viertl, D.; Gsponer, N.; Gasilova, N.; Denoel, T.; Schaefer, N.; Prior, J. O.; Hagens, G.; Lévy, F.; Garrouste, P.; Segura, J.-M.; Nyanguile, O., *Chem. Sci.* **2022,** *13*, 3965-3976.

[26] Yuan, D.; Zhang, Y.; Lim, K. H.; Leung, S. K. P.; Yang, X.; Liang, Y.; Lau, W. C. Y.; Chow, K. T.; Xia, J., *J. Am. Chem. Soc.* **2022,** *144*, 18494-18503.

[27] Tanriver, M.; Dzeng, Y.-C.; Da Ros, S.; Lam, E.; Bode, J. W., *J. Am. Chem. Soc.* **2021,** *143*, 17557-17565.

[28] Barltrop, J. A.; Plant, P. J.; Schofield, P., *Chem. Commun.* **1966**, 822-823.

[29] Klán, P.; Šolomek, T.; Bochet, C. G.; Blanc, A.; Givens, R.; Rubina, M.; Popik, V.; Kostikov, A.; Wirz, J., *Chem. Rev.* **2013,** *113*, 119-191.

[30] Matsuda, Y.; Malinao, M.-C.; Robles, V.; Song, J.; Yamada, K.; Mendelsohn, B. A., *J. Chromatogr. B* **2020,** *1140*, 121981.

[31] Ferraro, E.; Drago, J. Z.; Modi, S., *Breast Cancer Res.* **2021,** *23*, 84.

[32] Drake, P. M.; Rabuka, D., *BioDrugs* **2017,** *31*, 521-531.

[33] Amiri-Kordestani, L.; Blumenthal, G. M.; Xu, Q. C.; Zhang, L.; Tang, S. W.; Ha, L.; Weinberg, W. C.; Chi, B.; Candau-Chacon, R.; Hughes, P.; Russell, A. M.; Miksinski, S. P.; Chen, X. H.; McGuinn, W. D.; Palmby, T.; Schrieber, S. J.; Liu, Q.; Wang, J.; Song, P.; Mehrotra, N.; Skarupa, L.; Clouse, K.; Al-Hakim, A.; Sridhara, R.; Ibrahim, A.; Justice, R.; Pazdur, R.; Cortazar, P., *Clin. Cancer Res.* **2014,** *20*, 4436-4441.

[34] Kantner, T.; Watts, A. G., *Bioconjug. Chem.* **2016,** *27*, 2400-2406.

10.1002/anie.202401080

[35] Toda, N.; Asano, S.; Barbas III, C. F., *Angew. Chem. Int. Ed.* **2013,** *52*, 12592-12596.

[36] Hebieshy, A. e.; Gall, C. L.; Middelburg, J.; Fennemann, F.; Sluijter, M.; Hall, T. v.; Parren, P. P. W. H. I.; Noort, G. v. d. H. v.; Verdoes, M.; Ovaa, H.; Scheeren, F., *PREPRINT available at Research Square,* https://doi.org/10.21203/rs.3.rs-1835302/v1.

[37] Dovgan, I.; Koniev, O.; Kolodych, S.; Wagner, A., *Bioconjug. Chem.* **2019,** *30*, 2483-2501.

[38] Janssen, K. P.; Knez, K.; Spasic, D.; Lammertyn, J., *Sensors (Basel)* **2013,** *13*, 1353-84.

[39] Yarian, F.; Alibakhshi, A.; Eyvazi, S.; Arezumand, R.; Ahangarzadeh, S., *J. Cell. Physiol.* **2019,** *234*, 16724-16738.

[40] Sano, T.; Smith, C. L.; Cantor, C. R., *Science* **1992,** *258*, 120-2.

[41] Walker, I.; Irwin, W. J.; Akhtar, S., *Pharm. Res.* **1995,** *12*, 1548-53.

[42] Trads, J. B.; Tørring, T.; Gothelf, K. V., *Acc. Chem. Res.* **2017,** *50*, 1367-1374.

[43] Wiener, J.; Kokotek, D.; Rosowski, S.; Lickert, H.; Meier, M., *Sci. Rep.* **2020,** *10*, 1457.

[44] Konč, J.; Brown, L.; Whiten, D. R.; Zuo, Y.; Ravn, P.; Klenerman, D.; Bernardes, G. J. L., *Angew. Chem. Int. Ed.* **2021,** *60*, 25905-25913.

[45] Gong, H.; Holcomb, I.; Ooi, A.; Wang, X.; Majonis, D.; Unger, M. A.; Ramakrishnan, R., *Bioconjug. Chem.* **2016,** *27*, 217-225.

[46] Carvalho, A. M.; Manicardi, A.; Montes, C. V.; Gunnoo, S. B.; Schneider, R. J.; Madder, A., *Org. Biomol. Chem.* **2017,** *15*, 8923-8928.

[47] Dovgan, I.; Ehkirch, A.; Lehot, V.; Kuhn, I.; Koniev, O.; Kolodych, S.; Hentz, A.; Ripoll, M.; Ursuegui, S.; Nothisen, M.; Cianférani, S.; Wagner, A., *Sci. Rep.* **2020,** *10*, 7691.

[48] Dugal-Tessier, J.; Thirumalairajan, S.; Jain, N., *J. Clin. Med.* **2021,** *10*.

[49] Kazane, S. A.; Sok, D.; Cho, E. H.; Uson, M. L.; Kuhn, P.; Schultz, P. G.; Smider, V. V., *Proc. Natl. Acad. Sci. U.S.A.* **2012,** *109*, 3731-3736.

10.1002/anie.202401080

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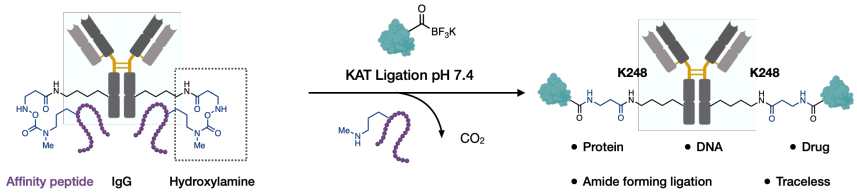

A cyclic peptide directs the covalent attachment of a hydroxylamine to specific lysine residues of the Fc region of antibodies, and is cleaved without a trace upon KAT ligation under physiological conditions. This convenient approach to the direct conjugation of commercial IgG antibodies is suitable for the formation of antibody-drug, antibody-protein, and antibody-DNA conjugates.